Pharmacological activation of cloned intermediate- and small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels

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Syme, Colin A., Aaron C. Gerlach, Ashvani K. Singh, and Daniel C. Devor. Pharmacological activation of cloned intermediate- and small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels. Am. J. Physiol. Cell Physiol. 278:C570–C581, 2000.—We previously characterized 1-ethyl-2-benzimidazolinone (1-EBIO), as well as the clinically useful benzoxazoles, chlorzoxazone (CZ), and zoxazolamine (ZOX), as pharmacological activators of the intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel, hIK1. The mechanism of activation of hIK1, as well as the highly homologous small-conductance, Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel, rSK2, was determined following heterologous expression in Xenopus oocytes using two-electrode voltage clamp (TEVC) and excised, inside-out patch-clamp techniques. 1-EBIO, CZ, and ZOX activated both hIK1 and rSK2 in TEVC and excised inside-out patch-clamp experiments. In excised, inside-out patches, 1-EBIO and CZ induced a concentration-dependent activation of hIK1, with half-maximal (K\textsubscript{1/2}) values of 84 µM and 98 µM, respectively. Similarly, CZ activated rSK2 with a K\textsubscript{1/2} of 87 µM. In the absence of CZ, the Ca\textsuperscript{2+}-dependent activation of hIK1 was best fit with a K\textsubscript{1/2} of 700 nM and a Hill coefficient (n) of 2.0. rSK2 was activated by Ca\textsuperscript{2+} with a K\textsubscript{1/2} of 700 nM and an n of 2.5. Addition of CZ had no effect on either the K\textsubscript{1/2} or n for Ca\textsuperscript{2+}-dependent activation of either hIK1 or rSK2. Rather, CZ increased channel activity at all Ca\textsuperscript{2+} concentrations (V\textsubscript{max}). Event-duration analysis revealed hIK1 was minimally described by two open and three closed times. Activation by 1-EBIO had no effect on t\textsubscript{o1}, t\textsubscript{o2}, or t\textsubscript{c3}, whereas t\textsubscript{o2} and t\textsubscript{c3} were reduced from 9.0 and 92.6 ms to 5.0 and 44.1 ms, respectively. In conclusion, we define 1-EBIO, CZ, and ZOX as the first known activators of hIK1 and rSK2. Openers of IK and SK channels may be therapeutically beneficial in cystic fibrosis and vascular diseases.

hIK1 channel; rSK2 channel; chlorzoxazone; zoxazolamine; 1-ethyl-2-benzimidazolinone

CALCIUM-DEPENDENT K\textsuperscript{+} CHANNELS have historically been subdivided into three distinct classes based on conductance and blocker pharmacology. These include the large-conductance (BK) channels, which are sensitive to block by charybdotoxin (CTX) and iberiotoxin (29), the intermediate-conductance (IK) channels, which are inhibited by CTX and clotrimazole (19, 21, 24), and the small-conductance (SK), apamin-sensitive (and -insensitive) K\textsuperscript{+} channels (23). Until recently, openers of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels had not been described. Olesen et al. (30, 31) identified a novel class of compounds, the benzimidazolones, as direct activators of BK channels, with the best studied being 1-(2-hydroxy-5-trifluoromethylphenyl)-5-trifluoroethyl-2-[\textsuperscript{3H}]benzimidazolone (NS-1619) and 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-[\textsuperscript{3H}]benzimidazole-2-one (NS-004) (27, 30, 31, 34). Based on the subsequent observation that NS-004 directly activated the cystic fibrosis transmembrane conductance regulator (CFTR) Cl\textsuperscript{−} channel (16), we determined whether NS-004 would modulate transepithelial Cl\textsuperscript{−} secretion. However, NS-004 failed to stimulate Cl\textsuperscript{−} secretion across the colonic cell line, T84, despite its ability to activate an apical membrane Cl\textsuperscript{−} conductance (5). In contrast, the structurally related benzimidazolone, 1-ethyl-2-benzimidazolinone (1-EBIO) induced a sustained Cl\textsuperscript{−} secretory response in these cells (5, 6). This difference was due to the ability of 1-EBIO to activate both the basolateral membrane intermediate conductance, Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel (IK), as well as the apical membrane Cl\textsuperscript{−} conductance, CFTR channel (5, 6). This coordinate activation of both membrane conductances is required to induce a sustained Cl\textsuperscript{−} secretory response. In contrast, NS-004 and NS-1619 failed to activate the IK channel in these epithelial cells (5, 6). Thus 1-EBIO represents the first characterized pharmacological opener of these IK channels.

Recently, we demonstrated that the structurally related compounds chlorzoxazone (CZ) and zoxazolamine (ZOX) similarly stimulate transepithelial Cl\textsuperscript{−} secretion across colonic and airway epithelia via an activation of both basolateral IK and apical CFTR (35). Based on these observations, we speculated that basolateral membrane K\textsuperscript{+} channels may represent novel pharmacological targets in cystic fibrosis therapy. CZ has been used clinically for over 30 years as a centrally acting skeletal muscle relaxant with limited knowledge regarding its mechanism of action. While 1-EBIO, CZ, and ZOX activate both IK and CFTR in epithelia, their mechanism of action is poorly understood. Thus we determined, in detail, the mechanism by which 1-EBIO, CZ, and ZOX activate IK and SK channels.

Recently, both Ishii et al. (19) (hIK1) and Joiner et al. (21) (hSK4) reported the cloning of a human intermediate-conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel. A nearly identical channel (2 amino acids different) has also been recently reported in human T-lymphocytes (22, 24). We previously demonstrated that the Ca\textsuperscript{2+}-
dependent K⁺ channel expressed in colonic and airway epithelia has biophysical and pharmacological characteristics identical to those of the recently cloned hIK1. That is, it exhibits inward rectification in symmetric K⁺ (4, 8) and is blocked with high affinity by CTX (Kᵢ = 3.5 nM; Refs. 6, 8) and clotrimazole (Kᵢ = 270 nM; Refs. 7, 8). Indeed, we have demonstrated the expression of hIK1 in both human colonic (TB4) and airway serous (Calu-3) cells by Northern blot analysis (14, 15). Based on the tissue distribution of hIK1, pharmacological modulators of these channels have been proposed as potential therapeutic agents for cystic fibrosis (5, 6), immunosuppression (11), sickle cell anemia (1, 19, 37), myotonic muscular dystrophy (32), and hypertension (25). Here, we demonstrate that the previously described pharmacological openers, 1-EBIO, CZ, and ZOX, activate hIK1, as well as the highly homologous rSK2, by increasing the opening rate, and hence open probability (Pₒ), of the channel while having no effect on the affinity of the channel for Ca²⁺.

METHODS

Channel expression. Xenopus laevis care and handling procedures were in accordance with University of Pittsburgh guidelines. X. laevis frogs were anesthetized with 3-aminobenzoic acid ethyl ester, the ovaries were surgically removed, and oocytes were dissected in modified Barth’s solution (MBS) containing (in mM) 88 NaCl, 2.4 NaHCO₃, 1 KCl, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 10 HEPES, and penicillin-streptomycin (1%) and defolliculated by digestion in calcium-free ND96 solution containing collagenase (Life Technologies). Oocytes were incubated at 19°C in MBS, pBF plasmid containing the genes for hIK1 or rSK2 (kindly supplied by J. P. Adelman, Oregon Health Sciences University) were linearized using Pvu I restriction enzyme (Boehringer Mannheim) and 5’ capped mRNAs generated using SP6 polymerase (mMESSAGE mMACHINE In Vitro Transcription Kit, Ambion). mRNAs were evaluated both spectrophotometrically and by agarose gel electrophoresis with ethidium bromide staining. Oocytes were injected with 5–50 ng of mRNA 1–2 days prior to recording.

Solutions. For two-electrode voltage clamp (TEVC) recordings, oocytes were continuously superfused with high-K⁺ ND96 containing (in mM) 96 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES (pH adjusted to 7.5 with KOH) at room temperature. During inside-out patch-clamp recording, the bath contained (in mM) 145 potassium gluconate, 5 KCl, 2 MgCl₂, 1 EGTA, and 10 HEPES (pH adjusted to 7.2 with KOH). Sufficient CaCl₂ was added to the bath solution to obtain the desired free [Ca²⁺]o (program kindly supplied by D.C. Dawson, Univ. of Michigan). The pipette solution contained (in mM) 140 potassium gluconate, 5 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES (pH adjusted to 7.2 with KOH).

TEVC recording. Oocytes were mounted in a recording chamber maintained at room temperature. Macroscopic currents were measured with the TEVC method using a GeneClamp 500B amplifier (Axon Instruments). Data were sampled at 100 Hz using Axoscope software (Axon Instruments). Electrodes were fabricated from borosilicate glass (Kimax-51, Kimble glass), pulled on a vertical puller (Narishige), and had resistances of 0.3–5 MΩ when filled with 3 M KCl. All recordings were performed at a holding voltage of −60 mV, such that activation of a K⁺ current resulted in an inward current flow. CTX and apamin were applied directly to the bath during continuous perfusion, at the indicated concentrations, such that inhibition of current was transient in nature.

Single-channel recording. Prior to patch-clamp recording, the vitelline membrane was carefully removed from the oocyte following cell shrinkage with hypertonic solution containing (in mM) 200 potassium gluconate, 20 KCl, 1 MgCl₂, 1 EGTA, and 10 HEPES (pH adjusted to 7.4 with KOH). Excised, inside-out single-channel currents were recorded using a List EPC-7 amplifier (Medical Systems) and were recorded on videotape for later analysis as described previously (4). Pipettes were fabricated from thin-walled borosilicate glass (World Precision Instruments). All recordings were performed at a holding voltage of −100 mV. The voltage is referenced to the extracellular compartment, as is the standard method for membrane potentials. Inward currents are defined as the movement of positive charge from the extracellular compartment to the intracellular compartment and are presented as downward deflections from the baseline in all recording configurations.

Single-channel analysis was performed on records sampled at 5 kHz after low-pass filtering at 1 kHz. The product of the number of channels and the channel open probability (Pₒ) of the channels was determined using Biopatch software (version 3.11; Molecular Kinetics). NPₒ was calculated from the mean total current (I) divided by the single-channel current amplitude (i), such that NPₒ = I/i. The i was determined from the amplitude histogram of the current record. For single-channel recordings, the channel open probability (Pₒ) was calculated as I/i. For determining mean open and closed times, an open-closed transition was considered valid if it remained in the state for at least two sample periods (0.4 ms). Event-duration histograms for both the open times (tₒ1, tₒ2) and fast closed times (t cbo1, t cbo2) were constructed by binning at 1-ms intervals from 1.0 to 30–50 ms (i.e., >5 times the time constant). To fit tₒ1, data were binned at 10-ms intervals. Event-duration histograms were fit to exponential functions to determine the open (tₒ) and closed (t cbo) time constants.

Chemicals. Both CZ and 6-hydroxychlorzoxazone (OH–CZ) were obtained from Research Biochemicals International. ZOX was purchased from Sigma Chemical. 1-EBIO was purchased from Aldrich. These compounds were made as 1,000-fold stock solutions in DMSO. Apamin and ionomycin were obtained from Calbiochem. CTX was obtained from Accurate Chemical and Scientific and was made as a 10 μM stock solution in standard bath solution. Ionomycin was made as a 5 mM stock solution in DMSO. The disodium salt of ATP was obtained from Boehringer Mannheim, and used at a final concentration of 1 mM in bath solution containing 2 mM MgCl₂ with free Ca²⁺ adjusted accordingly.

Data analysis. All data are presented as means ± SE, where n indicates the number of experiments. Statistical analysis was performed using the Student’s t-test. A value of P < 0.05 was considered statistically significant.

RESULTS

Effects of benzoxazoles on hIK1 in TEVC experiments. We previously characterized 1-EBIO, CZ, and ZOX as direct activators of the basolateral membrane IK in human colonic and airway epithelia (5, 6, 35). Northern blot analysis demonstrates this IK corresponds to the recently cloned intermediate conductance Ca²⁺-activated K⁺ channel, hIK1 (15, 19). This channel has also been given the name hSK4 (21), hKCa4 (24), and hIKCa1 (13) by other investigators. We further defined the mechanism by which these compounds activate hIK1.
following heterologous expression in Xenopus oocytes. Initial experiments were carried out using the TEVC technique. The effects of CZ and ZOX (300 µM) on hIK1 during TEVC are shown in Fig. 1. Initially, addition of the Ca\(^{2+}\) ionophore, ionomycin (1 µM), induced a variable increase in inward current, consistent with activation of hIK1 under our recording conditions (see METHODS). Addition of either CZ (Fig. 1A) or ZOX (Fig. 1B) resulted in a further activation of hIK1. The effect of ionomycin and the benzoxazoles was inhibited by the transient addition of CTX (50 nM) during continuous superfusion, a known blocker of hIK1 (19, 21). In a total of 24 experiments, ionomycin increased current from \(0.14 \pm 0.02\) to \(-1.69 \pm 0.18\) µA. In 10 of these experiments, addition of CZ induced a further increase in current to \(-3.30 \pm 0.31\) µA (\(P < 0.001\)), and this was reduced to \(-1.43 \pm 0.12\) µA by the addition of CTX (50 nM). In an additional 10 experiments, ZOX further increased current to \(-5.19 \pm 0.67\) µA (\(P < 0.001\)), and this was reduced to \(-1.73 \pm 0.41\) µA by CTX. We previously demonstrated that the primary metabolite of CZ, OH-CZ, failed to activate the IK in T84 cells (35). Similarly, as shown in Fig. 1C, OH-CZ (300 µM) failed to activate hIK1 following addition of ionomycin. However, the subsequent addition of CZ (300 µM), in the absence of OH-CZ, activated hIK1. The transient addition of CTX resulted in a significant inhibition of this response. In four experiments, OH-CZ did not activate hIK1 (\(-2.08 \pm 0.73\) µA, Fig. 1D), although the current was subsequently increased to \(-4.25 \pm 1.12\) µA by the addition of CZ (\(P < 0.05\)). Addition of CTX reduced the current to \(-1.17 \pm 0.25\) µA. CZ and ZOX failed to increase current in the absence of ionomycin (data not shown), suggesting that the benzoxazoles do not substitute for Ca\(^{2+}\) in activating hIK1. This is similar to what we previously described for the activation of endogenous hIK1 by 1-EBIO (6). No effect of either ionomycin or CZ was observed in uninjected (\(n = 3\)) or water-injected (\(n = 4\)) oocytes (data not shown), demonstrating that CZ does not activate channels endogenously expressed in Xenopus oocytes.

Fig. 1. Activation of intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channel, hIK1, by benzoxazoles during two-electrode voltage clamp (TEVC) recording. hIK1 was heterologously expressed in Xenopus oocytes, and the oocyte was depolarized using a high-K\(^+\) solution and voltage clamped to \(-60\) mV. Addition of the Ca\(^{2+}\) ionophore, ionomycin (1 µM), increased inward current flow, consistent with activation of hIK1. Subsequent to ionomycin, both chlorzoxazone (CZ, 300 µM) (A) and zoxazolamine (ZOX, 300 µM) (B) further increased current that was blocked by charybdotoxin (CTX, 50 nM). C: following addition of ionomycin, 6-hydroxychlorzoxazone (OH-CZ, 300 µM), a metabolite of CZ, failed to activate hIK1. However, subsequent addition of CZ (300 µM), in absence of OH-CZ, induced a CTX-sensitive activation of hIK1. D: summary (means ± SE; number of experiments shown in parentheses) of TEVC data. CZ, ZOX, and OH-CZ were added in the continued presence of ionomycin. Both CZ and ZOX caused a significant activation of hIK1 compared with ionomycin (*\(P < 0.05\)), whereas OH-CZ did not activate hIK1.
Effects of benzoxazoles on hIK1 in excised membrane patches. The above results indicate that CZ and ZOX activate hIK1. This was directly assessed using excised inside-out patch-clamp recordings on hIK1 heterologously expressed in Xenopus oocytes. As is shown in Fig. 2A, CZ (100 µM) induced a rapid activation of hIK1. Similarly, removal of CZ from the bath solution resulted in a rapid return of channel activity to control levels (Fig. 2A). This activation was not accompanied by a change in the single-channel current (i) as determined by gaussian fits of the amplitude histograms (Figs. 2, B and C). Expanded channel records corresponding to control and CZ-stimulated conditions are shown in Fig. 2, D and E, respectively. In six experiments, CZ increased channel activity, measured as $NP_\alpha$, from a control value of 0.22 ± 0.11 to 2.31 ± 0.98 with no change in $i$ (control = 3.47 ± 0.09 pA; CZ = 3.45 ± 0.12 pA). Also, ZOX (100 µM) caused a significant increase in $NP_\alpha$, from a control value of 0.29 ± 0.15 to 1.10 ± 0.38 with no change in $i$ (control = 3.41 ± 0.05 pA; ZOX = 3.45 ± 0.07 pA, n = 5). Similar to our TEVC data, OH-CZ (300 µM) had no effect on hIK1 $NP_\alpha$ (control = 0.25 ± 0.14, OH-CZ = 0.21 ± 0.12; n = 5) or $i$ (control = 3.37 ± 0.06, OH-CZ = 3.43 ± 0.13) in excised inside-out patches, whereas the subsequent addition of CZ (300 µM) increased $NP_\alpha$ to 7.77 ± 2.70 (n = 5). Also, following activation of hIK1 by CZ (100 µM), addition of OH-CZ (300 µM) in the continued presence of CZ had no effect on channel activity (data not shown), suggesting OH-CZ does not interact with the channel. These results demonstrate the direct activation of hIK1 by CZ and ZOX but not OH-CZ.

The concentration dependence of this activation was determined. Normalized concentration-response curves for CZ and ZOX on hIK1 are shown in Fig. 3A (bath free Ca$^{2+}$ = 400 nM). The CZ-dependent activation of hIK1 was fitted to the Hill equation, having a half-maximal ($K_{1/2}$) concentration of 98 µM and a Hill coefficient of 2.4. A $K_{1/2}$ value for ZOX was unattainable since $NP_\alpha$ did not saturate with 600 µM ZOX, the maximum soluble concentration.

Effect of CZ on the Ca$^{2+}$-dependent gating of hIK1. Our results demonstrate that CZ directly activates hIK1 in excised patches. As hIK1 is a Ca$^{2+}$-dependent K$^+$ channel, we determined the effect of CZ on the Ca$^{2+}$ affinity of hIK1 in excised, inside-out patches (Fig. 3B).
For these experiments, we used a concentration of 100 µM as this concentration correlates well with the K\textsubscript{1/2} obtained (Fig. 3A). Free Ca\textsuperscript{2+} was varied between 80 nM (normal resting level of Ca\textsuperscript{2+} in colonic cells; Ref. 3) and 10 µM (a saturating level of Ca\textsuperscript{2+}) in the absence and presence of CZ. Importantly, both Ca\textsuperscript{2+} concentration-response curves were carried out on the same patches. The average results of eight experiments are shown in Fig. 3B. In both the absence and presence of CZ, the data were fitted to the Hill equation, with a K\textsubscript{1/2} value of 700 nM and a Hill coefficient of 2.0. These Ca\textsuperscript{2+} affinity data are in agreement with previously reported values for hIK1 (19) and the homologous channel cloned from mouse (mIK1) expressed in Xenopus oocytes (37). These data suggest that CZ activates hIK1 by increasing V\textsubscript{max} without shifting the apparent affinity of the channel for Ca\textsuperscript{2+}. In addition, these data demonstrate that the CZ-dependent activation of hIK1 relies upon Ca\textsuperscript{2+} being elevated above resting levels (no activation was observed with 80 nM free Ca\textsuperscript{2+}), consistent with our TEVC data.

Effects of 1-EBIO on hIK1. We previously reported that the benzimidazolone, 1-EBIO modulates Cl\textsuperscript{−} secretion via the direct activation of a Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel in T84 and Calu-3 cells (5, 6, 8). Therefore, we assessed the effect of 1-EBIO on hIK1 expressed in Xenopus oocytes using TEVC and excised inside-out patch-clamp techniques. As described above, during TEVC recording, ionomycin (1 µM) increased current from −0.13 ± 0.03 to −2.30 ± 1.12 µA (n = 6), consistent with activation of hIK1. Addition of 1-EBIO (500 µM) further increased current to −9.53 ± 1.77 µA, which was blocked by CTX (50 nM; −2.80 ± 0.93 µA).

In contrast, CZ, 1-EBIO was capable of activating hIK1 during TEVC in the absence of ionomycin, albeit at very high concentrations (≥1 mM) (data not shown).

We next determined the effect of 1-EBIO on hIK1 in excised, inside-out patches. The results of one experiment are shown in Fig. 4A. In the presence of 400 nM free Ca\textsuperscript{2+} there is little channel activity. In agreement with the benzoxazole data, 1-EBIO (100 µM) markedly increased channel activity (NP\textsubscript{o}). In seven experiments, 1-EBIO increased NP\textsubscript{o} from 0.43 ± 0.22 to 7.62 ± 4.35. Similar to our results with CZ and ZOX, 1-EBIO had no effect on i (control = 3.41 ± 0.10 pA, 1-EBIO = 3.46 ± 0.13 pA; n = 7). A complete concentration-response curve for 1-EBIO is shown in Fig. 4B. The data were fitted to the Hill equation with a K\textsubscript{1/2} of 84 µM and a Hill coefficient of 1.8, similar to our results with CZ.

Effect of 1-EBIO on open and closed times of hIK1. Our results demonstrate that the benzimidazolones and benzoxazoles activate hIK1 via an increase in total current flow as opposed to changes in Ca\textsuperscript{2+} affinity or cooperativity. Total current flow is equal to the number of actively gating channels (N), the open probability of the channels (P\textsubscript{o}) and their single-channel current (i). Our results demonstrate that these pharmacological openers have no effect on i, similar to what we previously described for the endogenous channel (5, 6, 35). As channels were recorded in the excised patch-clamp configuration, the most likely explanation is an effect on P\textsubscript{o}. To verify this hypothesis, we determined the effect of 1-EBIO (100 µM) on single hIK1 channels. Addition of 1-EBIO resulted in a significant increase in the P\textsubscript{o} of hIK1 from 0.18 ± 0.03 to 0.65 ± 0.02 (n = 5). A portion of one of these recordings is shown in Fig 4A. In five experiments, 1-EBIO failed to affect the mean open time of the channel (control = 6.6 ± 1.1 ms, 1-EBIO = 7.0 ± 1.2 ms). In contrast, 1-EBIO significantly reduced the mean closed time of hIK1 from 39.5 ± 11.3 to 4.9 ± 0.6 ms.
We next determined which kinetic state of hIK1 1-EBIO affected, thereby resulting in the reduced closed time. This was evaluated by constructing open and closed time event-duration histograms from patches containing a single channel. The results from one recording are shown in Fig. 5. The channel records were minimally fit by two open ($\tau_{o1}$, $\tau_{o2}$) and three closed ($\tau_{c1}$, $\tau_{c2}$, and $\tau_{c3}$) times. As predicted by the mean open time data, 1-EBIO had no effect on $\tau_{o1}$ or $\tau_{o2}$ of hIK1 (Fig. 5, A and D; control, $\tau_{o1}$ = 1.3 ± 0.2 ms, $\tau_{o2}$ = 7.1 ± 1.2 ms; 1-EBIO, $\tau_{o1}$ = 1.1 ± 0.2 ms, $\tau_{o2}$ = 7.3 ± 0.8 ms; n = 5). In addition, 1-EBIO did not affect $\tau_{c1}$ (Fig. 5, B and E; control = 1.6 ± 0.2 ms, 1-EBIO = 1.6 ± 0.3 ms; n = 5). However, $\tau_{c2}$ was significantly reduced from 9.0 ± 1.0 to 5.0 ± 0.6 ms (n = 5, Fig. 5, B and E). Similar to $\tau_{c2}$, 1-EBIO caused a dramatic decrease in $\tau_{c3}$ from 92.6 ± 4.6 to 44.1 ± 7.5 ms (Fig. 5, C and F). The relative amplitudes of the open duration components for the control condition were 13% for $\tau_{o1}$ and 87% for $\tau_{o2}$, whereas with 1-EBIO these were 11% and 89% for $\tau_{o1}$ and $\tau_{o2}$, respectively. Relative amplitudes of the closed-duration components for the control condition were 31%, 50%, and 13% for $\tau_{c1}$, $\tau_{c2}$, and $\tau_{c3}$, respectively. Following addition of 1-EBIO, the relative amplitudes for $\tau_{c1}$, $\tau_{c2}$, and $\tau_{c3}$ were 66%, 31%, and 3%, respectively. For control and experimental conditions, the sum of the events accounted for by the individual time constants represented 94 and 99.5% of the total events, respectively. These values for open and closed times, coupled with their relative amplitudes, predict open probabilities of 0.30 ± 0.09 for control conditions and 0.65 ± 0.02 in the presence of 1-EBIO. These data accurately reflect the $P_o$ obtained in the presence of 1-EBIO (0.65 ± 0.02); however, the $P_o$ observed in control conditions was only 0.18 ± 0.03, suggesting a fourth long closed time which could not be resolved during our recordings (see Discussion). Based on our amplitude analysis, this time constant, which would represent 6% of our events, is predicted to be ~200 ms in duration. In summary, these data demonstrate that 1-EBIO increases the open probability of hIK1 by reducing the duration of the long closed times ($\tau_{c2}$ and $\tau_{c3}$) of the channel, thereby increasing the channel opening rate.

Effect of ATP on the activation of hIK1 by CZ. We recently determined that hIK1, heterologously expressed in Xenopus oocytes, is activated by ATP in excised, inside-out patches (14). This activation was reversed by alkaline phosphatase, suggesting an endogenous kinase was activating hIK1 in excised patches (14). We determined whether, in the presence of both saturating Ca$^{2+}$ (10 µM free Ca$^{2+}$) and ATP (1 mM, based on previous observations; Ref. 15), CZ could further potentiate the activity of hIK1. The effect of CZ (300 µM) on ATP-mediated activation of hIK1 in an inside-out patch is shown in Fig. 6A. ATP (1 mM) produced a large activation of hIK1, which was further activated by CZ, in the continued presence of ATP. The results of six experiments are summarized in Fig. 6B. 1 mM ATP increased $NP_o$ from a control value of 0.57 ± 0.18 to 3.39 ± 0.82. The subsequent addition of CZ further increased $NP_o$ to 5.70 ± 1.21. These data indicate that CZ and ATP activate hIK1 via independent mechanisms.

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1 A single exponential fit to our open time data shown in Fig. 5 yielded time constants ($\tau_o$) of 6.3 and 6.9 ms for control and 1-EBIO, respectively. A statistical analysis of the $\chi^2$ values obtained by fitting the data to a single exponential compared with the $\chi^2$ values obtained using a double exponential fit demonstrated a significantly greater goodness of fit to a double exponential ($P < 0.001$).
Effects of benzoazoles on rSK2 in TEVC experiments. Both CZ and ZOX are utilized clinically as centrally acting muscle relaxants and are known to affect the dopaminergic neurons (12, 28, 36). The neuronal, small-conductance, Ca$^{2+}$-activated K$^+$ channels (SK) possess 42–44% sequence homology to hIK1 (19). Thus we speculated that the benzoazoles may similarly activate SK channels. The effects of CZ, ZOX, and OH-CZ on rSK2 during TEVC experiments are shown in Fig. 7. Addition of ionomycin (1 µM) induced a variable inward current response, consistent with activation of a K$^+$ conductance in our recording conditions (see METHODS).

The further addition of apamin reduced this current to $-2.13 \pm 0.88 \mu$A. CZ and ZOX failed to increase current in the absence of ionomycin (data not shown). These results demonstrate that CZ and ZOX activate hIK1 as well as rSK2.

Effects of benzoazoles on rSK2 in excised membrane patches. The above results suggest that the benzoazoles are direct activators of rSK2. This was evaluated using excised, inside-out patch-clamp recordings. The effect of ZOX on rSK2 in an inside-out patch is shown in Fig. 8A. Under control conditions (400 nM free Ca$^{2+}$ in the bath), the activity of rSK2 was low. Addition of ZOX (100 µM) caused a significant increase in channel activity (NP$_o$), which was readily reversible. In seven patches, ZOX increased NP$_o$ from a control value of $0.58 \pm 0.21$ to $2.05 \pm 0.70$ with no change in single-channel amplitude ($t$; control $= 1.80 \pm 0.07$, ZOX $= 1.84 \pm 0.07$ pA). Similarly, CZ (100 µM) caused a significant increase in NP$_o$ from $5.19 \pm 1.82$ to $12.50 \pm 3.09$ with no change in $i$ (control $= 1.83 \pm 0.06$, CZ $= 1.84 \pm 0.05$ pA; $n = 7$). Consistent with our TEVC experiments, OH-CZ (300 µM) failed to activate rSK2 in excised inside-out patches (NP$_o$; control $= 1.97 \pm 0.97$, OH-CZ $= 2.77 \pm 1.21$; $n = 4$), whereas the subsequent addition of CZ caused an increase in NP$_o$ to $10.39 \pm 4.44$ ($n = 4$). OH-CZ also had no effect on $i$ (control $= 1.63 \pm 0.14$, OH-CZ $= 1.66 \pm 0.10$ pA; $n = 4$). Average concentration-response curves for CZ and ZOX.
on rSK2 are shown in Fig. 8B (bath free Ca\(^{2+}\) = 400 nM). The CZ data were fitted to the Hill equation with a \(K_{1/2}\) of 87 \(\mu\)M and a Hill coefficient of 3.3. The \(K_{1/2}\) value is similar to that obtained for the CZ-dependent activation of hIK1 (Fig. 3A); however, the Hill coefficient is greater for rSK2, indicating a greater level of cooperativity for agonist-dependent gating. Similar to our results on hIK1, a \(K_{1/2}\) value for ZOX on rSK2 was unattainable since \(N_{P0}\) did not saturate with 600 \(\mu\)M ZOX, the maximum soluble concentration. These results confirm our TEVC data and demonstrate that the benzoxazoles directly activate rSK2 in excised, inside-out patches.

Effects of CZ on the Ca\(^{2+}\)-dependent gating of rSK2. Our results demonstrate that CZ activates hIK1 without affecting either the Ca\(^{2+}\) affinity or cooperativity of hIK1 (Fig. 3B). To determine the effect of CZ on the Ca\(^{2+}\)-dependent gating of rSK2 in excised, inside-out patches, complete concentration-response curves for Ca\(^{2+}\) were generated in the absence and presence of CZ (100 \(\mu\)M). Importantly, both Ca\(^{2+}\) concentration-response curves were generated on the same patch. As shown in Fig. 8C, both in the absence and presence of CZ, the Ca\(^{2+}\)-dependent gating of rSK2 was fitted to the Hill equation with an apparent \(K_{1/2}\) of 700 nM and a Hill coefficient of 2.5. Similar to our findings with hIK1, these data suggest that CZ activates rSK2 by increasing \(V_{\text{max}}\) without shifting the apparent affinity of the channel for Ca\(^{2+}\). These data further demonstrate that the half-maximal Ca\(^{2+}\) affinities observed for hIK1 and rSK2 are not distinguishable, although the level of cooperativity appears to be greater for rSK2, in agreement with previous findings (18).

DISCUSSION

Pharmacological modulation of the intermediate conductance, Ca\(^{2+}\)-dependent K\(^+\) channels (IK) may prove therapeutically beneficial in a wide array of diseases. Blockers of this channel have long been proposed as a therapy in sickle cell anemia (1). More recently, clotrimazole, an inhibitor of the IK channel in red blood cells, has been utilized in clinical trials for sickle cell anemia (1) and has been proposed for use in \(\beta\)-thalassemia (2). The IK channel of T-lymphocytes was also recently cloned and shown to be nearly identical to hIK1 (22, 24). This channel is dramatically upregulated following antigenic or mitogenic stimulation, suggesting that it may play a critical role in the immune response (17, 22, 38). This has led to the suggestion that inhibitors of IK...
may be important as immunosuppressives. More recently, it was suggested that openers of IK and SK channels may be therapeutically beneficial in hypertension and peripheral vascular disease (10). Although several high-affinity blockers of IK have been described (19–21), openers of this class of channels have only recently been developed. We defined 1-EBIO as the only known opener of the IK family of channels in airway and colonic epithelia (5, 6). Subsequently, we demonstrated that the structurally related compounds CZ and ZOX also activate IK channels (35). In addition to activating IK channels, we also demonstrated that these compounds activated CFTR (5, 35). This coordinate regulation of both IK and CFTR resulted in a sustained Cl\(^{-}\) secretory response (5, 6, 35). Here, we define the mechanism by which these compounds activate the recently cloned intermediate-conductance Ca\(^{2+}\)-activated K\(^{+}\) channel, hIK1. We have also characterized these compounds as the first known pharmacological openers of the homologous, rSK2. Our findings suggest that rSK2 is modulated via an identical mechanism by these compounds.

Although CZ was ranked among the top 100 most widely prescribed drugs for many years, as a centrally acting muscle relaxant (36), its mechanism of action has remained largely obscure. Both CZ and ZOX were shown to decrease dopamine turnover in substantia nigra neurons, and this appeared to be due to a decrease in neuronal firing rate resulting in a pacemaker-like firing pattern (26). Interestingly, SK channels have been localized to the substantia nigra by in situ hybridization (23). Thus a possible mechanism of action of these muscle relaxants is the activation of neuronal SK channels, resulting in a hyperpolarization of the membrane potential and hence decreased firing rate. Supporting this hypothesis is the observation that OH-CZ had no muscle relaxant activity (36); OH-CZ failed to activate either hIK1 or rSK2 (Figs. 1 and 7). Importantly, the peak plasma concentrations achieved during oral administration of CZ in patients (100–200 µM; Refs. 12 and 36) is \(\approx \) the \(K_{1/2}\) we obtain for CZ on both hIK1 and rSK2 (80–90 µM), indicating these channels can serve as pharmacological targets. However, our results do not allow us to rule out the
possibility that these compounds affect alternate ion channels resulting in their known biological effect.

Pharmacological modulation of hIK1 and rSK2 by benzimidazolones and benzoxazoles. Recently, the benzimidazolones NS-1619 and NS-004 were shown to activate maxi-K channels in a variety of tissues (30, 31, 34). This activation of BK channels by NS-004 and NS-1619 required the presence of cytoplasmic Ca\(^{2+}\), i.e., the benzimidazolones could not simply substitute for Ca\(^{2+}\) as an activator of the channel (27, 30, 31). We similarly demonstrated that the 1-EBIO- and CZ-dependent activation of endogenous IK channels required the presence of a slightly elevated Ca\(^{2+}\) concentration (6, 35). Here, we confirm these results for CZ on hIK1 and rSK2 heterologously expressed in Xenopus oocytes (Figs. 3 and 8). However, the interaction of benzimidazolones and benzoxazoles with Ca\(^{2+}\) appears to be distinct between the BK and IK channels. The benzimidazolones induce a parallel shift in the current (P_{o})-voltage relationship for activation of BK channels with no apparent shift in V_{max}; analogous to the activation of BK by Ca\(^{2+}\) (27, 30, 31). However, to our knowledge, a complete characterization of the interaction among Ca\(^{2+}\), benzimidazolones, and P_{o} has not been undertaken for the BK channels. We demonstrate that CZ activates both hIK1 and rSK2 via an increase in P_{o} with no change in the Ca\(^{2+}\) dependence of gating, i.e., both Ca\(^{2+}\) affinity and cooperativity were unchanged (Figs. 3 and 8). Thus the activation of hIK1 and rSK2 by benzimidazolones and benzoxazoles appears to be distinct from the mechanism described previously for BK channels.

Recently, others have shown that the Ca\(^{2+}\)-binding protein, calmodulin (CAM) exists tightly bound to the COOH-terminal tail of both hIK1 and rSK2 in a Ca\(^{2+}\)-independent manner and acts as a common mechanism for Ca\(^{2+}\)-dependent gating of these channels (13, 39). Ca\(^{2+}\)-CAM binding initiates a conformational change in the \(\alpha\)-subunit of the channel proteins, resulting in gating (39). In addition, recent evidence from comparison of native IK channels and heterologously expressed hIK1 (hSK4) suggests that accessory molecules such as CAM-kinase (22) or protein kinaseA (14) may be involved in channel regulation. These findings
suggest that the Ca\(^{2+}\)-dependent activation of hIK1 and rSK2 by 1-EBIO, CZ, and ZOX may result from an indirect effect via interaction of these compounds with CAM or other undescribed accessory molecules rather than direct binding to the ion-conducting α-subunit of the channel proteins.

Our results demonstrate that pharmacological openers of hIK1 function by increasing NP\(_o\) in excised, inside-out patches. To further define the mechanism of action of these compounds, a kinetic analysis was undertaken. During control recordings (400 nM Ca\(^{2+}\)), our data demonstrate that hIK1 is minimally described by two open (O\(_1\) and O\(_2\)) and three closed (C\(_1\), C\(_2\), and C\(_3\)) states. This is consistent with previous reports on hIK1 endogenously expressed in human erythrocytes (9, 33). We demonstrate that 1-EBIO has no effect on the open time of the channel but causes a dramatic decrease in the mean closed time. This effect on the open time of the channel could not be attributed to an affect on the fast closed state (C\(_1\)). Rather, 1-EBIO decreased both long closed times (t\(_{c2}\) and t\(_{c3}\)) of hIK1, thereby increasing the opening rate of the channel and hence P\(_o\). From our individual channel records, we were able to calculate the P\(_o\) predicted from the relative number of events accounted for by our fitted time constants. For 1-EBIO, we predicted a P\(_o\) of 0.65, which accurately reflected the observed P\(_o\) of 0.65. However, for the control condition, our predicted P\(_o\) of 0.30 was substantially higher than the P\(_o\) of 0.18 actually recorded. These data suggest that an additional long-lived closed state exists which contributes to channel gating. In fact, 6% of the closed-duration events from the control record could not be accounted for by the three closed time constants. To obtain the actual P\(_o\) of 0.18, this time constant, t\(_{cd}\), would be predicted to be \(~200\) ms.

Our results suggest that 1-EBIO and the benzoxazoles activate both hIK1 and rSK2 via a similar mechanism, i.e., an increase in total current flow (P\(_o\)) with no effect on Ca\(^{2+}\)-dependent gating. However, a detailed kinetic analysis has not been undertaken to determine whether CZ similarly affects the opening rate of rSK2. Recently, the Ca\(^{2+}\)-dependent gating of both rSK2 (39) and hIK1 (13) have been shown to be due to a direct interaction of the channel with calmodulin, suggesting these two channels may be described by similar kinetic schemes. rSK2 was recently demonstrated to have six kinetic states, two open and four closed (18), consistent with our observations. In contrast to our results on hIK1, the benzimidazolones NS-004 and NS-1619 have been shown to increase the P\(_o\) of BK via both an increase in the opening rate as well as a decrease in the closing rate of the channel (30, 31).

We recently demonstrated that ATP activates hIK1 in excised, inside-out patches and that both alkaline phosphatase and the protein kinase A pseudosubstrate, PK1-(5–24), could reverse this effect (14). These results suggest that hIK1 can be activated by an endogenous kinase that associates with the excised patch. These results are similar to what has recently been reported for endogenous hIK1 expressed in red blood cells (33).

Indeed, this ATP-dependent activation produced identical effects on the Ca\(^{2+}\)-dependent gating of hIK1, an increase in P\(_o\) with no change in either Ca\(^{2+}\) affinity or cooperativity. Thus we determined whether the effects of ATP and CZ were additive, suggesting divergent mechanisms of action, or mutually exclusive, suggesting similar mechanisms of action. In the presence of both saturating levels of Ca\(^{2+}\) (10 µM) and ATP (1 mM, based on previous observations; Refs. 14 and 15), the activity of hIK1 was potentiated by CZ in excised patches. These data suggest that CZ acts via a mechanism that is independent of ATP-mediated phosphorylation of the channel.

In conclusion, we have defined the mechanism of action for the pharmacological activation of the recently cloned intermediate-conductance (hIK1) and small-conductance (rSK2) Ca\(^{2+}\)-activated K\(^{+}\) channels by the benzimidazolone, 1-EBIO, and the benzoxazoles, CZ and ZOX. We demonstrate these openers act by increasing channel open probability via a decrease in the mean duration of at least two long-lived closed states of hIK1 without affecting open-channel kinetics. These effects are independent of both ATP-mediated phosphorylation and the Ca\(^{2+}\)-dependent gating (K\(_o\), Hill coefficient) of hIK1. Thus these compounds represent the first defined openers of the IK and SK family of channels and as such represent lead compounds upon which higher affinity agonists may be defined. It is expected that the development of compounds that activate hIK1 with greater specificity and affinity will be useful in defining these channels as potential therapeutic targets in diseases such as cystic fibrosis and hypertension.

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