Chlorpromazine binding to the PAS domains uncovers the effect of ligand modulation on EAG channel activity

Ze-Jun Wang‡, Stephanie M. Soohoo‡, Purushottam B. Tiwari§, Grzegorz Piszczek§, and Tinatin I. Breidze‡*†

From the ‡Departments of Pharmacology and Physiology, Georgetown University Medical Center, Washington, D.C., 20057, the §Department of Oncology, Georgetown University Medical Center, Washington, D.C., 20057, and the ¶Biophysics Core, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Ether-a-go-go (EAG) potassium selective channels are major regulators of neuronal excitability and cancer progression. EAG channels contain a Per—Arnt—Sim (PAS) domain in their intracellular N-terminal region. The PAS domain is structurally similar to the PAS domains in non-ion channel proteins, where these domains frequently function as ligand-binding domains. Despite the structural similarity, it is not known whether the PAS domain can regulate EAG channel function via ligand binding. Here, using surface plasmon resonance, tryptophan fluorescence, and analysis of EAG currents recorded in Xenopus laevis oocytes, we show that a small molecule chlorpromazine (CH), widely used as an antipsychotic medication, binds to the isolated PAS domain of EAG channels and inhibits currents from these channels. Mutant EAG channels that lack the PAS domain show significantly lower inhibition by CH, suggesting that CH affects currents from EAG channels directly through the binding to the PAS domain. Our study lends support to the hypothesis that there are previously unaccounted steps in EAG channel gating that could be activated by ligand binding to the PAS domain. This has broad implications for understanding gating mechanisms of EAG and related ERG and ELK K+ channels and places the PAS domain as a new target for drug discovery in EAG and related channels. Up-regulation of EAG channel activity is linked to cancer and neurological disorders. Our study raises the possibility of repurposing the antipsychotic drug chlorpromazine for treatment of neurological disorders and cancer.

This work was supported by the NIGMS, National Institutes of Health Grant R01GM124020 (to T. I. B.). The Biacore Molecular Interaction Shared Resource is supported by National Institutes of Health Grants P30CA10081 and S10OD019982-01. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence should be addressed: Dept. of Pharmacology and Physiology, Georgetown University Medical Center, Washington, D.C. 20057. Tel.: 202-687-6178; E-mail: tib5@georgetown.edu.

2 The abbreviations used are: EAG, ether-a-go-go; PAS, Per—Arnt—Sim; SPR, surface plasmon resonance; ERG, EAG-related gene; HERG, human ERG; VS, voltage sensor; CNBH, cyclic nucleotide-binding homology; CNG, cyclic nucleotide-gated; HCN, hyperpolarization-activated cyclic nucleotide-gated; mEAG, mouse EAG; NTA, nitriiotsiracetic acid; TEVC, two-electrode voltage-clamp.
In this study, we used surface plasmon resonance (SPR) and tryptophan fluorescence to show that a small molecule chlorpromazine hydrochloride (CH) binds to the PAS domain of EAG channels. CH inhibited currents recorded from EAG channels in a concentration-dependent manner. Deletion of the PAS domain significantly decreased the apparent affinity and potency of CH inhibition. These results suggest that the PAS domain can regulate EAG channel function by binding small molecule ligands. Importantly, our findings indicate that CH, which is a widely used antipsychotic drug, can be repurposed for treatment of cancer and neurological disorders associated with increased EAG channel activity.

Results

Chlorpromazine binds to the PAS domain of EAG channels in a concentration-dependent manner

In a recent study, we screened small molecules in the Spectrum Library of chemical compounds for binding to the intracellular PAS and CNBH domains of KCNH channels using the SPR method (27). From this screen, CH (structure shown in Fig. 1b) emerged as a potential EAG PAS domain small molecule binder. To further investigate the concentration dependence and specificity of CH binding, we immobilized the isolated PAS and CNBH domains of mouse EAG (mEAG) and human ERG (hERG) channels on a NTA sensor chip (Fig. 1c) and determined the SPR
response over a range of CH concentrations for the four target proteins. Application of CH to the PAS domain of mEAG channels increased the SPR response in a concentration-dependent manner (Fig. 1, d and e) via direct binding to the immobilized proteins. It is noteworthy that the PAS domains of mEAG and human EAG1 channels are 99% identical. Therefore, we expect our findings to hold for human EAG channels as well. No substantial direct binding was observed for the PAS domain of hERG channels or for the CNBH domains of mEAG and hERG channels (Fig. 1, f–h). The PAS and CNBH domains have different structural folds (Fig. 1a) (14–16). Therefore, CH binding is PAS domain structure-specific. At concentrations of >30 μM, CH displayed a nonspecific binding to the NTA chip, resulting in a higher SPR response for the control surface than for the surfaces with immobilized proteins. Nonspecific bindings of analytes to the control surface of the Ni²⁺-NTA sensor chip can limit the applicability of the SPR-based experiments (28). Because of this limitation at higher CH concentrations, we were unable to obtain a full dose-response and determine the CH binding affinity for the isolated mEAG PAS domain with SPR.

To further examine CH binding with a method that does not require PAS domain immobilization, we used tryptophan fluorescence as a reporter of CH binding. Binding of a ligand in the vicinity of a Trp residue could change tryptophan fluorescence because of its environmental sensitivity (21, 29, 30). The PAS domain of mEAG channels contains two endogenous Trp residues (Fig. 2a). Therefore, we tested whether CH binding to the PAS domain can be detected by changes in the fluorescence of the Trp residues. Upon excitation at 290 nm, a commonly used wavelength for Trp excitation, the emission spectra of the mEAG PAS domain displayed a strong fluorescence signal with a peak at 343 nm. Application of CH decreased the peak fluorescence intensity in a concentration-dependent manner (Fig. 2b). To test whether the changes in the fluorescence signal are specific to the PAS domain protein rather than a nonspecific effect such as an inner filter effect, we acquired the emission spectra of free tryptophan in solution. Unlike for the PAS domain, for free tryptophan application of CH had no concentration-dependent effect on the fluorescence signal (Fig. 2c). Therefore, the observed decrease in the fluorescence intensity of the mEAG PAS domain is due to the changes in the environment of Trp residues caused by a specific binding of CH to the PAS domain. To determine CH-binding affinity, plots of the changes in the peak fluorescence intensity versus total CH concentration were fitted with Equation 2. This analysis revealed the binding affinity of 1 ± 0.7 μM for CH (Fig. 2d). Taken together, the results of the SPR and fluorescence-based experiments indicate that CH is a small molecule binder of the PAS domain of mEAG channels that binds in a concentration-dependent and structure-specific manner.

CH inhibits currents from EAG channels in a concentration-dependent manner without affecting kinetics of deactivation

To determine the functional effect of CH on EAG channels, full-length mEAG channels were expressed in Xenopus laevis oocytes, and currents from mEAG channels were recorded in the absence and presence of 50 μM CH (Fig. 3a). The inside-out con-
The configuration of the patch-clamp technique was used for the current recordings so that the ligand can be directly applied to the intracellular side of the channels containing the PAS domains. CH dramatically inhibited steady-state and tail currents from mEAG channels \((\text{Fig. 3a})\). The inhibition was concentration-dependent with an \(I_{50}\) of \(3.7 \pm 0.7 \, \mu M\), and Hill coefficient \((n)\) of 1. \(\text{c}\), a representative tail current recorded at \(-100 \, \text{mV}\) after a voltage step to \(+70 \, \text{mV}\) in the absence (black) and presence (red) of \(10 \, \mu M\) CH. The gray lines represent fits of the tail currents with a single exponential function with the time constant of deactivation of 3.3 ms in the absence and 4.1 ms in the presence of CH. \(\text{d}\), plots of the averaged deactivation time constants for tail currents recorded at \(-100 \, \text{mV}\) after a voltage step to \(+70 \, \text{mV}\) versus CH concentration. \(n = 4\) for each condition.

**Figure 3. Concentration dependence of EAG channel current inhibition by CH.** \(\text{a}\), representative mEAG current traces recorded in the inside-out configuration in the absence (black) and presence (red) of \(50 \, \mu M\) CH. \(\text{b}\), plots of the percentage of inhibition of tail currents versus the CH concentration. Tail currents were recorded at \(-100 \, \text{mV}\) following a voltage step to \(+70 \, \text{mV}\). The lines represent fits of the data with the Hill equation with the \(I_{50}\) of \(3.7 \pm 0.7 \, \mu M\), and Hill coefficient \((n)\) of 1. \(\text{c}\), a representative tail current recorded at \(-100 \, \text{mV}\) after a voltage step to \(+70 \, \text{mV}\) in the absence (black) and presence (red) of \(10 \, \mu M\) CH. The gray lines represent fits of the tail currents with a single exponential function with the time constant of deactivation of 3.3 ms in the absence and 4.1 ms in the presence of CH. \(\text{d}\), plots of the averaged deactivation time constants for tail currents recorded at \(-100 \, \text{mV}\) after a voltage step to \(+70 \, \text{mV}\) versus CH concentration. \(n = 4\) for each condition.

The effect of CH on the tail–current deactivation kinetics was not statistically significant (Fig. 3c and d). The averaged deactivation time constant was \(3.6 \pm 0.3\) ms in the absence and \(4.2 \pm 0.1\) ms in the presence of \(10 \, \mu M\) CH for tail currents recorded following a \(+30\)-mV test pulse and \(3.5 \pm 0.3\) ms in the absence and \(4.7 \pm 0.2\) ms in the presence of \(10 \, \mu M\) CH for tail currents recorded following a \(+70\)-mV test pulse \((p = 0.5\) for both \(+30\) mV and \(+70\) mV by Student’s \(t\) test). The tail currents recorded in the presence of \(50 \, \mu M\) CH were too small to be included in the analysis of the deactivation kinetics. The lack of CH effect on the kinetics of tail currents suggests that CH does not affect the return of the VS domain to the resting state.

**Voltage dependence of CH inhibition of currents from EAG channels**

In the absence of CH, EAG channels activated with voltage with the \(V_{1/2}\) of \(-35.8\) mV. Application of CH decreased the channel conductance and shifted the conductance versus voltage plots to more depolarized potentials (Fig. 4a). Importantly, current inhibition by CH was mostly voltage-independent (Fig. 4b). For instance, for tail currents recorded in the presence of \(10 \, \mu M\) CH, the inhibition was \(45.3 \pm 7.2\%\) following a \(0\)-mV test.
Mechanism of EAG channel inhibition by chlorpromazine

Figure 4. Voltage dependence of EAG current inhibition by CH. a, plots of the averaged normalized tail currents versus test voltage obtained in the absence (black symbols) and presence (red symbols) of 10 μM CH. The line represents fit with Equation 3, with $V_{1/2}$ of −35.8 ± 1.2 mV and s of 15.6 ± 1.1. b, plots of the averaged percentage of current inhibition versus voltage for tail-currents in the presence of 10 μM CH. n ≥ 4 for each condition.

pulse and 59.6 ± 3.8% following a +70-mV test pulse, which are not statistically significantly different values ($p = 0.13$ by Student’s $t$ test). The voltage independence of the inhibition suggests that the EAG current inhibition by CH is caused by changes in the gating mechanism rather than a pore block of EAG channels.

Deletion of the PAS domain significantly decreases CH inhibition of EAG currents

To determine whether binding of CH to the PAS domain is directly involved in the inhibition of CH currents, we examined the effect of CH on a mutant mEAG channel with a deletion of the PAS domain (ΔPAS). In our hands, deletion of the PAS domain substantially decreased the surface expression of mEAG channels, making it difficult to study the inhibition by recording currents from excised inside-out patches. To circumvent this issue, we recorded currents from the WT and ΔPAS mutant mEAG channels using two-electrode voltage-clamp (TEVC) technique. Taking advantage of the membrane permeability of CH, we then studied the effect of CH on mEAG channel currents over a range of CH concentrations applied to the bath solution. MEG tail currents recorded with TEVC deactivated too fast to be used for a tail current–based channel gating analysis (Fig. 5a), consistent with the previous report (31). Therefore, only steady-state currents were analyzed for experiments carried out using TEVC.

Consistent with the results for tail currents obtained using excised inside-out configuration of the patch-clamp technique, steady-state currents from WT mEAG channels recorded with TEVC were also inhibited by CH (Fig. 5, a and c). Notably, the IC_{50} of the CH inhibition for WT channels was ~10-fold higher than the IC_{50} for the excised inside-out patches. Most likely this difference reflects the decrease in the effective concentration of CH caused by the oocyte volume and various intracellular molecules that could bind CH, decreasing the effective concentration available to affect EAG channels.

Similar to the results for excised patches, CH shifted the conductance versus voltage plots to more depolarized potentials and decreased the channel conductance, which was largely voltage-independent from 0 to +50 mV. At voltages higher than +50 mV, both in the absence and in the presence of CH, EAG channel conductance started to decrease (Fig. 5d). Most likely this reflects a voltage-dependent block by various potential channel pore-blocking molecules present in the oocytes. This voltage-dependent decrease was absent for currents recorded in the excised inside-out patch-clamp configuration, where the intracellular and extracellular solutions were devoid of impurities (Fig. 4a).

Deletion of the PAS domain uncovered an inactivation-like behavior at high voltages not seen in WT channels and shifted the current–voltage relationship to more depolarized potentials (Fig. 5, b, d, and e), as reported previously (32, 33). Importantly, deletion of the PAS domain significantly decreased CH inhibition of mEAG currents (Fig. 5, b and c) and eliminated the shift of the conductance versus voltage plots to more depolarized potentials (Fig. 5e). The IC_{50} of CH inhibition was 29.7 ± 0.7 μM for WT and 53.6 ± 8.2 μM for ΔPAS mEAG channels. In addition, the magnitude (potency) of the current inhibition significantly decreased in the absence of the PAS domain. Although the averaged current inhibition observed at 300 μM CH was 63.3 ± 4.9% for WT channels, it decreased to 25.1 ± 2.9% for ΔPAS channels. Moreover, the residual inhibition of ΔPAS channels that is apparent only at voltages higher than +20 mV (Fig. 5, e and f) could be due to the voltage-dependent pore block caused by various intracellular molecules present in oocytes, as suggested by the results in Fig. 5d. Overall, the current inhibition by CH was very weakly voltage-dependent (Fig. 5f), consistent with the results obtained for currents recorded from excised inside-out membrane patches (Fig. 4b). The voltage dependence of the current inhibition was the same for the WT and ΔPAS channels, suggesting that the mutation did not cause drastic changes in the pore energetics and CH pore accessibility. Taken together, the results of the TEVC experiments demonstrate that the PAS domain deletion significantly decreased the effect of CH on EAG currents, strongly suggesting that the PAS domain is directly involved in the CH regulation of EAG currents.

Discussion

Using a combination of SPR and tryptophan fluorescence, we show that a small molecule CH directly binds to the PAS domain of EAG channels. When applied to the excised inside-
out patches of membranes expressing EAG channels, CH inhibits currents from EAG channels. Deletion of the PAS domain substantially decreases the potency and apparent affinity of CH inhibition. These results strongly suggest that CH binding to the PAS domain modulates EAG channel currents. To our knowledge, this is the first indication of the PAS domain function as a ligand-binding domain in EAG channels and its capacity to modulate EAG channel currents via the ligand binding. CH is clinically used as an antipsychotic medication. Therefore, our study demonstrates a potential of repurposing CH for treatment of cancer and neurological disorders associated with increased EAG channel activity.

PAS domains of EAG and other KCNH channels contain a cavity formed by the β-strands and flanking α-helices (Fig. 2a, gray mesh). It is tempting to speculate that the cavity might provide the CH-binding site. However, a structural analysis of the PAS domains suggested that the cavity lacks polar residues necessary for supporting small molecule binding (24, 34). Structural studies are necessary to determine whether CH binds inside or outside of the PAS domain cavity.
CH directly inhibited currents from EAG channels in a concentration-dependent and weakly voltage-dependent manner. CH inhibition of EAG channels could result from the effect of CH on EAG channel gating via the PAS domain or a voltage-dependent channel pore block. We feel that our data favor the former mechanism. A voltage-dependent pore block would decrease channel conductance as membrane voltage increases. However, our results indicate that for currents recorded from excised inside-out patches, in the presence of CH, EAG channel conductance does not decrease with increases in membrane voltage (Fig. 4a). In fact, for voltages of −30 mV and higher, EAG channel conducance is essentially constant and voltage-independent in the presence of CH. Additional evidence supporting the effect of CH through the binding to the PAS domain comes from experiments on the mutant EAG channels with the deletion of the PAS domain (ΔPAS). The deletion of the PAS domain essentially removed CH inhibition of EAG channels at voltages of <30 mV (Fig. 5e) and significantly decreased the effect of CH on EAG currents at voltages of ≥30 mV (Fig. 5c). We think that the residual CH inhibition of EAG currents at voltages of >30 mV is due to a voltage-dependent pore block by various small molecules, other than CH, present intracellularly in oocytes. This explanation is further strengthened by the voltage-dependent decline of currents from WT EAG channels detected at high voltages in the absence of CH (Fig. 5d). Although it is possible that the ΔPAS mutation could directly or allosterically affect the energetics of the pore, similar voltage dependence of the CH inhibition for the WT and ΔPAS channels suggests that drastic changes to the pore energetics are unlikely. It is noteworthy that CH also inhibits hERG channels in a voltage-dependent manner with the current inhibition increasing with membrane depolarization (35). hERG channels are notorious for being susceptible to inhibition by various compounds (36). Unlike for hERG channels, very few inhibitors (especially clinically relevant ones) have been identified for EAG channels. Our SPR-based results indicate that CH does not bind to the PAS domain of hERG channels (Fig. 1f), suggesting that the proposed PAS domain-dependent CH inhibition is unique to EAG channels.

How can CH binding to the PAS domain inhibit EAG currents? In KCNH channels, PAS domains form intersubunit interactions with the CNBH domains, where a PAS domain from one subunit interacts with the CNBH domain from the adjacent subunit (Fig. 1a) (15, 37–39). In EAG and ERG channels, the PAS/CNBH domain interaction favors the open state of the channel (31, 40) (Fig. 6, left half). CH binding to the PAS domain, either directly or allosterically, could weaken the PAS/CNBH domain interaction, causing decreases in EAG channel currents (Fig. 6, right half). In KCNH channels the PAS domain also forms interactions with the VS domain (Figs. 1a and 6, left half) (15). The PAS/VS domain interactions are functionally important because deletion of the PAS domain, either directly or allosterically, shifts the current–voltage relationship of EAG channels to more depolarized potentials (Ref. 33 and Fig. 5, d and e). Therefore, CH binding, either directly or allosterically, could change the PAS/VS domain interactions, causing changes in the pore energetics (Fig. 6, right half). Future studies are necessary to test the possible pathways of CH action in EAG channels.

Inhibition of the EAG channel activity using RNAi and EAG channel-specific antibody blockers decreases tumor growth (12, 13). It is noteworthy that all known and functionally examined mutations in EAG channels associated with epilepsy and Zimmerman–Laband and Temple–Baraitser syndromes are gain-of-function mutations that increase EAG channel activity (3, 4, 41). Therefore, EAG channel inhibitors have high therapeutic potential for treatment of both cancer and neurological disorders. CH is an Food and Drug Administration–approved drug that has been extensively used as an antipsychotic medication (42). Our study presents a strong rational for repurposing CH for treatment of cancer and neurological disorders.

In summary, we show that CH is a novel ligand of EAG channels. To our knowledge, our study presents the first evidence that a ligand can bind to the PAS domain and regulates EAG channel activity via this binding. Therefore, our study sets a precedent of KCNH channel regulation via ligand binding to the PAS domain and indicates that this mechanism should be taken into consideration for future studies of KCNH channel gating and drug discovery efforts targeting KCNH channels.
Mechanism of EAG channel inhibition by chlorpromazine

Experimental procedures

Protein purification

PAS and CNBH domains of mEAG1 and hERG1 channels were purified with Ni²⁺-NTA and size-exclusion chromatography, as previously described (21, 22, 27). The start and end of the amino acid sequence of the domains used in this study and their gene identifier numbers are indicated in the Table S1.

Briefly, the genes encoding the domains were subcloned into pETM11 bacterial expression vector containing an N-terminal His6 affinity tag and expressed in BL21 (DE3) cells. Protein expression in BL21 (DE3) cells was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside at 18 °C. The cells were harvested by centrifugation and resuspended in buffer A (150 mM KCl, 10% glycerol, 1 mM tris(2-carboxyethyl)phosphine, 30 mM HEPES, pH 7.5) supplemented with 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride and 2.5 mg/ml DNase I. The cells were lysed with an Emulsiflex C-5 homogenizer (Avestin), and insoluble protein was separated by centrifugation at 30,000 rpm for 1 h at 4 °C in a Beckman 45 Ti rotor. The supernatant was loaded onto His-Trap HP column (GE Healthcare). The column was washed with buffer A, and the proteins were eluted with buffer A with added 500 mM imidazole. The proteins were further purified with size-exclusion chromatography on a Superdex 200 Increase column (GE Healthcare) equilibrated with buffer A. The molecular weight of the purified proteins was verified with Coomassie Blue–stained gels and with MS (electrospray) at Georgetown Proteomics and Metabolomics Core Facility. The purified proteins were stored at −80 °C in small aliquots before use.

Protein immobilization for SPR

A Biacore 4000 SPR instrument was used in all SPR experiments. The purified PAS and CNBH domains of mEAG and hERG channels were immobilized on a NTA chip (GE Healthcare), as previously described (27, 43). Immobilizations of the proteins were performed in HBS-P buffer (150 mM NaCl, 10 mM HEPES, 0.05% (v/v) surfactant P20, pH 7.4). The NTA sensor surface was first activated with a 1-min injection of 0.5 mM NiCl₂. The coupling of the Ni²⁺-NTA chip surface groups with the His6-tagged proteins was then achieved by 2.5-min injections of the proteins at 10–200 nM concentrations. After the initial coupling, the proteins were covalently cross-linked via 20-s injections of NHS-EDC carbodiimide solution. The coupling of the proteins to the chip surface was confirmed by the absence of CH concentration-dependent changes in the free tryptophan fluorescence, the inner filter effect was negligible in our experiments. Therefore, no inner-filter correction was performed.

To estimate the binding affinity (K_d), plots of the change in the peak fluorescence intensities versus total CH concentration for the mEAG PAS domain were analyzed using the equations below, as previously described (21, 29),

\[
\Delta F = RL^*x
\]

(Eq. 2)

where RL is the concentration of the free receptor-ligand complex, R and L, are total receptor and ligand concentration, and x is a scaling factor.

Fluorescence measurements

Fluorescence intensity was recorded in a quartz cuvette with a 100-µl chamber using a PTI QuantaMaster spectrofluorometer (Horiba Jobin Yvon) and Felix GX 4.9 software. The sample was excited at 290 nm, and the emission spectra were recorded from 300 to 450 nm for 5 µM mEAG PAS and 5 µM free tryptophan in the absence and presence of various concentrations of CH, and also for CH alone over the range of the examined CH concentrations. In subsequent analysis, the fluorescence intensities for mEAG PAS and free tryptophan samples were background subtracted. For the background subtraction, the fluorescence intensity of CH at a given concentration was subtracted from the fluorescence intensity of the sample with the same CH concentration. Observed emission intensity could be smaller than expected because of the optical density of the sample—the so-called inner filter effect. However, as indicated by the absence of CH concentration-dependent changes in the free tryptophan fluorescence, the inner filter effect was negligible in our experiments. Therefore, no inner-filter correction was performed.

To estimate the binding affinity (K_d), plots of the change in the peak fluorescence intensities versus total CH concentration for the mEAG PAS domain were analyzed using the equations below, as previously described (21, 29),

\[
RL = \frac{1}{2}(R + L + K_d) - \frac{1}{4}(R - L - K_d)^2 - R^*L^*
\]

(Eq. 1)

where RL is the concentration of the free receptor-ligand complex, R and L, are total receptor and ligand concentration, \( \Delta F \) is the peak fluorescence change, and x is a scaling factor.

The data analysis and fitting of the plots was performed in Origin (Microcal Software, Inc.). Each of the experiments was repeated at least three times. The error bars on the figures correspond to the S.E.

Electrophysiology

The cDNA encoding mEAG1 channels in pGH19 vector was kindly provided by G. Robertson (University of Wisconsin-Madison, Madison, WI). The mutant mEAG1 channel with the PAS domain deletion (Δ2–173) in pGH19 was generated by Bio Basic Inc. (Canada) and verified by sequencing (Genewiz). The cRNA was transcribed using the T7 mMessage mMachine kit (Thermo Fisher Scientific). Defolliculated *X. laevis* oocytes were purchased from Ecoway Biosciences (Austin, TX) and injected with the cRNA using a Nanoinject II oocyte injector (Drummond).

For current recordings in the inside-out patch configuration (44), following a manual removal of the vitelline membrane oocytes were transferred to a handmade chamber containing bath solution for current recording. Currents were recorded with Axopatch 200A patch-clamp amplifier (Molecular Devices) and pClamp10 software (Molecular Devices). The signals were digitized using Digidata 1550 (Molecular Devices). Patch pipettes were pulled from borosilicate glass and had resistances of 0.5–1.2 MΩ after fire polishing. The intracellular (bath) and extracellular (pipette) solutions contained 130 mM KCl, 10 mM HEPES, 0.2 mM EDTA, pH 7.2.
Mechanism of EAG channel inhibition by chlorpromazine

The EAG currents were elicited by applying a series of 0.1-s voltage pulses (ranging from -100 to +70 mV in 10-mV increments) from a holding potential of -80 mV, followed by a 0.15-s voltage pulse to -100 mV. The currents were not leak-subtracted.

To analyze voltage dependence of the tail currents, peak tail-current amplitudes recorded in the absence or presence of CH were normalized to the largest peak tail-current amplitude recorded in the absence of CH for the given membrane patch ($G_{\text{max}}$). These normalized data were then plotted against the test voltage and were fit with a Boltzmann equation,

$$\frac{G}{G_{\text{max}}} = \frac{1}{1 + e^{\left(\frac{V_s - V}{s}\right)}}$$

(Eq. 3)

where $V$ represents the test voltage (mV), $V_s$ is the midpoint activation voltage (mV), and $s$ is the slope of the relation (mV). To determine the deactivation time constant, tail currents were recorded with OC-725C amplifier (Warner Instruments) and fit with a single-exponential function.

To determinethe deactivation time constant, tail currents were recorded with TEVC, the conductance ($G$) was calculated as $G = I_{\text{ss}}/(V - V_{\text{rev}})$, where $I_{\text{ss}}$ is the steady-state current recorded at the end of the 0.1-s voltage pulses, $V$ is the test voltage, and $V_{\text{rev}}$ is the membrane reversal potential for $K^+$ selective channels. For our experiments $V_{\text{rev}}$ was -83.9 mV, calculated based on the bath concentration of $K^+$ of 4 mM and the intracellular $K^+$ concentration of 109.5 mM (45). The conductance in the absence or presence of CH was normalized to the largest conductance in the absence of CH for the given oocyte ($G_{\text{max}}$). The normalized conductance was then plotted against the test voltage, and the plots were fit to the Boltzmann equation (Equation 3).

CH was purchased from Alfa Aesar. CH stock was prepared in high purity water and then diluted with the bath solution to obtain the range of concentrations used for the dose-response experiments. The bath solution was changed using a gravity-fed solution changer for both patch-clamp and TEVC experiments. To determine the $IC_{50}$, the concentration of the compound at half-maximal current inhibition, for both excised patch-clamp and TEVC experiments, the plots of the percentage of current inhibition versus the concentration of CH were fitted with a Hill equation,

$$Y[x] = Y_o + \frac{(Y_{\infty} + Y_o)}{1 + (IC_{50}/x)^n}$$

(Eq. 4)

where $Y_0$ represents the minimum percentage of inhibition, $Y_{\infty}$ is the maximum percentage of inhibition, and $n$ is the Hill coefficient.

The data analysis and fitting of the plots were performed in Clampfit (Molecular Devices) and Origin (Microcal Software, Inc.). The error bars on the figures correspond to the S.E. Statistical analysis was performed using Student’s $t$ tests. $p$ values $< 0.05$ were considered significant. $n$ represents the number of recordings from different oocytes.

Author contributions—T. I. B. conceptualization; Z.-J. W., S. M. S., P. B. T., G. P., and T. I. B. data curation; Z.-J. W., S. M. S., P. B. T., and T. I. B. formal analysis; Z.-J. W., G. P., and T. I. B. supervision; T. I. B. funding acquisition; Z.-J. W., S. M. S., P. B. T., G. P., and T. I. B. validation; Z.-J. W., S. M. S., P. B. T., G. P., and T. I. B. visualization; T. I. B. writing-original draft; T. I. B. project administration; Z.-J. W., S. M. S., P. B. T., G. P., and T. I. B. writing-review and editing.

Acknowledgments—We thank Dr. William N. Zagotta for comments and discussion of an earlier version of the manuscript. We thank the Biocore Molecular Interaction Shared Resource at the Lombardi Comprehensive Cancer Center at Georgetown University.

References

1. Veeramah, K. R., Johnstone, L., Karafet, T. M., Wolf, D., Spriessler, R., Salogiannis, J., Barth-Maron, A., Greenberg, M. E., Stuhlmann, T., Weinstein, S., Jungens, T., Pazi, M., Ristivo, L. L., Tabbar, D., Erickson, R. P., et al. (2013) Exome sequencing reveals new causal mutations in children with epileptic encephalopathies. *Epilepsia* 54, 1270–1281 CrossRefMedline

2. Mastrangelo, M., Scheffer, I. E., Bramsng, N. C., Nair, L. D., Myers, C. T., Dentici, M. L., Korenke, G. C., Schoo, K., Campeau, P. M., White, S. M., Shashi, V., Kansagra, S., Van Essen, A. J., and Leuzzi, V. (2016) Epilepsy in KCNHI-related syndromes. *Epileptic Disorders* 18, 123–136 Medline

3. Kortium, F., Caputo, V., Bau, C. K., Stella, L., Ciolfi, A., Alawi, M., Bocchinisou, G., Flex, E., Paolo, S., Dentici, M. L., Grammatico, P., Korenke, G. C., Leuzzi, V., Mowitz, D. N., Nair, L. D., et al. (2015) Mutations in KCNHI and ATP6V1B2 cause Zimmermann–Laband syndrome. *Nat. Genet.* 47, 661–667 CrossRefMedline

4. Simons, C., Rash, L. D., Crawford, J., Ma, L., Cristofori-Armstrong, B., Miller, D., Rok, K., Baillie, G. J., Alany, Y., Jaccquet, A., Debray, F. G., Verloes, A., Shen, J., Yesil, G., Guler, S., et al. (2015) Mutations in the voltage-gated potassium channel gene KCNH1 cause Temple–Baraitser syndrome and epilepsy. *Nat. Genet.* 47, 73–77 CrossRefMedline

5. Hemmerlein, B., Wenseloh, R. M., Mello de Queiroz, F., Knörgen, H., Sánchez, A., Rubio, M. E., Martin, S., Schluepcke, T., Jenke, M., Heinz, J. R., Stühmer, W., and Pardo, L. A. (2006) Overexpression of Eag1 potassium channels in clinical tumours. *Mol. Cancer* 5, 41 CrossRefMedline

6. Stühmer, W., Alves, F., Hartung, F., Zientkowska, M., and Pardo, L. A. (2006) Potassium channels as tumour markers. *FEBS Lett.* 580, 2850–2852 CrossRefMedline

7. Ding, X. W., Luo, H. S., Jin, X., Yan, J. J., and Ai, Y. W. (2007) Aberrant expression of Eag1 potassium channels in gastric cancer patients and cell lines. *Med. Oncol.* 24, 345–350 CrossRefMedline

8. Mello de Queiroz, F., Suarez-Kurtz, G., Stühmer, W., and Pardo, L. A. (2006) Ether a go-go potassium channel expression in soft tissue sarcoma patients. *Mol. Cancer* 5, 42 CrossRefMedline
9. Ousingsawat, J., Spitzner, M., Puntheeranurak, S., Terracciano, L., Torrillo, L., Bubendorf, L., Kunzelmann, K., and Schreiber, R. (2007) Expression of voltage-gated potassium channels in human and mouse colonic carcinoma. Clin Cancer Res. 13, 824–831 CrossRef Medline

10. Pardo, L. A., del Camino, D., Sánchez, A., Alves, F., Brüggemann, A., Beckh, S., and Stühmer, W. (1999) Oncogenic potential of EAG K⁺ channels. EMBO J. 18, 5540–5547 CrossRef Medline

11. Patt, S., Preussat, K., Beetz, C., Kraft, R., Schrey, M., Kalff, R., Schönherr, K., and Heinemann, S. H. (2004) Expression of ether a go-go potassium channels in human gliomas. Neurosci. Lett. 368, 249–253 CrossRef Medline

12. Weber, C., Mello de Queiroz, F., Downie, B. R., Suckow, A., Stühmer, W., and Pardo, L. A. (2006) Silencing the activity and proliferative properties of the human Eagl potassium channel by RNA interference. J. Biol. Chem. 281, 13030–13037 CrossRef Medline

13. Hartung, F., Stühmer, W., and Pardo, L. A. (2011) Tumor cell-selective apoptosis induction through targeting of K(V)1.1 via bifunctional TRAIL antibody. Mol. Cancer 10, 109 CrossRef Medline

14. Ganetzky, B., Robertson, G. A., Wilson, G. F., Trudeau, M. C., and Titus, S. A. (1999) The eag family of K⁺ channels in Drosophila and mammals. Ann. N.Y. Acad. Sci. 868, 356–369 CrossRef Medline

15. Whicher, J. R., and MacKinnon, R. (2016) Structure of the voltage-gated K⁺ channel Eag1 reveals an alternative voltage sensing mechanism. Science 353, 664–669 CrossRef Medline

16. Wang, W., and MacKinnon, R. (2017) Fryo-EM structure of the open human Ether-a-go-go–related K⁺ channel hERG. Cell 169, 422–430.e10 CrossRef Medline

17. Bamford, S., Dawson, E., Forbes, S., Clements, J., Pettett, R., Dogan, A., Flanagan, A., Teague, J., Futeal, P. A., Stratton, M. R., and Wooster, R. (2004) The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. Br. J. Cancer 91, 355–358 CrossRef Medline

18. Chen, J., Zou, A., Splawski, I., Keating, M. T., and Sanguinetti, M. C. (1999) condol. Long QT syndrome-associated mutations in the Per–Arnt–Sim (PAS) domain of HERG potassium channels accelerate channel deactivation. J. Biol. Chem. 274, 10113–10118 CrossRef Medline

19. Splawski, I., Chen, J., Timothy, K. W., Lehmann, M. H., Priori, S., Robinson, J. L., Moss, A. J., Schwartz, P. J., Towbin, J. A., Vincent, G. M., and Keating, M. T. (2000) Spectrum of mutations in long-QT syndrome genes: KV-LQT1, HERG, SCN5A, KCNE1, and KCNE2. Circulation 102, 1178–1185 CrossRef Medline

20. Zagotta, W. N., Olivier, N. B., Black, K. D., Young, E. C., Olson, R., and Guo, A. (2003) Structural basis for modulation and agonist specificity of HCN pacemaker channels. Nature 425, 200–205 CrossRef Medline

21. Breidte, T. I., Carlson, A. E., and Zagotta, W. N. (2009) Absence of direct cAMP binding to KCNQ1. J. Gen. Physiol. 141, 347–358 CrossRef Medline

22. Codding, S. J., and Trudeau, M. C. (2019) The hERG potassium channel intrinsic ligand regulates N- and C-terminal interactions and channel activation in hERG channels. J. Gen. Physiol. 142, 351–366 CrossRef Medline

23. Hartung, F., and Stühmer, W. (2019) On the role of voltage-gated potassium channels in human and mouse colonic carcinoma. J. Clin. Gastroenterol. 53, 3–12 CrossRef Medline

24. Morais-Cabral, J. H., and Robertson, G. A. (2015) The enigmatic cytoplasmic PAS domains of KCN channels. J. Mol. Biol. 427, 67–76 CrossRef Medline

25. Henry, J. T., and Crosson, S. (2011) Ligand-binding PAS domains in a genomic, cellular, and structural context. Annu. Rev. Microbiol. 65, 261–286 CrossRef Medline

26. Mögli, A., Ayers, R. A., and Moffat, K. (2009) Structure and signaling mechanism of Per–ARNT–Sim domains. Structure 17, 1282–1294 CrossRef Medline

27. Wang, Z. J., Tiwari, P. B., Üren, A., and Breidte, T. I. (2019) Identification of undecylenic acid as EAG channel inhibitor using surface plasmon resonance-based screen of KCN channels. BMC Pharmacol. Toxicol. 20, 42 CrossRef Medline

28. Nguyen, H. H., Park, J., Kang, S., and Kim, M. (2015) Surface plasmon resonance: a versatile technique for biosensor applications. Sensors (Basel) 15, 10481–10510 CrossRef Medline

29. Cukkemane, A., Grütler, B., Novak, K., Gensch, T., Böninger, W., Gruber, T., Kaupp, U. B., and Seifert, R. (2007) Subunits act independently in a cyclic nucleotide-activated K⁺ channel. EMBO Rep. 8, 749–755 CrossRef Medline

30. Lakowicz, J. R. (2006) Principles of Fluorescence Spectroscopy, 3rd ed., pp. 56 and 551, Springer, New York

31. Zhao, Y., Goldschen-Ohm, M. P., Morais-Cabral, J. H., Chanda, B., and Robertson, G. A. (2017) The intrinsically liganded cyclic nucleotide-binding homology domain promotes KCN channel activation. J. Gen. Physiol. 149, 249–260 CrossRef Medline

32. Terlau, H., Heinemann, S. H., Stühmer, W., Pongs, O., and Ludwig, J. (1997) Amino terminal-dependent gating of the potassium channel rat eag is compensated by a mutation in the S4 segment. J. Physiol. 502, 537–543 CrossRef Medline

33. Carlson, A. E., Breidte, T. I., and Zagotta, W. N. (2013) Flavonoid regulation of hEAG1 channels. J. Gen. Physiol. 141, 347–358 CrossRef Medline

34. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording and voltage-clamp analysis of epilepsy and autism mutation Kv10.2-R327H in cardionicity and cardiac arrhythmia. Nature 440, 463–469 CrossRef Medline

35. Gianulis, E. C., Liu, Q., and Trudeau, M. C. (2013) Direct interaction of eag domains and cyclic nucleotide-binding homology domains regulate deactivation gating in hERG channels. J. Gen. Physiol. 142, 351–366 CrossRef Medline

36. Carelli, V., and Trudeau, M. C. (2011) The hERG potassium channel is meditated by N- and C-terminal region interactions. J. Gen. Physiol. 137, 315–325 CrossRef Medline

37. Hainin, Y., Carlson, A. E., and Zagotta, W. N. (2013) The structural mechanism of KCN-channel regulation by the eag domain. Nature 501, 444–448 CrossRef Medline

38. Geddis, K. J., and Trudeau, M. C. (2019) The hEAG1 channels. J. Gen. Physiol. 141, 347–358 CrossRef Medline

39. Yang, Y., Vasylyev, D. V., Dib-Hajji, F., Veeramah, K. R., Hammer, M. F., Dib-Hajji, S. D., and Waxman, S. G. (2013) Multistate structural modeling and voltage-clamp analysis of hERG channel. J. Gen. Physiol. 142, 878–894 CrossRef Medline

40. López-Muñoz, F., Alamo, C., Cuenca, E., Shen, W. W., Cervero, P., and Rubio, G. (2005) History of the discovery and clinical introduction of chlorpromazine. Annu. Clin. Psychiatry 17, 113–135 CrossRef Medline

41. Hayoz, C., Tiwari, P. B., Piczczek, G., Üren, A., and Breidte, T. I. (2017) Investigating cyclic nucleotide and cyclic dinucleotide binding to HCN channels by surface plasmon resonance. PLoS One 12, e0185359 CrossRef Medline

42. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 391, 85–100 CrossRef Medline

43. Costa, P. F., Emilio, M. G., Fernandes, P. L., Ferreira, H. G., and Ferreira, K. G. (1989) Determination of ion permeability coefficients of the plasma membrane of Xenopus laevis oocytes under voltage clamp. J. Physiol. 413, 199–211 CrossRef Medline