Combinatorial augmentation of voltage-gated KCNQ potassium channels by chemical openers

Qiaojie Xiong*, Haiyan Sun*, Yangming Zhang†, Fajun Nan†, and Min Li**

*Departments of Neuroscience, Physiology and High Throughput Biology Center, School of Medicine, Johns Hopkins University, 733 North Broadway, Baltimore, MD 21205; and †National Center for Drug Screening, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China

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Noninactivating potassium current formed by KCNQ2 (Kv7.2) and KCNQ3 (Kv7.3) subunits resembles neuronal M-currents which are activated by voltage and play a critical role in controlling membrane excitability. Activation of voltage-gated potassium channels by a chemical opener is uncommon. Therefore, the mechanisms of action are worthy further investigation. Retigabine and zinc pyrithione are two activators for KCNQ channels but their molecular interactions with KCNQ channel remain largely elusive. Here we report that retigabine and zinc pyrithione recognize two different sites of KCNQ2 channels. Their agonistic actions are noncompetitive and allow for simultaneous binding of two different activators on the same channel complex, hence giving rise to combinatorial potentiation with characteristic properties of both openers. Examining their effects on mutant channels, we showed zinc pyrithione is capable of opening nonconductive channels and coapplication of zinc pyrithione and retigabine could restore a disease mutant channel similar to wild type. Our results indicate two independent activator binding sites present in KCNQ channels. The resultant combinatorial potentiation by multiple synthetic chemical openers indicates that KCNQ channels are accessible to various types and combinations of pharmacological regulation.

channel opener | epilepsy | genetic mutants

M-currents are noninactivating, subthreshold potassium currents (1–3). They are suppressed by activation of G protein-coupled receptors (GPCRs), particularly muscarinic acetylcholine receptor (mAChR) (4–6). M-currents are encoded by heteromultimeric potassium channels involving KCNQs. KCNQ channels have five subtypes, KCNQ1 to KCNQ5, also known as Kv7.1 to Kv7.5 (7–12). KCNQ2 and KCNQ3 are widely distributed in the nervous system, and these heteromultimeric channels display typical biophysical and pharmacological properties resembling M currents (1, 3, 9, 10), that are further supported by both biochemical and anatomical evidence (13, 14). A number of mutations in either KCNQ2 or KCNQ3 subunits resulting in reduced currents lead to a form of epilepsy in newborns, benign familial neonatal convulsion (BFNC) (11, 15). This is in agreement with the notion that M-type channels play a key role in dampening neuronal excitability.

KCNQ channels are gated by transmembrane voltage, and they are members of the Kv superfamily with six transmembrane segments, S1–S6. Compared with other Kv channels, KCNQ channels are unique in that they may be activated by small synthetic chemical ligands including a clinically tested drug, *N*-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester, also known as retigabine (RTG) (16–21). RTG activates KCNQ2 to KCNQ5 channels by causing a hyperpolarizing shift of the G-V curve, with an EC₅₀ value of 1.4 μM at –30 mV on the basis of current potentiation (22). Its potential clinical applications include treatment of anxiety, neuropathic pain, neurodegenerative disorders, cancer, inflammation, and ophthalmic diseases. Structural and functional studies have identified several interesting features of RTG-mediated activation. In particular, RTG most potently activates KCNQ3 but has no detectable effect on KCNQ1 (20, 23).

More recently, zinc pyrithione (ZnPy) was reported to potentiate KCNQ channels, causing both a hyperpolarizing shift in the G-V curve and marked increase in overall current amplitude, with an EC₅₀ value of 1.5 μM for KCNQ2 (24). The activation appears to be different from RTG in several aspects. Most noticeably, ZnPy activates all KCNQ channels except KCNQ3. In contrast, KCNQ3 is most sensitive to RTG. Furthermore, ZnPy increases channel open probability at the saturated voltage (24). In contrast, RTG has little effects at the saturated voltage. These data are consistent with the idea that ZnPy and RTG recognize two different sites. However it remains unknown whether one KCNQ channel complex may possess two classes of agonistic sites simultaneously functional for both RTG and ZnPy.

One critical question concerning the mechanisms of action for these channel openers is to understand the molecular determinants critical for conferring the augmentation. Several different classes of openers have been identified (25). However, very little is known about their sites of action. Acrylamide (S)-1, for example, was shown to augment KCNQ by recognizing the same site as retigabine (26). Meclofenamate was also shown as a potentiator with EC₅₀ of 25 μM. However, when KCNQ2/KCNQ3 is treated with RTG in the presence of 25 μM meclofenamate, there is an additive effect shifting maximal ΔV_{1/2} from –24 to –32 mV (27). The observation of additional augmentation by coapplication of two agonists, despite of different interpretations, raises the question of both number and type of binding sites for activators.

Heteromultimeric KCNQ2/KCNQ3 channels are thought to represent a major component of M-current. However, growing evidence from anatomic localization and TEA blockade studies have argued for the presence of KCNQ2 homomultimer *in vivo* (28–30). Because of considerable difference in augmenting KCNQ2 and KCNQ3 homomultimers, ZnPy, but not RTG, is much more effective on KCNQ2 homomultimers than typical KCNQ2/KCNQ3 heteromultimers (24). The knowledge of molecular determinants and isoform-specific potency when combined with structure–activity relationship (SAR) studies of different chemical openers should ultimately facilitate development of new structures with isoform or heteromultimer specificity.

To examine any functional interaction between ZnPy and RTG, we performed a series of electrophysiological analyses testing whether effects by one drug may affect the affinity and efficacy of the other. In addition, we have identified and characterized different mutants, including a functionally null mutant that can only be activated by an agonistic ligand. The experimental results provide strong evidence that ZnPy and RTG are noncompetitive. A given KCNQ2 homomultimer channel complex has at least two classes of

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†To whom correspondence should be addressed. E-mail: minli@jhmi.edu.

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Fig. 1. Retigabine (RTG) and zinc pyruithione (ZnPy) display differential specificity to KCNQ subtypes. (A) KCNQ2 currents elicited by depolarization from -70 mV to $+50$ mV in 10 mV incremental steps, in the presence and absence of drugs as indicated. (B) G-V curves of KCNQ1 and KCNQ3 channels for control ($V_{1/2}$ was -24 mV for KCNQ1 and -29 mV for KCNQ3), $10 \mu\text{M}$ RTG ($V_{1/2}$ was -28 mV for KCNQ1 and for -53 mV for KCNQ3) and $10 \mu\text{M}$ ZnPy ($V_{1/2}$ was -28 mV for KCNQ1 and -28 mV for KCNQ3) are shown. (C) Histogram summary of RTG and ZnPy effects on currents of KCNQ2, KCNQ3 and KCNQ2/KCNQ3 channels is shown. Effects at $+50$ and -30 mV testing voltages are as indicated. ZnPy at $10 \mu\text{M}$ caused current increase of 5.2 ± 1.3 fold at $+50$ mV and 13.6 ± 2.3 fold at -30 mV for KCNQ2 homomultimers, significantly higher than the effects of $10 \mu\text{M}$ RTG, 1.2 ± 0.2 and 4.3 ± 0.9 , respectively. RTG caused current increase of 1.1 ± 0.1 fold at $+50$ mV and 4.9 ± 1.7 fold at -30 mV for KCNQ3 homomultimers, whereas ZnPy caused no change. When KCNQ2 and KCNQ3 were coexpressed in CHO cells to form heteromultimers, they responded to RTG and ZnPy similarly, with current increase by 1.6 ± 0.1 fold at $+50$ mV and 4.3 ± 0.7 fold at -30 mV by RTG, and 1.9 ± 0.1 fold at $+50$ mV and 5.6 ± 0.9 fold at -30 mV by ZnPy. Dash lines indicate value 1, i.e., no change to control ($n \geq 4$; $P < 0.001$).

agonistic sites that, when simultaneously occupied depending on the concentrations and ratios of agonistic ligands, cause combinatorial activation with characteristics that are contributed by both openers.

Results

Differential Specificity of Zinc Pyruithione (ZnPy) and Retigabine (RTG) for KCNQ Channels. Difference in augmenting KCNQ channels by RTG and ZnPy were observed in the context of isoform specificity and biophysical properties (20, 24). KCNQ2 is sensitive to both activators, but the resultant effects exhibit considerable difference (Fig. 1A). In addition to the different degree of current increase, the augmentation by ZnPy required much longer pulse duration to reach saturation [supporting information (SI) Fig. 6]. Furthermore, individual homomultimers of five KCNQ channels respond differently to the two activators. Most prominently, KCNQ1 is insensitive to RTG, whereas KCNQ3 shows no response to ZnPy (Fig. 1B and

Fig. 2. Different molecular determinants confer sensitivity to RTG and ZnPy. (A) topology of a KCNQ2 subunit. (B–F) RTG and ZnPy caused changes of G–V curves on wild-type KCNQ2 (B), W236L (C), L249A (D), L275A (E), and L249A/L275A (F). Conductance at different voltages was plotted after division by the maximum conductance in control (no drug). Dash lines are curves in the presence of $10 \mu\text{M}$ ZnPy after being normalized to 1, and the gray lines are curves in the presence of $10 \mu\text{M}$ RTG after being normalized to 1 ($n \geq 4$).

SI Fig. 7). Potentiation of KCNQ current by either RTG or ZnPy was observed within minutes after compound application and rapidly reversible, indicating drug effects are on channel function but not expression, consistent with the earlier report (24).

Native M-current is thought to be mediated by coassembly of KCNQ2/KCNQ3 subunits (1, 3). We heterologously coexpressed KCNQ2 and KCNQ3 that resulted in M-like current as judged by both biophysical properties and pharmacological sensitivities (24). When ZnPy and RTG are applied to KCNQ2/KCNQ3 heteromultimeric channels, the difference between the two activators was less pronounced compared with homomultimeric channels (Fig. 1C and SI Fig. 8). This is consistent with the fact that both KCNQ2 and KCNQ3 are sensitive to RTG. In contrast, only KCNQ2, but not KCNQ3, is sensitive to ZnPy. These results suggest that in addition to the differential effects of the two activators, the number of sensitive subunits within a given channel complex is proportional to the overall level of response.

Molecular Determinants for Retigabine (RTG) and Zinc Pyruithione (ZnPy) Modulation. Earlier studies have identified residues important for conferring sensitivity to either ZnPy (24) or RTG (31, 32) (Fig. 2A). Whether and to what extent these residues are involved in determining the sensitivity to the other activators remains largely unknown. We compared these mutants individually by examining the G–V (conductance–voltage) relationship in the presence of ZnPy or RTG. Wild-type KCNQ2 homomultimers display half

Fig. 4. RTG and ZnPy activate a conductively null KCNQ2 mutant. (A) Current traces of the KCNQ2 (I238A) mutant are shown at indicated conditions. Currents were induced by depolarization from -70 mV to $+50$ mV at 10 mV incremental steps from holding potential -80 mV. (B) Peak current of KCNQ2 (I238A) $+50$ mV induced by indicated drug applications. (C) $I-V$ curves of KCNQ2 (I238A) channel at the indicated drug applications. (D) $G-V$ curves of KCNQ2 (I238A) channel at the indicated drug applications. The $V_{1/2}$ of I238A in the presence of $10 \mu\text{M}$ of ZnPy is -13 ± 2 mV, similar to that of KCNQ2 wild type. Gray line is the curve obtained from wild-type KCNQ2 channel without any drug application ($n \geq 4$).

conductive upon voltage depolarization. In the presence of 100 μM RTG, membrane depolarization remains ineffective (Fig. 4A). In contrast, in the presence of 10 μM ZnPy, the mutant channel displays KCNQ2-like currents similar to that of wild-type KCNQ2 channel (Fig. 4A and D). Noticeably, the $V_{1/2}$ of I238A in the presence of ZnPy is similar that of wild-type KCNQ2 in the absence of any drug. In the presence of ZnPy, the resultant conductive I238A mutant became sensitive to RTG, revealing a hyperpolarizing shift of the $V_{1/2}$, accompanied by a slight reduction of current amplitudes (Fig. 4B–D). Together, these data indicate that there are two independent binding sites and modulation pathways for ZnPy and RTG on KCNQ2 channels.

Combinatorial Modulation of Disease Mutants by Retigabine (RTG) and Zinc Pyrithione (ZnPy). More than 30 mutations in the KCNQ2 gene have been identified from patients with benign familial neonatal convulsions (BFNC); most have displayed reduced channel activity (7, 11, 15, 33). We previously showed that at the saturated voltages, ZnPy could increase the overall currents of all three BFNC mutants: R207W, Y284C and A306T (24). In contrast, under the same condition RTG displayed much reduced potentiation. Whether and to what extent other channel properties being

affected by RTG and ZnPy remains largely unknown. Both Y284C and A306T homomultimers have reduced currents but retained much of their biophysical properties when compared with wild-type channels. Therefore, inefficient trafficking may contribute to the disease phenotype. ZnPy at 10 μ M induces current increases at +50 mV by 3.6 ± 0.5 fold for Y284C and 1.5 ± 0.1 fold for A306T. By comparison, 10 μ M RTG induces increases of 1.3 ± 0.2 fold for Y284C and no change for A306T. In addition, ZnPy caused delay of activation and deactivation of both mutants, whereas RTG accelerated activation and delayed deactivation of both mutants (Fig. 5A). Both ZnPy and RTG caused a hyperpolarizing shift in activation curves, with $V_{1/2}$ changing from -20 to -42 mV for Y284C and from -28 to -52 mV for A306T (Fig. 5B).

In contrast to Y284C and A306T, R207W homomultimers have smaller currents, and much slower activation and deactivation than wild-type channels. Most significantly, the mutant, in the absence of any drugs, displays a right-shift activation curve with $V_{1/2}$ changed from -18 to 31 mV (Fig. 5 *A* and *B*). ZnPy increases current by 3.9 ± 0.4 fold (at $+50$ mV), further delayed channel activation and deactivation, and causes a left shift in the activation curve with $V_{1/2}$ at 19 mV. By comparison, $10 \mu\text{M}$ RTG alone increases current at $+50$ mV by 1.5 ± 0.1 fold, accelerates channel activation and delayed deactivation, and left shifts the activation curve dramatically ($V_{1/2}$ of -37 mV) (Fig. 5 *A* and *B* and **SI Fig. 10**). When RTG and ZnPy are combined, different compound ratios afford a wide spectrum of changes when applied to R207W. In addition to enhancing the current amplitude, the mutant channel voltage dependence and activation kinetics can be restored. When appropriate ratio of compounds mixture is applied, the resultant current resembles that of wild-type KCNQ2 channel (Fig. 5 *C* and *D* and **SI Fig. 10**). These results suggest that mutant channels have differential deficiencies and sensitivities to activators. Combined application of noncompetitive activators, ZnPy and RTG, could cause a variety of changes in channel properties. For certain genetic mutants, such as KCNQ2 (R207W), a fine-tuned ratio of the two compounds could essentially restore channel properties resembling wild-type channel.

Discussion

Activation of voltage-gated potassium channels by synthetic openers is an intriguing phenomenon that may be exploited both for understanding of channel gating and for therapeutic development. This report presents a series of evidence demonstrating that ZnPy and RTG interact with two different sites on KCNQ channels, and their effects require different molecular determinants. Importantly, one channel complex can simultaneously house two different ligands and consequently, display a hybrid response that varies according to the ratios of the two compounds applied. Therefore, a desired modulating effect may be achieved by tuning the ratios of two agonistic compounds. KCNQ channels belong to the superfamily of Shaker-type voltage-gated potassium (Kv) channels. The identification of two different modulation sites and the hybrid effects by co-application of two activators raises the question of whether these features are general to other KCNQ-related channels.

Several scenarios could account for the two classes of binding sites on one channel complex. One likely possibility is that each tetrameric channel complex has a total of eight binding sites: four for ZnPy and four for RTG. However, for a saturated response, only a fraction of these sites may need to be occupied. This model clearly requires additional investigation, although the Hill constants are consistent with two or three bound molecules per channel complex (Fig. 3). In the presence of a saturated concentration for one compound, the EC_{50} and Hill constant for the other compound remain largely unchanged. This finding supports the conclusion that binding of one compound does not significantly affect the affinity for the other compound, consistent with a noncompetitive mode of action. One intriguing question is whether the ZnPy and RTG

Fig. 5. Combinatorial application of RTG and ZnPy rescues KCNQ2(BFNC) mutants. (A) Current traces of selected BFNC mutants in the presence of 10 μ M ZnPy (Upper) or RTG (Lower). The specific mutations in KCNQ2 are indicated. Currents were elicited by depolarizing to +50 mV and then hyperpolarizing to -120 mV. (B) RTG and ZnPy effects on G-V curves for each mutant. Dash lines are curves for wild-type KCNQ2 channel. (C) Normalized outward current traces of KCNQ2 currents were elicited by depolarizing to +50 mV from holding voltage -80 mV. Different drug concentrations are as indicated. Gray line is the trace of wild-type KCNQ2 channel. (D) G-V curves of KCNQ2 (R207W) at different drug concentrations as indicated. Dashed line is the curve of wild-type KCNQ2 channel ($n \geq 4$).

binding sites within one subunit can be simultaneously occupied. This possibility requires further investigation.

The chemistry to achieve activation of voltage-gated ion channels includes direct binding of small molecules, covalent modification, and enzymatic modulation. The potentiation of KCNQ channels by covalent modification is a prominent phenomenon (34–37). However, it is not clear whether or to what extent the effect triggered by modification of cysteine residues is similar to that by either RTG or ZnPy. In addition to the hyperpolarizing shift of voltage dependence, several factors are known to cause a positive (depolarizing) shift, for example, through modulation by protein kinase C (38).

Although KCNQ channels are gated primarily by transmembrane voltage, several compounds, such as RTG and ZnPy, could potentially activate the channel by causing a hyperpolarizing shift. Thus, the channel could begin to open at the resting potential in the absence of membrane depolarization. For example, incubation of ZnPy with native neurons results in hyperpolarization (24). In this report, the intriguing mutant KCNQ2 (I238A) was described (Fig. 4). Different from other mutants, I238A is functionally null when tested by membrane depolarization. Thus, membrane depolarization alone is not sufficient to activate the mutant channel. In the presence of ZnPy, but not RTG, the I238A channel becomes functional and responds to membrane depolarization. Remarkably, the mutant channel when bound to ZnPy displays almost identical biophysical properties to those of wild type, reminiscent of a

ligand-gated channel (Fig. 4 C and D). The ZnPy-bound functional I238A channel regains its sensitivity to RTG. Although the molecular details concerning this mutant and ZnPy effects are of great interest for further investigation, the activation of null KCNQ channels by a small molecule raises the exciting possibility of manipulating membrane excitability using an artificial ligand.

Experimental Procedures

Cell Culture and Transient Transfection. CHO cells were grown in 50:50 DMEM/F12 (Cellgro) with 10% FBS (Gibco), 100 units ml^{-1} penicillin (Cellgro), 100 $\mu\text{g ml}^{-1}$ streptomycin (Cellgro) and 2 mM L-glutamine (Gibco). At 24 h before transfection, cells were split and plated in 60-mm dishes and were transfected with Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, cells were split and replated onto coverslips coated with poly(L-lysine) (Sigma-aldrich). Plasmid expressing CD4⁺ complementary DNA as a marker was cotransfected with the channel cDNAs kindly provided by our colleagues: human KCNQ1 (from Michael Sanguinetti, University of Utah, Salt Lake City), rat KCNQ2 (from David McKinnon, State University of New York, Stony Brook), rat KCNQ3 (from Mark Shapiro, University of Texas, San Antonio), human KCNQ4 (from Vitya Vardanyan, University of Hamburg, Hamburg, Germany), and human KCNQ5 (from Mark Shapiro and Thomas Jentsch, University of Hamburg). Before recording, anti-CD4⁺ Dynabeads (Dyna Beads) were added into the medium to allow for identification of the transfected cells. Stable lines expressing KCNQ2 were generated by standard protocols using pcDNA3.1 and selected in complete medium supplemented with 500 $\mu\text{g ml}^{-1}$ G418.

Mutagenesis. The KCNQ2 point mutants were constructed by PCR using standard protocol and verified by sequencing.

Electrophysiological Recording. Standard whole-cell recording was used. Pipettes were pulled from borosilicate glass capillaries (TW150-4, World Precision Instruments). When filled with the intracellular solution, the pipettes have resistances of 3–5 M Ω . During the recording, constant perfusion of extracellular solution was maintained by using a BPS perfusion system (ALA Scientific). Pipette solution contained 150 mM KCl, 1 mM MgCl₂, 5 mM EGTA, 10 mM Hepes, and 5 mM MgATP (pH 7.3); extracellular solution contained 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.5 mM MgCl₂, 10 mM Hepes, and 10 mM glucose (pH 7.4). Current and voltage were recorded by using an Axopatch-200A amplifier (Molecular Devices), filtered at 1 kHz and digitized by using a DigiData 1322A with pClamp 9.2 software (Molecular Devices). Series resistance compensation was also used and set to 60–80%. Duration of test pulse was adjusted to make certain steady state has been reached for conductance measurements. Zinc pyrithione is purchased from Sigma-Aldrich. Retigabine dihydrochloride was synthesized according to ref. 39.

Data and Statistical Analysis. Patch-clamp data were preprocessed by using Clampfit 9.2 (Molecular Devices) and then analyzed in Origin 7 (OriginLab).

The activation curve was fitted by the Boltzmann equation: $G = (G_{\max} - G_{\min}) / (1 + \exp((V - V_{1/2})/S)) + G_{\min}$, where $G_{\max}$ is the maximum conductance, $G_{\min}$ is the minimum conductance, $V_{1/2}$ is the voltage for half of the total number of channels to open and S is the slope factor. The dose–response curve was fitted by the Hill equation: $E = E_{\max} / (1 + (EC_{50}/C)^P)$, where $E_{\max}$ is the maximum response, C is the drug concentration, EC_{50} is the drug concentration producing half of the maximum response, and P is the Hill coefficient. The deactivation trace was fitted by the standard exponential equation $I(t) = \Sigma I_i \times \exp(-t/\tau_i)$, where I is the current, t is the time and τ is the time constant. Data are presented as means $\pm$ SEM. Significance was estimated by using the paired two-tailed Student's t test.

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