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Activation of peripheral KCNQ channels relieves gout pain

Yueming Zheng, Haiyan Xu, Li Zhan, Xindi Zhou, Xueqin Chen, Zhaobing Gao*

Abstract

Intense inflammatory pain caused by urate crystals in joints and other tissues is a major symptom of gout. Among therapy drugs that lower urate, benzbromarone (BBR), an inhibitor of urate transporters, is widely used because it is well tolerated and highly effective. We demonstrate that BBR is also an activator of voltage-gated KCNQ potassium channels. In cultured recombinant cells, BBR exhibited significant potentiation effects on KCNQ channels comparable to previously reported classical activators. In native dorsal root ganglion neurons, BBR effectively overcame the suppression of KCNQ currents, and the resultant neuronal hyperexcitability caused by inflammatory mediators, such as bradykinin (BK). Benzbromarone consistently attenuates BK-, formalin-, or monosodium urate–induced inflammatory pain in rat and mouse models. Notably, the analgesic effects of BBR are largely mediated through peripheral and not through central KCNQ channels, an observation supported both by pharmacokinetic studies and in vivo experiments. Moreover, multiple residues in the superficial part of the voltage sensing domain of KCNQ channels were identified critical for the potentiation activity of BBR by a molecular determinant investigation. Our data indicate that activation of peripheral KCNQ channels mediates the pain relief effects of BBR, potentially providing a new strategy for the development of more effective therapies for gout.

Keywords: KCNQ, Benzbromarone, Peripheral nervous system, Gout, Pain

1. Introduction

Intense inflammatory pain induced by urate crystals in joints is a major complication of gout, a disease affecting approximately 1% to 2% of the Western population.[48,49] Nonsteroidal anti-inflammatory drugs and cyclooxygenase (COX)-2 agents are used to treat acute flare-ups but have several adverse effects.[45] Benzbromarone (BBR) is a potent urate-lowering therapy (ULT) drug for chronic treatment.[71] It reduces serum urate by inhibiting URAT-1 and SLC2A9, 2 urate transporters.[12,18] Based on a benefit–risk assessment, BBR is superior to allopurinol (ALO) and probenecid (PRB), 2 alternative first-line ULT drugs, because it is well tolerated and highly effective.[29] Currently, according to data from IMS health, BBR is widely used and holds the lead position in the antigout drug market in many countries, including China.

Voltage-gated KCNQ channels have 5 isoforms, KCNQ1 to KCNQ5. Of the 5 known isoforms, the neuronal KCNQ2-5 is expressed throughout the central nervous system (CNS) and peripheral nervous system (PNS) and mediates a subthreshold activated M-current.[8,47,67,69] Activation of neuronal KCNQ channels would dampen membrane excitability[28,58]; a few novel KCNQ activators have been reported.[1,6,19,25,27,43,64,69] In 2011, retigabine (RTG), an activator of KCNQ channels was approved to treat human epilepsy.[60] It is reasonable to deduce that the antiepileptic activity reported for KCNQ activators occurs by their activity on CNS KCNQ channels. Increasing evidence indicates that neuronal KCNQ channels are also important analgesic targets. Being a painkiller that acts on KCNQ channels in the market, flupirtine (FLP) has been used for treatment of various pain.[14,59] Systematic administration of other small chemical activators has also been demonstrated to be effective in various pain models.[5,19,36] Although these studies shed light on the application of KCNQ activators in pain treatment, whether the analgesic effects are mediated through CNS or PNS KCNQ channels is not clear. A study by Bi et al.[2] showed that suppression of KCNQ channels expressed in the forebrain results in visceral hyperalgesia, and that this hyperexcitability can be reversed by central application of RTG. Consistently, central intracerebroventricular administration of RTG resulted in reduced allodynia in rats.[70] These data suggest that CNS KCNQ channels have important roles in pain treatment. However, local intraplantar injections of RTG or ICA-27243, another previously reported KCNQ activator, were found to relieve the pain induced by formalin, which suggests that activation of PNS KCNQ channels is also effective for pain relief.[21] Notably, the activation of CNS KCNQ channels may increase the CNS side effects that accompany the therapeutic effects. For example, in humans, the reported neuronal side effects of RTG or FLP include dizziness,

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somnolence, and a confused state, which have limited their use in clinic.[23,50,57] Thus, peripheral KCNQ channels are preferable targets for pain treatment.

In this study, we provide evidence that BBR is a KCNQ activator that primarily acts on peripheral KCNQ channels, which indicates that activation of peripheral KCNQ channels contributes to the pain relief effects of BBR. Our study provides a new strategy for the development of more effective therapies for gout.

2. Materials and methods

2.1. cDNA, cell culture, and transfection

The KCNQ and KCNE1 cDNAs are gifts from Drs T. Jentsch (Zentrum für Molekulare Neurobiologie, Hamburg), D. Makinnon (State University of New York, Stony Brook), M. Sanguinetti (University of Utah), M. Shapiro (University of Texas Health Science Center, San Antonio), V. Vardanyan (Universität Hamburg), and Kenneth L. Byron (Loyola University Chicago). Chinese Hamster ovary cells were grown in 50/50 DMEM/F-12 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Life Technology, Carlsbad, CA). To transiently express the channels for electrophysiological studies, cells were seeded at 24 hours before transfection in 60-mm dishes and then transfected with 3.6 μg of the cDNA using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The molar ratio of KCNQ1 and KCNE1 for expressing KCNQ1/KCNE1 complex was 1:1. A GFP cDNA construct (0.4 μg, Amaxa, Gaithersburg, MD) was cotransfected to aid identification of transfected cells by fluorescence microscopy. CHO cells stably expressing rat KCNQ2 (CHO-KCNQ2) were maintained in DMEM/F12 medium, supplemented with 10% FBS and 500 μg/mL G418.

2.2. Preparation of dorsal root ganglion neurons

Dorsal root ganglia from all spinal levels were dissected out from male adult Sprague-Dawley rats and pooled to prepare neurons using the standard enzymatic dissociation procedure as described previously.[42] Briefly, the ganglia were sequentially placed into collagenase type IA solution (3 mg/mL for 45 minutes) and trypsin solution (2 mg/mL for 15 minutes) at 37°C. The digested fragments were subsequently suspended with DMEM growth medium supplemented with 10% FBS and 2 mM glutamine plus 1% penicillin and streptomycin to stop digestion. Then, the ganglia were mechanically triturated into single cells with a fire-polished glass Pasteur pipette. The neurons were centrifuged and rinsed 3 times with the growth medium. The washed cells were resuspended in cell culture medium. For electrophysiological recordings, the dissociated neurons were plated onto glass coverslips coated with poly-D-lysine and cultured for 2 to 5 days in wells of sterile 6-well tissue culture plates in a humidified incubator at 37°C, 5% CO_2, until use.

2.3. Preparation of hippocampus neurons

Hippocampus neurons were isolated from postnatal day 0 Sprague-Dawley rats. Briefly, the dissected hippocampus tissues were enzymatically dissociated with trypsin (2.5 mg/mL) and incubated at 37°C for 30 minutes. The solution was then added with DMEM/F12 growth medium containing 10% FBS and 2 mM glutamine plus 1% penicillin and streptomycin. The tissues were then triturated into single cells using a fire-polished glass Pasteur pipette. The neurons were collected through centrifugation, resuspended, and transferred onto glass coverslips precoated with poly-D-lysine and cultured at 37°C. After the neurons had attached to the coverslips, all media were replaced by Neurobasal containing 2% B-27, 0.5 mM GlutaMAX, and 1% penicillin and streptomycin. The hippocampus neurons were cultured in a humidified incubator at 37°C, 5% CO_2, for 12 to 15 days before they were used for electrophysiology recording.

2.4. Electrophysiology

For current measurements in CHO cells and hippocampus neurons, a standard whole-cell voltage-clamp technique was used. For recording action potential (AP) firing, the current-clamp mode was used. Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) to resistances of 3 to 5 MΩ when filled with the intracellular solution and placed in the bath. For CHO cells, the intracellular solution contained (in mM): 145 KCl, 1 $MgCl_2$, 5 MgATP, 5 EGTA, and 10 HEPES (pH 7.3 adjusted by KOH); bath or extracellular solution contained (in mM): 140 NaCl, 3 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 adjusted by NaOH). For hippocampus neurons, the intracellular solution contained (in mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 20 EGTA, and 10 HEPES (pH 7.3 adjusted by KOH); extracellular solution contained (in mM): 135 NaCl, 5 KCl, 2 $MgCl_2$, 10 glucose, 0.001 tetrodotoxin, and 10 HEPES (pH 7.3 adjusted by NaOH). During the recording, the bath solution was continuously perfused using a BPS perfusion system (ALA Scientific Instruments, Westburg, NY). Perforated patch clamp was performed for voltage and current clamp experiments in dorsal root ganglion (DRG) neurons. Amphotericin B (250 μg/mL) was used to perforate the patch. The pipette solution contained (in mM): 80 K acetate, 30 KCl, 1 $CaCl_2$, 3 $MgCl_2$, 3 EGTA, and 40 HEPES (pH 7.4 adjusted by KOH); extracellular solution contained (in mM): 144 NaCl, 2.5 KCl, 2 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES, and 10 glucose (pH 7.4 adjusted by NaOH). Current injection and voltage command were given by and data acquisition was achieved using an Axopatch-700B amplifier, with the data filtered at 2 kHz and digitized using a DigiData 1440A with pClamp 10.3 software (Molecular Devices, Sunnyvale, CA). Series resistance compensation was used and set to 60% to 80%. All recordings were made at the room temperature.

2.5. Animal experiments

All animal procedures were performed in accordance with the National Institutes of Heath Guide for the Care and Use of Laboratory Animals, under protocols approved by and strictly following guidelines of the Institutional Animal Care and Use Committees (IACUC). The IACUC checked all protocols and approved this study. The animals were obtained from Shanghai SLAC Laboratory Animal Co, Ltd (Shanghai, China). All animal experiments were performed in a blinded manner, ie, administration of drugs and behavioral assessments were performed by different investigators.

2.5.1. Formalin-induced inflammatory model

Male Sprague-Dawley rats or KM mice were randomly grouped and allowed to habituate for at least 20 minutes in a transparent observation chamber before the experiment. The animals were given a subcutaneous injection of 4% formalin (30 μL per site) into the plantar of the left hind paw. Formalin-induced nociception was assessed by scoring pain behaviors and licking time during a 60-minute observation. The score represents the sum of weighted pain behaviors: 1 = flinching, 2 = shaking, and 3 = licking or biting of the injected paw. Phases were defined as

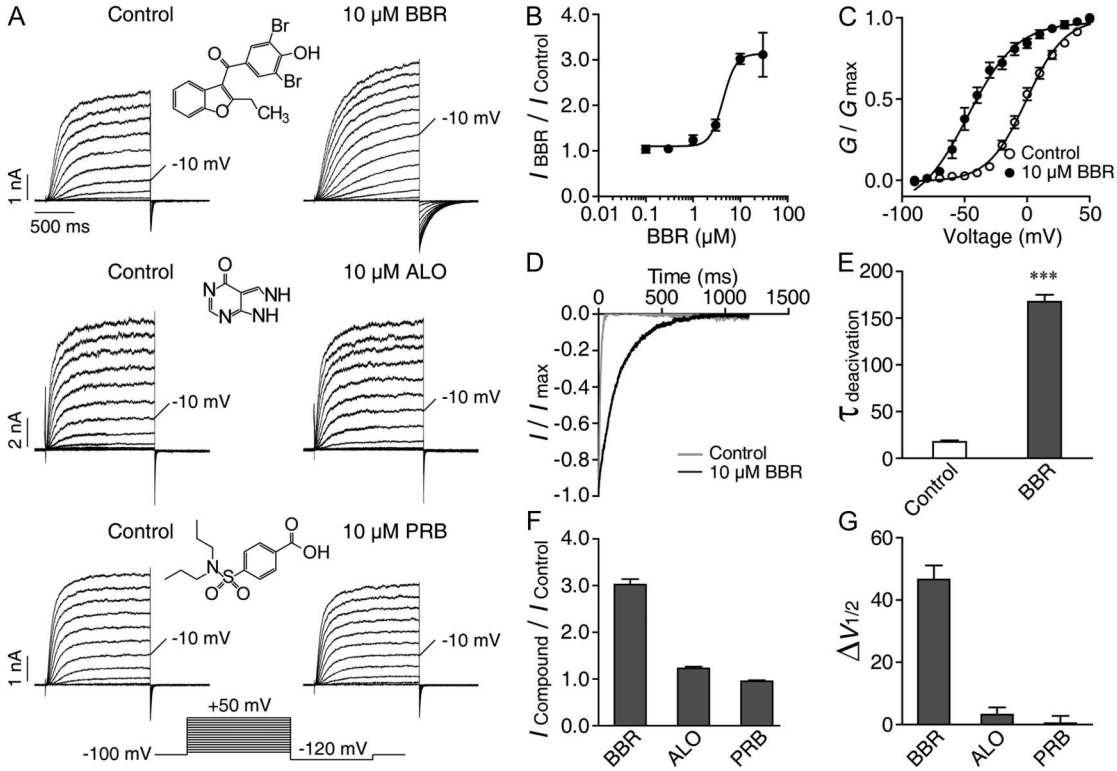

Figure 1. Potentiation effects of benzbromarone (BBR) on KCNQ2 channels. (A), Representative traces of KCNQ2 currents elicited by a voltage step protocol in the absence (left) and presence (right) of 10 μM drug as indicated. The holding potential was −100 mV. The currents were elicited by a series of depolarization from −90 mV to +50 mV with 10 mV increments. The tailed currents were elicited by a hypopolarization step to −120 mV followed the +50 mV depolarization. The chemical structures of the drugs are shown as inset. (B), Concentration dependence of BBR of KCNQ2 currents. The outward currents were elicited by stepping to −10 mV from the holding potential −100 mV. I_{BBR} and $I_{control}$ represent current amplitudes before and after BBR application, respectively. (C), Voltage-dependent activation curves of KCNQ2 in the absence or presence of 10 μM BBR. (D), The normalized deactivation phases after the +50 mV depolarization in the absence (control, gray line) and presence of 10 μM BBR (black line). (E), Bar graph of time constants of deactivation in the absence or presence of 10 μM BBR. (F and G), Bar graphs of ratios ($I_{compound}/I_{control}$) of outward current at −10 mV in the absence ($I_{control}$) and presence ($I_{compound}$) of 10 μM drugs (F) and difference in $V_{1/2}$ ($\Delta V_{1/2}$) (G) in the absence and presence of the drugs as indicated.

follows: phase I (0-10 minutes), and phase II (11-60 minutes). Benzbromarone could be administrated by 2 ways, intraperitoneal (i.p.) injection or local injection.

2.5.2. Monosodium urate crystal–induced arthritic model

Male Sprague-Dawley rats were used for the arthritic gout model. According to the method described by Coderre and Wall previously,[13] monosodium urate (MSU) crystals (5 mg, 50 μL per site) were injected into the tibiotarsal joint (ankle) on the isoflurane-anesthetized animals. The nociception assessment was performed 20 hours after the injection of MSU. The paw pressure, ie, the amount of weight that the rat was willing to put on the hind paw of the injected limb, was evaluated and categorized according to the scale described previously,[13,15] with some modifications. Score 0: no visible impairment of stance and gait, equal weight on both hind paws; score 1: slight impairment of stance and gait; score 2: moderated impairment of stance, moderate limp; score 3: foot elevated completely, severe limp. The highest score maintained for at least 10 seconds was assigned as the final score. Intermediate scores (0.5, 1.5, and 2.5) were used for the animals that displayed a behavior in between the above-described definitions. The Δpaw pressure score indicates a decrease in nociceptive behavior after and before drugs administration. The ankle edema was assessed as an increase in ankle thickness after MSU injection and measured with a digital caliper,[15,24] compared with baseline values.

2.6. Homology modeling of KCNQ2 channels

The modeling of KCNQ2 structure at the open state had been described in our previous study.[30] Briefly, KCNQ2 structures were modeled based on the structural information of the open-state Kv1.2 channel (Protein Data Bank code: 2A79) using Discovery studio 2.6 (Accelry Inc, San Diego, CA). The corresponding sequence alignment between KCNQ2 and Kv1.2 were generated using CLUSTALW Web server (http://www.ebi.ac.uk/Tools/msa/clustalw2).

2.7. Drugs

Formalin and bradykinin (BK) were purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). Benzbromarone, XE991, ALO, and PRB were obtained from Sigma-Aldrich Chemicals (St Louis, MO). Retigabine was synthesized by the laboratory of Professor Fajun Nan (Shanghai Institute of Materia Medica). For animal tests, drugs were formulated in 5% dimethyl sulfoxide (DMSO)/95% (1% Tween 80). Administration of drugs and behavior tests were conducted in a blinded and randomized manner.

2.8. Data analysis

Patch clamp data were processed using Clampfit 10.3 (Molecular Devices, Sunnyvale, CA) and then analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Voltage-dependent activation curves were fitted with the Boltzmann

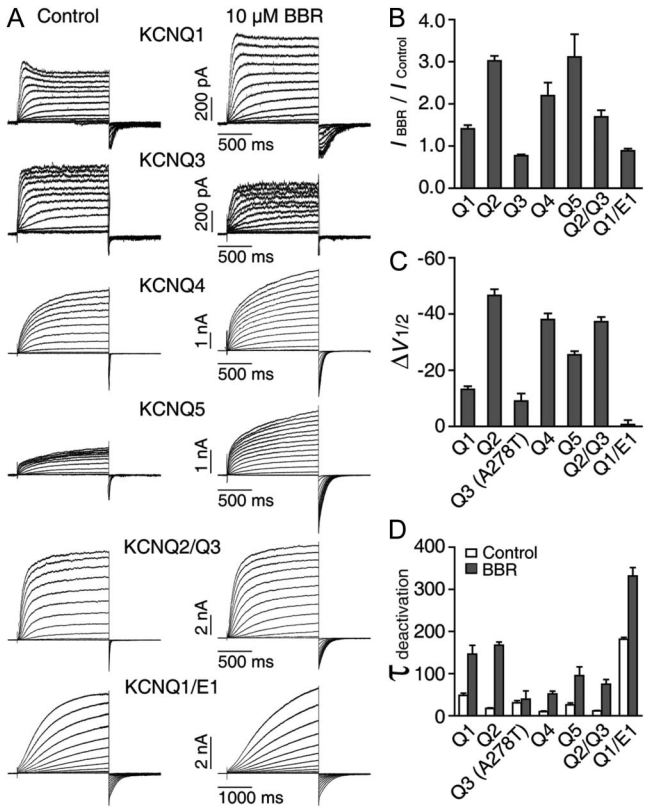

Figure 2. Subtype selectivity of benzbromarone (BBR) on KCNQ channels. (A), Representative current traces (left) and voltage-dependent activation curves (right) of KCNQ isoforms as indicated in the absence and the presence of 10 μM BBR. (B-D), Bar graphs showing effects of 10 μM BBR on the outward current at -10 mV (B), $\Delta V_{1/2}$ (C), and deactivation of tail currents at -120 mV developed after depolarization to +50 mV (D) for different KCNQ isoforms.

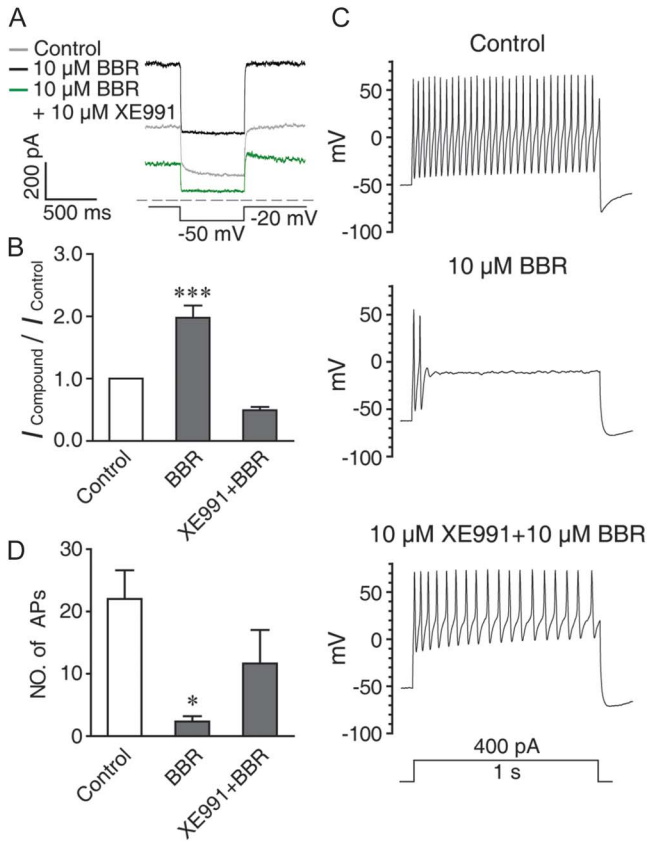

Figure 3. Potentiation effects of benzbromarone (BBR) on native M-current in dorsal root ganglion (DRG) neurons. (A), Representative traces of M-current recorded from cultured DRG neurons in the absence (gray) and presence of 10 μM BBR. XE991 (10 μM) was applied at the end of the experiments to inhibit M-current (green). Voltage protocol used for recording native M-current was shown at the bottom. (B), Bar graph showing augmentation of the current amplitude at −20 mV by 10 μM BBR and inhibition by XE991. (C), Representative traces of action potential firing recorded from a DRG neuron before (top) and during (middle) application of 10 μM BBR. XE991 (10 μM) was applied at the end of the recording to inhibit M-current (bottom). (D), Bar graph showing the suppression of action potential (AP) firing by 10 μM BBR and its reversal by XE991.

equation, $G = G_{min} + (G_{max} - G_{min})/(1 + \exp[V - V_{1/2}]/S)$, where G_{max} is the maximum conductance, G_{min} is the minimum conductance, $V_{1/2}$ is the voltage for reaching 50% of maximum conductance, and S is the slope factor. Dose–response curves were fitted with the Hill equation, $E = E_{max}/[1 - (EC_{50}/C]^P)$, where EC_{50} is the drug concentration producing the half-maximum response, and P is the Hill coefficient. The data are presented as mean ± SEM, and the significance was estimated using unpaired 2-tailed Student *t* tests unless otherwise stated. Statistical significance: $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

3. Results

3.1. Identification of benzbromarone as an activator of KCNQ2 channels

In a screen evaluating the effects of marked drugs or drug candidates on KCNQ2 channels, we screened 1280 compounds from the US Drug Collection library against a stable CHO cell line expressing the homomeric KCNQ2 channel using a thallium assay. This fluorescence-based assay was used to identify hexachlorophene, a potent KCNQ1/KCNE1 potassium channel activator, in our previous report.[72] From the primary screen, BBR was found to significantly increase the fluorescence signal at 10 μM (Supplemental Figure 1A, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). In addition, the potentiation of the fluorescent signal by BBR was concentration dependent, with 30 μM causing a 2-fold increase in fluorescence over that measured at 10 μM (Supplemental Figure 1B, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48).

To confirm the findings of the fluorescence assay, we examined the effects of BBR on KCNQ2 channels using whole-cell voltage clamping, the gold standard for studying ion channels. The application of 10 μM BBR caused a 3.02 ± 0.12-fold (n = 4) increase in the KCNQ2 current at −10 mV test potential (**Fig. 1A**). Further analysis of the concentration dependence of BBR on current amplitudes at −10 mV revealed an EC_{50} value of 4.32 ± 1.40 μM (n = 4) (**Fig. 1B**). We then examined the influence of BBR on the voltage dependent activation and kinetics of KCNQ2. Similar to other reported activators, such as RTG, NH29, and ICA-27243, etc,[7,25,43,64,69] BBR significantly left-shifted the voltage dependent activation curve (G-V curve) and slowed the deactivation kinetics. With 10 μM BBR, the $V_{1/2}$ of the KCNQ2 channel was left-shifted approximately 45.6 ± 4.5 mV from −0.5 ±1.2 mV in the absence of BBR to −47.1 ± 3.3 mV in the presence of BBR (n = 4, $P < 0.0001$) (**Fig. 1C**). The deactivation time constant for the tail current at −120 mV, stepped back from the +50 mV

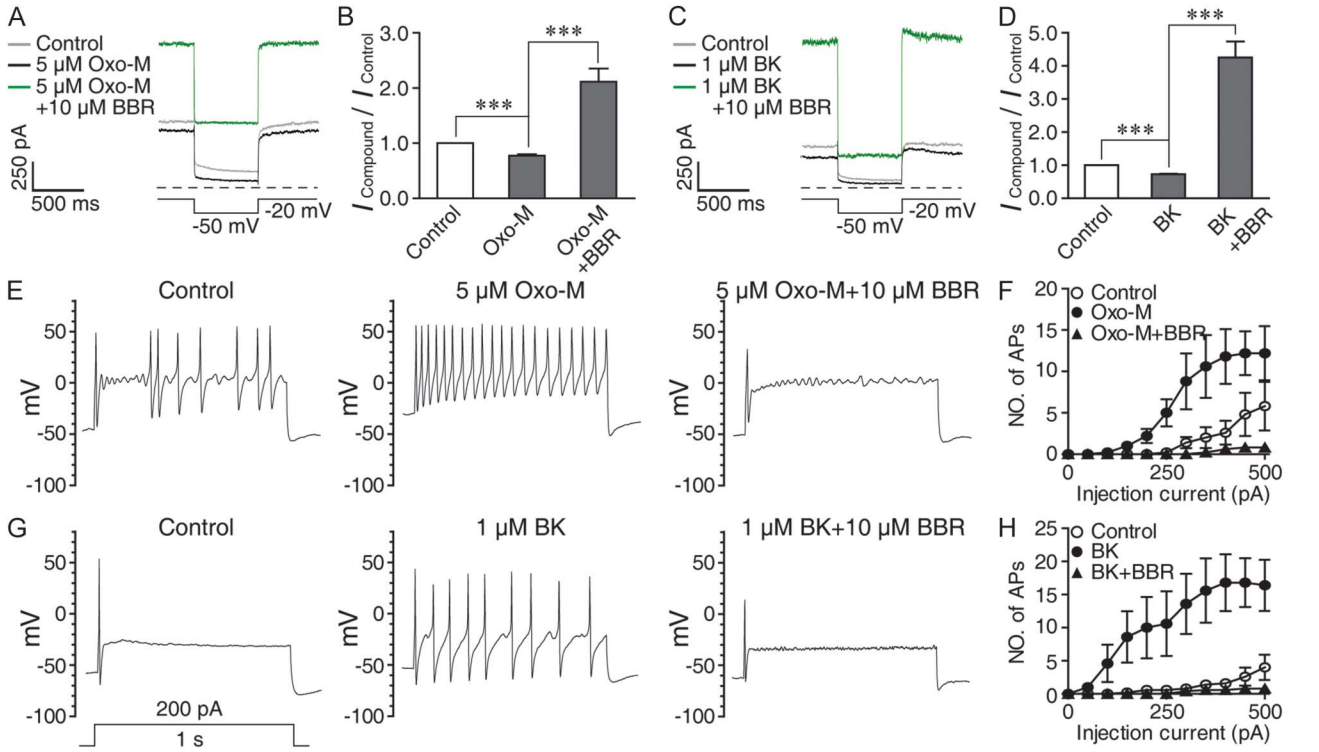

Figure 4. Benzbromarone (BBR) reverted inhibition of M-current and overexcitability of dorsal root ganglion (DRG) neurons induced by Oxo-M and bradykinin (BK). (A), Representative traces of M-current in a DRG neuron before (Control) and during successive applications of Oxo-M (5 μM) and Oxo-M + BBR (10 μM). Voltage protocol used for recording native M-current was shown at the bottom. (B), Bar graph showing that Oxo-M (5 μM) suppressed the native M-current and BBR (10 μM) overcame the suppression. (C), Similar to (A), but BK (1 μM) was used in place of Oxo-M. (D), Bar graph showing that BK (1 μM) suppressed the native M-current and BBR (10 μM) overcame the suppression. For both (B) and (D), *** $P < 0.001$, n = 5. (E and G), Representative current clamp traces of membrane potential responses of DRG neurons to the injection of a +200 pA depolarizing current before (control) and during successive applications of Oxo-M (E) or bradykinin (BK) (G) and then Oxo-M (E) or BK (G) plus BBR. (F and H), Dependence of action potential (AP) firing on the amount of injected currents in the absence (control) and presence of Oxo-M (F) and BK (H) without or with BBR. Note also the dramatic changes in the threshold of action potential (AP) firing induced by the drugs.

depolarization pulse, increased from 17.5 ± 1.7 milliseconds in the absence of BBR to 167.6 ± 7.5 milliseconds in the presence of BBR (n = 4, $P < 0.001$) (**Fig. 1D and E**). Unlike BBR, the 2 alternative ULT drugs used as a first-line treatment, ALO and PRB, did not exhibit potentiation effects on KCNQ2 channels (**Fig. 1A, F, and G**). Taken together, these results demonstrate that BBR is an activator of KCNQ2 channels.

3.2. Subtype selectivity of benzbromarone

KCNQ members share considerable sequence homology. To obtain a better understanding of BBR activity on KCNQ channels, we examined its effects on KCNQ1, KCNQ1/KCNE1, KCNQ3, KCNQ4, and KCNQ5 channels using a whole-cell patch clamp with transiently transfected CHO cells. At 10 μM, BBR potentiated KCNQ1, KCNQ4, and KCNQ5 but not KCNQ3. Interestingly, the potentiation effects of BBR on KCNQ1/KCNE1 complex seems were weaker than those on KCNQ1 alone (**Fig. 2**). Although all neuronal KCNQ isoforms contribute to the native neuronal KCNQ current, heteromultimers of KCNQ2/KCNQ3 are thought to represent a major component of the M-current.[3,44,54,55,63] To test the effects of BBR on KCNQ2/KCNQ3 heteromultimers, the KCNQ2 and KCNQ3 cDNA was cotransfected at an equimolar ratio into CHO cells, and currents were elicited using the same protocol used for KCNQ2 and KCNQ3 homomeric channels. At the −10 mV testing potential, BBR (10 μM) increased the outward currents of KCNQ2/KCNQ3 by approximately 1.64 ± 0.08-fold (n = 7), which was slightly weaker than that observed for KCNQ2 channels. The left-shift of the G-V curve and slowing of deactivation were observed in KCNQ2/KCNQ3 channels. The $V_{1/2}$ values before and during application of 10 μM BBR were 0.4 ± 1.1 mV and −36.9 ± 2.2 mV (n = 7), respectively. The time constants of deactivation were 11.8 ± 1.1 milliseconds (n = 7) in the absence and 74.9 ± 11.2 milliseconds (n = 7) in the presence of BBR. These results suggest that KCNQ2 plays a dominant role in conferring BBR sensitivity to KCNQ2/KCNQ3 heteromultimers.

3.3. Benzbromarone potentiates the native M-current and dampens neuronal excitability

After confirming the potentiation of KCNQ2/KCNQ3 heteromultimer channels by BBR in the heterologous system (**Fig. 2**), we examined the effects of BBR on the native M-current in cultured rat DRG and hippocampal neurons. The native M-current was recorded in small-diameter DRG neurons, which represent mostly sensory neurons.[4] With the application of 10 μM BBR by bath perfusion, the current was increased by 197% ± 19% (n = 6). Both the basal and BBR-enhanced currents were inhibited by XE991, a specific inhibitor of KCNQ channels, confirming the specific effect of BBR on the M-current (**Fig. 3A and B**). As potentiation of the M-current is known to downregulate neuronal excitability,[40] we tested the effect of BBR on action potentials evoked by injection of a +400 pA current. As expected, 10 μM BBR suppressed the generation of action potential firing, and this effect was reversed by coapplication of the neurons with 10 μM XE911 (**Fig. 3C and D**). Similar results were obtained from cultured hippocampal neurons (Supplemental Figure 2, available

online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). These data indicate that BBR can potentiate native KCNQ channels and dampen the excitability of neurons.

3.4. Benzbromarone suppresses neuronal overexcitability induced by inflammatory mediators

It has been suggested that the inhibition of KCNQ channels by inflammatory mediators coupled to phospholipase C (PLC) activation results in overexcitability of sensory neurons and inflammatory pain.[31–33] We reasoned that by enhancing KCNQ function, BBR could overcome the inhibitory action of inflammatory mediators and thereby prevent overexcitation of DRG neurons. We first used OXO-M, an activator of muscarinic receptors, to activate the inhibitory PLC signaling. Five micormolar OXO-M significantly suppressed the M-current in small diameter neurons, but 10 μM BBR not only reverted the suppression but also further augmented the current (**Fig. 4A and B**). The average potentiation by BBR was 2.11 ± 0.24-fold (n = 10) at −20 mV. Second, we tested BK, an inflammatory mediator known to inhibit the M-current through the PLC pathway.[33] Similarly to OXO-M, 1 μM BK significantly suppressed the M-current in DRG neurons, but 10 μM BBR completely overcame the inhibitory effect of BK and further potentiated the current by 4.25 ± 0.48-fold (n = 6) (**Fig. 4C and D**). Moreover, both OXO-M and BK caused overexcitability of DRG neurons, as shown by the increased numbers of action potentials in response to the injection of +200 pA current (**Fig. 4E and G**). The application of BBR (10 μM) completely abolished the upregulated firing (**Fig. 4E and G**). The quantification of the number of action potentials firing elicited by different amounts of current injection revealed that BBR not only reduced the firing rate but also increased the firing threshold (**Fig. 4F and H**). Consistent with the in vitro experiments above, in the BK-induced pain behavioral test in mice, administration of BBR (50 mg/kg body weight, i.p.) significantly suppressed the nociceptive responses of BK-injected animals, including both licking time and the pain behavioral score (Supplemental Figure 3, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). These results indicate that BBR can effectively overcome overexcitability of DRG neurons induced by inflammatory mediators.

3.5. Analgesic effects of benzbromarone in formalin-induced inflammatory pain

The formalin test is a classical inflammatory pain model. Intraplantar injection of formalin results in a biphasic nociceptive behavior characterized by licking and flinching of the affected hind paw in rats. The first phase (0-10 minutes) reflects mostly nociceptive pain, whereas the second phase (11-60 minutes) represents the inflammatory responses.[37,61] To examine the effects of BBR on inflammatory pain, we administered i.p. BBR, and the 2 alternative first-line treatment drugs, ALO and PRB, all at 50 mg/kg body weight individually 30 minutes before intraplantar injection of formalin. Consistent with the in vitro experiments, BBR but not ALO or PRB, significantly attenuated pain behaviors, including the licking time and overall pain score in both phases of nociceptive behavior in formalin-injected rats. The attenuation of the pain score was more pronounced than the licking time (**Fig. 5**). Supporting the essential role of KCNQ channels in the BBR effect, the anti-inflammatory pain action of BBR on the pain score was blocked by XE991 (3 mg/kg body weight, i.p.) (**Fig. 5C**). The anti-inflammatory pain activity of BBR was also observed in mice. In the formalin-induced mouse inflammatory pain model, BBR dose-dependently suppressed the pain behavior in the measured parameters, ie, the licking time and overall pain score (Supplemental Figure 4, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). The anti-inflammatory pain activity of BBR is consistent with its potentiation activity on KCNQ channels.

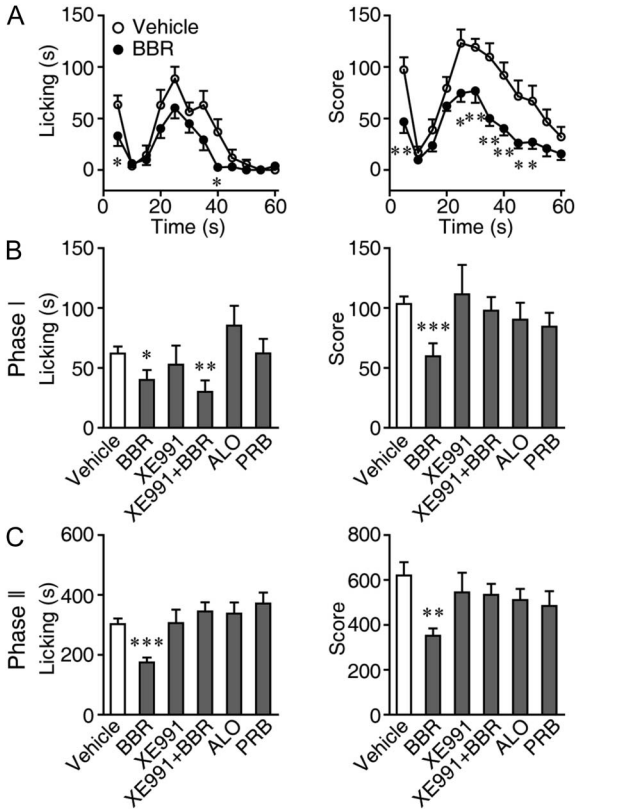

Figure 5. Analgesic effects of benzbromarone (BBR) in the formalin-induced rat inflammatory pain model. (A), BBR attenuated the biphasic pain responses, including both licking time (left) and score (right) throughout the 60-minute trial. (B and C), Bar graph showing the effects of BBR, allopurinol (ALO), and probenecid (PRB) on the formalin-induced inflammatory pain. Phase I (B) and phase II (C) were displayed in separated panels for clarity. In the phase II, coapplication of XE991 (3 mg/kg, i.p.), a KCNQ channel blocker reversed the reduction of both the licking time and the score caused by BBR. In the phase I, application of XE991 only reversed the reduction of score. In all groups, n = 6 to 17 animals.

3.6. Benzbromarone attenuates monosodium urate–induced articular nociception in rats

Monosodium urate crystals are the etiological agents of gout.[45] Therefore, the potential effects of BBR on MSU-induced acute inflammatory pain were evaluated. Injection of needle-shaped MSU (1.25 mg) in the rat ankle caused significant nociception behavioral responses characterized by limping, paw pressure reduction on the injected limb, and ankle edema (**Fig. 6A and B**). Benzbromarone was administered 20 hours after the MSU injection, and subsequently the paw pressure of the injected limb was assessed. Indomethacin (IMC), a NASID, was set as the positive control. The diluent solution was used as the negative control (vehicle). We found that BBR (50 mg/kg, i.p.) exhibited

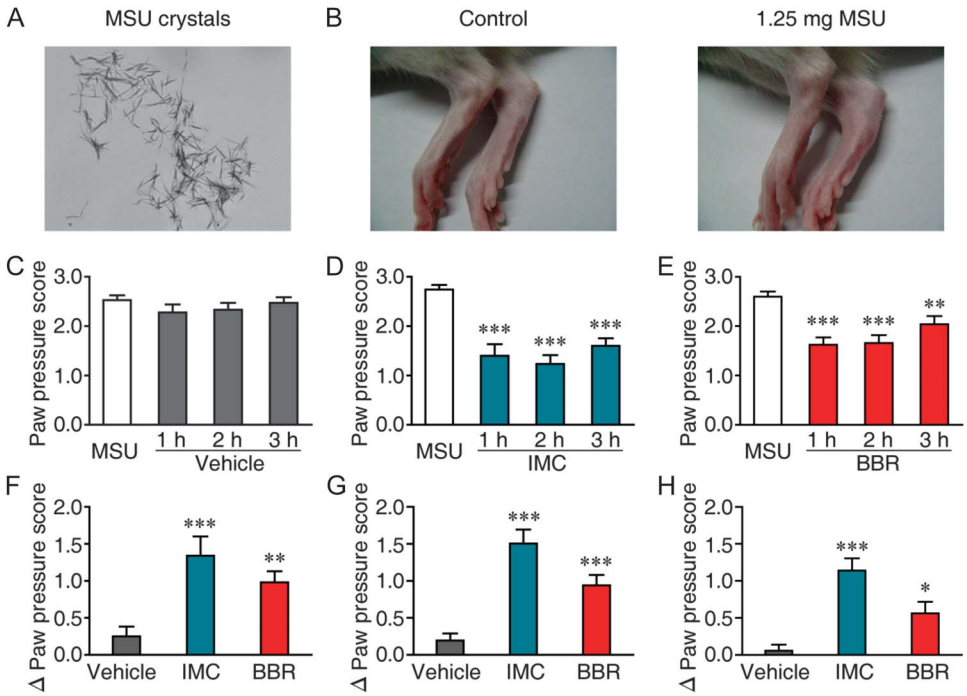

Figure 6. Benzbromarone (BBR) attenuated the nociception induced by intra-articular monosodium urate (MSU) in rats. (A), Needle-shaped MSU crystals. (B), Graphs showing the rat ankles without (control) and with 1.25 mg MSU injection. (C-E), Bar graphs showing the paw pressure score of injected rat limbs intraperitoneally treated with vehicle (C), 10 mg/kg indomethacin (IMC) (D) and 50 mg/kg BBR (E). (F-H), Bar graphs showing the Δpaw pressure score at 1 hour (D), 2 hours (E), and 3 hours (F), respectively. The Δpaw pressure score represented a decrease in nociceptive behavior before and after the administration as indicated. In all groups, n = 12 to 24 animals.

comparable analgesic effects to 10 mg/kg IMC. Compared with the values before BBR, the paw pressure scores at all examined time points (1 hour, 2 hours, and 3 hours) were significantly reduced by BBR (**Fig. 6E**). In contrast, the MSU-induced ankle edema was not affected (Supplemental Figure 5, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). In a parallel experiment, RTG (12.5 mg/kg, i.p.) and FLP (20.0 mg/kg, i.p.) effectively attenuated paw pressure in more than 50% of MSU-injected animals (Supplemental Table 1, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). However, the central sedation and impaired motor coordination induced by the 2 drugs prevented further analgesic assessment at higher doses. Being a ULT drug, BBR is only used for chronic treatment in the clinic.[71] The attenuation of MSU-induced pain by BBR suggests that potentiation of KCNQ channels is beneficial for gout pain relief.

3.7. Benzbromarone primarily acts on peripheral KCNQ channels

KCNQ channels are expressed in both the CNS and PNS. Activation of CNS KCNQ channels may cause CNS side effects. Unlike other KCNQ activators on the market, such as RTG and FLP, BBR has few reported CNS side effects. We did not observe any severe CNS side effects, such as sedation or impaired motor coordination, from BBR at or below 50 mg/kg (i.p.) in our experiments. Pharmacokinetic analysis revealed that the brain/plasma ratio of BBR was as low as 0.1 (**Table 1**). In vitro binding assays revealed that the brain protein binding ratio of BBR was almost 100.0% (Supplemental Table 2, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). Given that BBR is bound to protein with a similar ratio in vivo, free BBR in the brain would be rather low. Benzbromarone at doses at or below 50 mg/kg body weight, which were effective to relieve pain in BK, formalin, and MSU tests, consistently lacked antiepileptic activity in a seizure model (Supplemental Figure 6, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). Furthermore, the anti-inflammatory pain activity of local treatment with BBR was only observed when it was injected into the ipsilateral, and not the contralateral, plantar with formalin. As shown in **Figure 7**, BBR (100 nmol per site) effectively attenuated rat pain behaviors in both phase I and phase II. However, the same dose of BBR was ineffective when it was injected into the contralateral plantar. Taken together, these data suggest that peripheral KCNQ channels are the major targets of BBR in relieving inflammatory pain.

Table 1

Pharmacokinetic parameters of mice orally (20 mg/kg) given benzbromarone (BBR).

Compound	Sample	T_{max}, h	C_{max}, ng/mL (g)	AUC_{0-t}, ng·h/mL (g)	$AUC_{0-\infty}$, ng·h/mL (g)	MRT, h	$t_{1/2}$, h	Ratio of AUC_{0-t}, brain:plasma
BBR	Plasma	0.25	21,270	38,182	38,186	2.23	1.85	0.10
	Brain	0.50	3689	3820	3850	0.80	0.40	

$AUC_{0-\infty}$, area under the plasma concentration–time curve from time zero to infinity; AUC_{0-t}, area under the plasma concentration–time curve from time zero to the last measurable concentrations calculated by the trapezoidal method; C_{max}, maximum observed plasma concentration; MRT, the mean residence time; $t_{1/2}$, half-life; T_{max}, time to reach C_{max}.

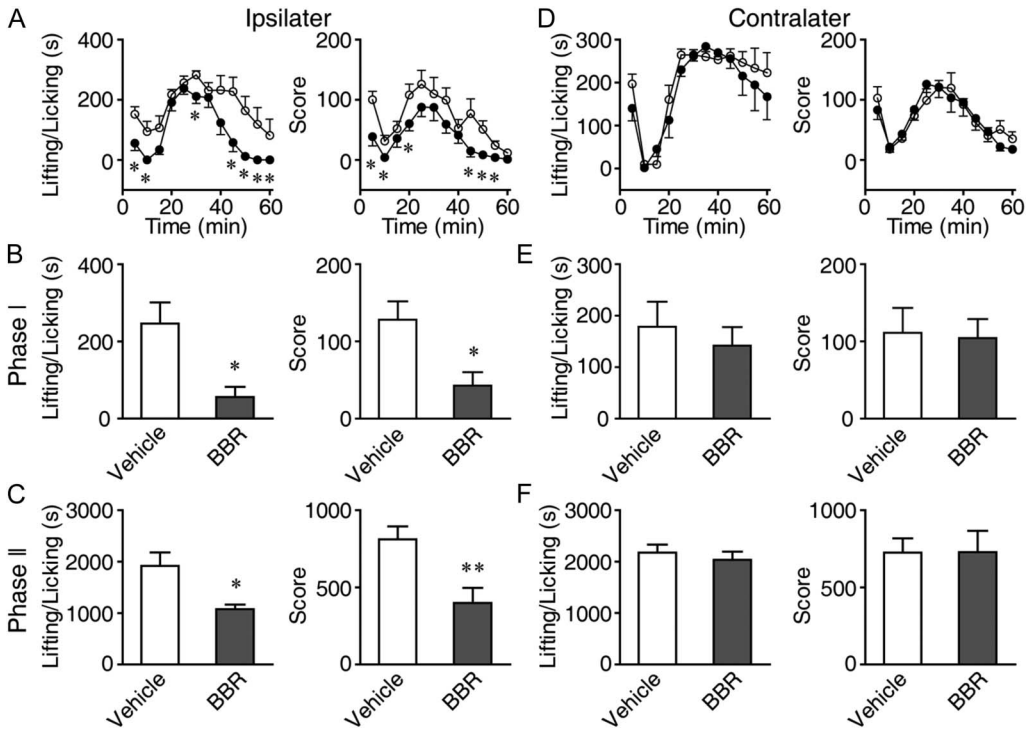

Figure 7. Analgesic effects of local injection of benzbromarone (BBR) in the formalin-induced rat inflammatory pain model. (A-C), Intraplantar administration of BBR (100 nmol) attenuated ipsilateral formalin-induced inflammatory pain, including both the lifting/licking time (left) and score (right). (D-E), Intraplantar administration of BBR (100 nmol) could not attenuate contralateral formalin-induced inflammatory pain. The influence of BBR on the lifting/licking time (left) and score (right) were evaluated, respectively. In all groups, n = 6 animals.

3.8. Essential role of voltage sensing domain for potentiation activity of benzbromarone

It has been reported that the pore region and voltage sensing domain (VSD) may be involved in activator effects. For example, the tryptophan residue (W236) in S5 of KCNQ2 was reported to be critical for RTG activity, whereas 2 leucine residues (L245 and L279) in S5 and the S5-S6 loop of KCNQ2 were critical for zinc pyrithione (ZnPy).[51,68,69] In addition, some residues (eg, R198 of KCNQ2) are critical for the action of NH29, another KCNQ activator, and also have been identified in the superficial part of VSD.[43] Most recently, the hydrophobic residue, F137 in the middle of KCNQ2 S2, was demonstrated to be critical for ztz240 binding in a deep, water-accessible pocket in the VSD.[30] To explore the potential site of BBR action, the effects of mutating these previously identified residues on the potentiation effects of BBR were individually tested. We found that mutations of the essential residues in the pore region did not affect the activity of BBR (Supplemental Figure 7, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). The mutation F137A, which largely reduced KCNQ2 sensitivity to ztz240, did not change BBR activity (Supplemental Figure 7, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). However, the R198A substitution, which lacks sensitivity to NH29, resulted in the loss of responses to BBR, including an increase of the current amplitude, a slowing of deactivation and a left shift of the G-V curve (**Fig. 8A and C**; Supplemental Table 3, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). Importantly, the concentration response curve of R198A to BBR was largely right shifted (**Fig. 8B**). The pronounced reduction in the apparent affinity of R198A compared with the wild-type KCNQ2 (EC_{50} = 28.20 ± 1.32 μM vs 4.32 ± 1.40 μM of wild type) suggests that the R to A mutation in position 198 may affect BBR interactions with the channel or a weaker response of gating to the same BBR–channel interaction.

R198 represents the first positive gating charge located at the external end of S4. If BBR recognizes the superficial portion of VSD, other residues besides R198 in this neighboring region would influence BBR's effects. Therefore, residues in the external part of the KCNQ2 VSD were individually mutated to alanines and tested for their response to BBR. Indeed, alanine scanning revealed multiple essential residues for potentiation by BBR (**Fig. 8** and Supplemental Table 3, available online as Supplemental Digital Content at http://links.lww.com/PAIN/A48). In addition to R198A, the mutations C106A, V111A, S121A, L126A, F137A, G186A, T194A, and L200W were found to significantly reduce potentiation on the outward currents (**Fig. 8D**, upper panel). Six mutations, C106A, Y127C, N190A, L197A, R201A, and R207W, reduced the left-shift of G-V curves by BBR to varying degrees. Noticeably, distinctly from R198A, the left-shifts of G-V curves in the 6 mutants above were all significant, with a $\Delta V_{1/2}$ larger than 13 mV (**Fig. 8D**, lower panel). To further evaluate the contributions of these residues, 6 pairs of double mutations were constructed and tested individually. All 6 mutants exhibited reduced responses to BBR. Particularly, 2 pairs of double mutants, C106AR207W and C106AN190A, almost completely abolished the left-shift of G-V curves. Additionally, C106AR207W and Y127CR207W lost augmentation of outward currents by BBR (**Fig. 8D**). Taken together, these data reveal an essential role of VSD for the potentiation activity of BBR on KCNQ2 channels.

4. Discussion

Gout is a disorder of purine metabolism characterized by elevated urate levels and deposition of MSU crystals in joints and other

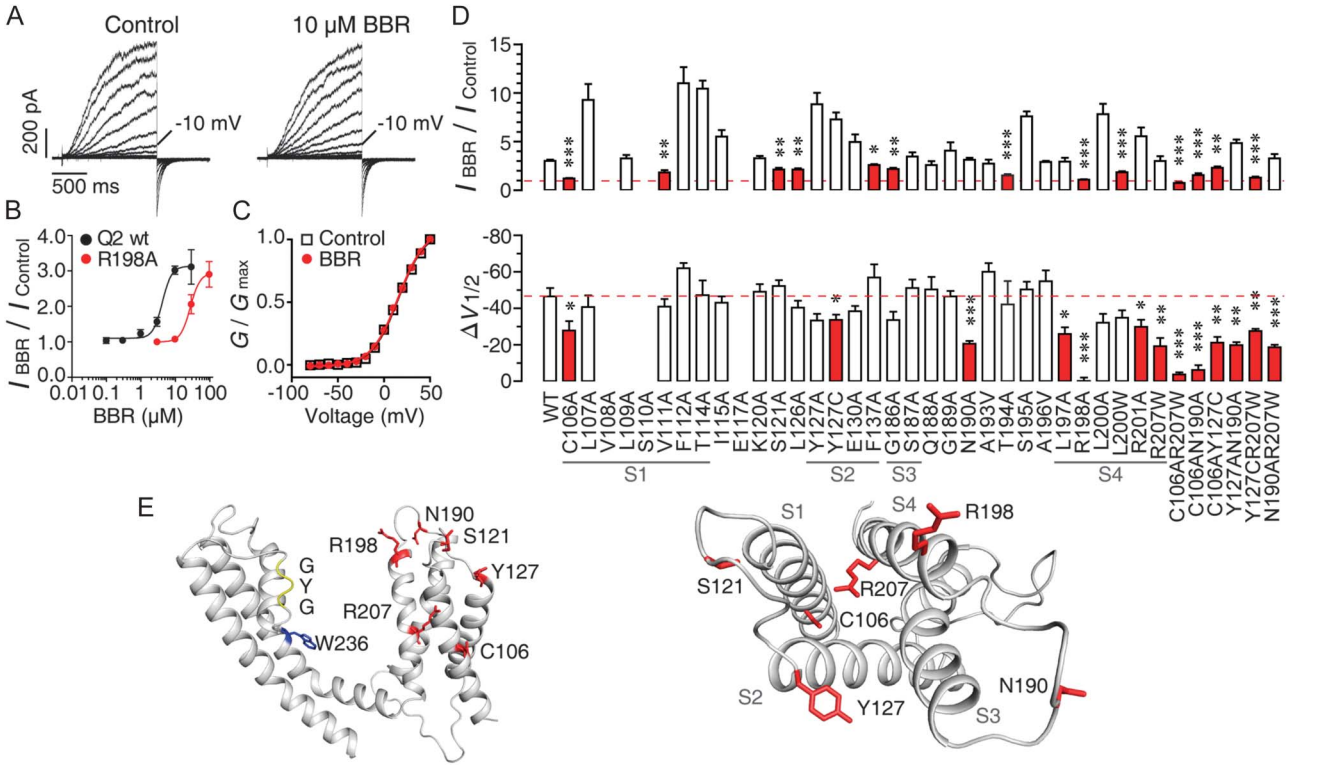

Figure 8. Critical residues for benzbromarone (BBR) potentiation activity. (A), Representative traces of R198A mutant with or without BBR. (B), Dose–response curve of BBR on wild-type and R198A KCNQ2 channels. (C), Activation curve of R198A in the presence or absence of BBR. (D), Bar graphs showing the effect of 10 μM BBR on outward current amplitude (upper panel) and $\Delta V_{1/2}$ (lower panel) of voltage sensing domain (VSD) mutants. (E), Modeling 1 subunit (left) and the VSD (right) of KCNQ2 channels. G (glycine), Y (tyrosine), and G (glycine) represented the residues forming the selectivity filter. W236 in the pore region was the reported interaction site for retigabine. Key residues for BBR potentiation activity were highlighted in red.

tissues. In humans, the upper end of the urate range is 360 to 400 μmol/L. An elevated urate level above 420 μmol/L slowly results in MSU crystals and induces serious complications in the tissues where the crystals are deposited.[45] In addition to lifestyle modifications, the treatments for gout can be split into 2 areas.[45] For an acute attack, the aim of the treatment is to relive pain promptly and safely. Nonsteroidal anti-inflammatory drugs, colchicines, and corticosteroids are 3 general medicines used for pain relief. However, these medicines are not helpful in preventing pathological damage. For chronic gout, the aim of long-term treatment is to reduce blood urate levels to 360 μmol/L and the saturation point of MSU and to prevent new crystal formation and accelerating dissolution of existing crystals. Benzbromarone has been reported to be the inhibitor of URAT-1 and SLC2A9, 2 urate transporters.[12,18] URAT-1 is a urate–anion exchanger located at the apical brush of the proximal nephron, whereas SLC2A9 is a urate transporter facilitated by glucose. Although BBR was withdrawn by its original manufacturer, Sanofi-Synthélabo, after reports of serious hepatotoxicity in 2003, a benefit–risk assessment reported that based on the limited reported cases, there was no clear relationship between the drug and liver injury and that the withdrawal was unwarranted.[12] Except for possible hepatotoxicity, adverse effects associated with BBR have been relatively infrequent because the drug was approved in the 1970s. Therefore, BBR is still widely used because it is well tolerated and highly effective. At the usual dose of 100 mg, the peak plasma concentration of BBR in healthy adults is around 7 to 8 μM.[62] Clearly, BBR at this concentration effectively potentiated KCNQ channels in both recombinant cells and native neurons (**Figs. 1-3**). Interestingly, celecoxib, a NASID, which is effective and well tolerated for acute gout pain, has also been demonstrated to be an activator of KCNQ channels.[10,17,52] Therefore, this indicates that BBR activity is involved in more than just MSU crystal clearance. The dual effects of BBR, ie, the inhibition of urate transporters and potentiation of KCNQ channels, may underlie its superior pain relief activity in gout treatment.

A growing body of evidence supports the notion that BBR primarily acts on the peripheral KCNQ channels. It has been reported that PNS KCNQ channels play important roles in the regulation of sensory nerve activity.[9,22,26,46,53,63,65,66] For example, activation of KCNQ channels by FLP downregulates the excitability of myelinated axons in isolated rat sural nerves.[56] Furthermore, RTG was demonstrated to produce long-lasting and robust hyperpolarization of primary afferents in rat and reduce the excitability of sensory fibers in isolated fascicles of human sural nerves.[46] Focal application of RTG to DRG reduces peripheral nociceptive transmission in rats in vivo.[16] In contrast, suppression of peripheral KCNQ channels may upregulate neuronal excitability. Electrophysiology recordings in single afferents in vitro show that XE991 significantly sensitizes A delta-fibers to noxious heat stimulation and induces spontaneous ongoing activity in many A delta-fibers.[41] Consistent with the in vitro experiments above, intraplantar injection of XE991 into the hind paw produces acute pain while local treatment with RTG effectively attenuates pain behaviors induced by formalin in rats.[21,32] In addition, inflammatory mediators, such as protease and BK, show inhibitory effects on peripheral KCNQ channels, which may represent one of the general mechanisms underlying inflammatory pain.[32,33] Notably, an increasing number of studies have reported upregulated production of inflammatory mediators in MSU affected tissues.[11] In addition to the possible

inhibition of KCNQ channels by inflammatory mediators, the reduction of peripheral nerve fibers and sensitization of nociceptors may also contribute to intense gout pain.[20,34] Although the expression or function of KCNQ channels in joint tissue is rarely investigated, the data above suggest that BBR's activation of peripheral KCNQ channels contributes to its pain relief activity.[31,41]

Compared with the pore region, the VSD has not been targeted for therapeutic purposes.[35,38,39,43] NH29 is the first demonstrated KCNQ activator acting on the VSD. It binds to the superficial portion of the VSD, augmenting KCNQ channel activity through a VSD trapping mechanism, and thus is defined as a gating modifier.[43] Recently, we reported that ztz240 binds within a much deeper pocket in the VSD than NH29. ztz240 resides in the VSD filling the space between the fourth and fifth gating charge, thereby preventing arginine from going down to its resting state.[30] Notably, although the VSD has been proven to be an effective target for epilepsy and neuropathic pain in animal models, none of its activators achieved therapeutic effects in human diseases. Being a widely used drug on the market, BBR may provide the first practical example that the VSD of the KCNQ channels is targetable.

In summary, our study demonstrates that BBR is a KCNQ activator that primarily acts on peripheral KCNQ channels. The discovery of the analgesic activity of BBR in inflammatory pain models is significant given that PNS KCNQ channels are preferable analgesic targets that can reduce the potential for CNS side effects. The activation of KCNQ channels alone or in combination with other treatments may provide an alternative choice for patients suffering acute attacks or recurrent flare-ups of gout who are less sensitive to or not tolerant of current medications.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental Digital Content

Supplemental Digital Content associated with this article can be found online at http://links.lww.com/PAIN/A48.

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