Note

Effects of Bepridil and Pimozide, Existing Medicines Capable of Blocking T-Type Ca^{2+} Channels, on Visceral Pain in Mice

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Received September 14, 2020; accepted December 21, 2020

T-Type Ca^{2+} channels (T-channels), particularly $\text{Ca}_v3.2$, are now considered as therapeutic targets for treatment of intractable pain including visceral pain. Among existing medicines, bepridil, a multi-channel blocker, used for treatment of arrhythmia and angina, and pimozide, a dopamine D_2 receptor antagonist, known as a typical antipsychotic, have potent T-channel blocking activity. We thus tested whether bepridil and pimozide could suppress visceral pain in mice. Colonic and bladder pain were induced by intracolonic administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) and systemic administration of cyclophosphamide (CPA), respectively. Referred hyperalgesia was assessed by von Frey test, and colonic hypersensitivity to distension by a volume load with intracolonic water injection and spontaneous bladder pain were evaluated by observing nociceptive behaviors in conscious mice. The mice exhibited referred hyperalgesia and colonic hypersensitivity to distension on day 6 after TNBS treatment. Systemic administration of bepridil at 10–20 mg/kg or pimozide at 0.1–0.5 mg/kg strongly reduced the referred hyperalgesia on the TNBS-induced referred hyperalgesia and colonic hypersensitivity to distension. CPA treatment caused bladder pain-like nociceptive behavior and referred hyperalgesia, which were reversed by bepridil at 10–20 mg/kg or pimozide at 0.5–1 mg/kg. Our data thus suggest that bepridil and pimozide, existing medicines capable of blocking T-channels, are useful for treatment of colonic and bladder pain, and serve as seeds for the development of new medicines for visceral pain treatment.

Key words bepridil; pimozide; visceral pain; $\text{Ca}_v3.2$; T-type calcium channel

INTRODUCTION

Among three isoforms of T-type Ca^{2+} channels (T-channels), $\text{Ca}_v3.2$ participates in pathological pain including visceral pain, and is now considered a target for treatment of intractable pain.^{1,2)} A number of selective T-channel blockers have been developed.³⁾ Apart from ethosuximide, a classic T-channel blocker used for treatment of absence seizures, some of existing medicines including bepridil, an anti-arrhythmic and anti-anginal medicine, and pimozide, an antipsychotic agent, have potent T-channel blocking activity. Bepridil, a multi-channel blocker, inhibits $\text{Ca}_v3.2$ channel-currents in a state-dependent manner; the IC_{50} (μM) being 0.4, 1.4, 7.7 and 10.6 at the holding potentials of -70 , -80 , -90 and -100 mV, respectively.⁴⁾ Pimozide, a dopamine D_2 receptor antagonist, blocks $\text{Ca}_v3.2$ -currents; the IC_{50} being $0.058 \mu\text{M}$ at a holding potential of -100 mV.⁵⁾ The potency of bepridil and pimozide in inhibiting T-channel-dependent currents is comparable to well-known selective T-channel blockers.³⁾ Given the essential role of $\text{Ca}_v3.2$ T-channels in visceral pain,^{1,2,6,7)} we tested whether bepridil and pimozide could reduce the visceral pain accompanying colitis caused by 2,4,6-trinitrobenzene sulfonic acid (TNBS) or cystitis caused by cyclophosphamide (CPA) in mice.

MATERIALS AND METHODS

Animals Male and female ddY mice (4–5 weeks old) were purchased from Kiwa Laboratory Animals Co. Ltd. (Wakayama, Japan). The animals were housed in a room maintained around 24°C under a 12-h day–night cycle, and had free access to food and water before the use for experiments. All experimental protocols were approved by Kindai University's Committee for the Care and Use of Laboratory Animals and were in accordance with the Guiding Principles approved by The Japanese Pharmacological Society.

Major Chemicals TNBS was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and CPA, bepridil, pimozide and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). TNBS was dissolved in 50% ethanol, and CPA was in saline. Pimozide was dissolved in saline containing 2% Tween-20 and 3.3% DMSO, and bepridil was in saline containing 5% ethanol and 50% polyethylene glycol.

Creation of a TNBS-Induced Colitis-Related Colonic Pain Model and Assessment of Colonic Pain/Hypersensitivity in Mice Essentially as reported elsewhere,⁶⁾ male mice were anesthetized with inhalation of isoflurane, and received intracolonic administration of TNBS at 2 mg/mouse in a volume of $50 \mu\text{L}$. Referred hyperalgesia and colonic hypersensitivity to distension were evaluated on day 6 after intracolonic TNBS, according to our previous report.⁶⁾ Briefly, referred hyperalgesia was evaluated by stimulating the lower abdomen of mice with von Frey filaments (strength: 0.008, 0.02, 0.16 and 1.0 g).

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Fig. 1. Effects of Bepridil (A, B) and Pimozide (C, D), Existing Medicines Capable of Blocking T-Type Ca^{2+} Channels, on the Referred Hyperalgesia and Behavioral Colonic Hypersensitivity to Distention by a Volume Load in Mice Treated with TNBS

Mice received intracolonic (i.col.) administration of TNBS at 2mg/mouse or vehicle (V; saline) in a volume of 50 μ L under isoflurane anesthesia. On day 6 after the TNBS treatment, referred hyperalgesia in the lower abdomen was assessed by von Frey test (A, C). Thereafter, the freely moving mice received intracolonic infusion of 200 μ L water for application of a volume load, and the number of nociceptive behaviors was then counted for 30 min (B, D). Bepridil at 10 or 20 mg/kg (A, B) and pimozide at 0.1 or 0.5 mg/kg (C, D) were administered intraperitoneally (i.p.) 15 and 30 min, respectively, before the von Frey test. Statistical significance was analyzed by ANOVA followed by Tukey's test for (B, D) and by the Kruskal–Wallis H-test followed by LSD-type test for (A, C). Data show the mean with standard error of the mean (S.E.M.) for 7 (A, B) or 4–5 (C, D) mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. [(A): 0.008 g; Kruskal–Wallis chi-squared (χ^2) = 9.87, degree of freedom (DF) = 3, $p = 0.020$, 0.02 g; $\chi^2 = 13.50$, DF = 3, $p = 0.004$, 0.16 g; $\chi^2 = 7.88$, DF = 3, $p = 0.049$, 1.0 g; $\chi^2 = 8.10$, DF = 3, $p = 0.044$. (B): F value = 4.25, DF = 3, $p = 0.015$. (C): 0.008 g; $\chi^2 = 6.95$, DF = 3, $p = 0.073$, 0.02 g; $\chi^2 = 7.89$, DF = 3, $p = 0.048$, 0.16 g; $\chi^2 = 6.95$, DF = 3, $p = 0.074$, 1.0 g; $\chi^2 = 7.50$, DF = 3, $p = 0.058$. (D): F value = 11.26, DF = 3, $p < 0.001$].

Mice were stimulated 10 times with each filament, and the nociceptive responses were scored, as follows: score 0 = no response; score 1 = licking/biting of the site stimulated with the filaments, or immediate escape; score 2 = strong retraction of the abdomen or jumping. The scores in responses to 10-times stimulation with each filament were summed and are shown as parameters for referred pain. Thereafter, the conscious mice received intracolonic infusion of water in 200 μ L, which would induce temporary colonic distention by a volume load, and nociceptive behaviors, such as licking the lower abdomen, were counted for 30 min.

Creation of a CPA-Induced Cystitis/Bladder Pain Model and Assessment of Bladder Pain in Mice We used female mice in creation of the cystitis/bladder pain model, considering that the prevalence of cystitis/bladder pain is much higher in women than in men.⁸⁾ As reported previously,⁷⁾ CPA at 400 mg/kg was administered intraperitoneally (i.p.) to female mice, and bladder pain-like nociceptive behaviors, such as licking the skin of the lower abdomen close to the bladder or pressing the lower abdomen against the floor, were counted for a 30-min period starting 3.5 h after CPA treatment. Afterwards, the skin between the anus and urethral openings of mice was stimulated with von Frey filaments with four different strengths (0.008, 0.07, 0.4, and 1.0 g). Nociceptive responses to 10-times stimulation with each filament were scored and

summed, as described above. The mice were then killed by cervical dislocation and the wet weight of the excised bladder was measured as an indicator of bladder swelling.

Drug Administration Bepridil at 10 or 20 mg/kg^{9,10)} and pimozide at 0.1, 0.5 or 1 mg/kg^{11,12)} were administered i.p. 15 and 30 min, respectively, before the von Frey test on day 6 after TNBS treatment, and administered i.p. 3 h 15 min and 3 h, respectively, after CPA treatment. Injection volume was 10 mL/kg.

Statistics Data are shown as the mean $\pm$ standard error of the mean (S.E.M.). Statistical significance was analyzed by ANOVA followed by Tukey's test for multiple comparisons of parametric data using Excel-Toukei Ver.5.0 (ESUMI Co., Ltd., Tokyo, Japan), and by the Kruskal–Wallis H-test followed by a least significant difference (LSD)-type test for multiple comparisons of non-parametric data using EXSUS Ver. 10.0 (CAC Croit Corporation, Tokyo, Japan). Significance was set at a level of $p < 0.05$.

RESULTS

Effects of Bepridil and Pimozide on the Colonic Pain and Hypersensitivity to Distension in the Mice Treated with TNBS Six days after intracolonic administration of TNBS, the mice exhibited referred hyperalgesia on the skin of

Fig. 2. Effects of Bepridil (A–C) and Pimozide (D–F), Existing Medicines Capable of Blocking T-Type Ca^{2+} Channels, on the Bladder Pain-Like Nociceptive Behavior (A, D), Referred Hyperalgesia (B, E) and Bladder Swelling (C, F) in Mice Treated with CPA

Mice received i.p. administration of CPA at 400 mg/kg or vehicle (V). Bladder pain-like nociceptive behavior was observed 3.5–4 h after i.p. CPA (A, D), followed by the evaluation of referred hyperalgesia (B, E), and then the mice were killed for measurement of the bladder weight (C, F). Bepridil at 10 or 20 mg/kg (A–C) and pimozide at 0.5 or 1.0 mg/kg (D–F) were administered i.p. 3 h 15 min and 3 h after i.p. CPA, respectively. Statistical significance was analyzed by ANOVA followed by Tukey's test for (A, C, D, F) and by the Kruskal–Wallis H-test followed by LSD-type test for (B, E). Data show the mean with S.E.M. for 5 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. [(A): F value = 101.0, degree of freedom (DF) = 3, $p < 0.001$. (B): 0.008 g; Kruskal–Wallis chi-squared (χ^2) = 16.40, DF = 3, $p < 0.001$, 0.07 g; χ^2 = 14.58, DF = 3, $p = 0.002$, 0.4 g; χ^2 = 12.49, DF = 3, $p = 0.006$, 1.0 g; χ^2 = 14.81, DF = 3, $p = 0.002$. (C): F value = 9.10, DF = 3, $p < 0.001$. (D): F value = 22.21, DF = 3, $p < 0.001$. (E): 0.008 g; χ^2 = 12.06, DF = 3, $p = 0.007$, 0.07 g; χ^2 = 13.33, DF = 3, $p = 0.004$, 0.4 g; χ^2 = 16.99, DF = 3, $p < 0.001$, 1.0 g; χ^2 = 16.74, DF = 3, $p < 0.001$. (F): F value = 20.21, DF = 3, $p < 0.001$]

the lower abdomen in response to mechanical stimulation with von Frey filaments, and colonic hypersensitivity to distension, as assessed by counting nociceptive responses following a volume load by intracolonic injection of 200 μL water (Fig. 1), in agreement with our previous report.⁶⁾ Bepridil, when administered i.p. at 10 or 20 mg/kg on day 6 after TNBS treatment, significantly reduced the referred hyperalgesia and colonic hypersensitivity to distension in the mice (Figs. 1A, B). Similarly, pimozide, administered at 0.1–0.5 mg/kg in the same manner, significantly suppressed the TNBS-induced referred hyperalgesia and colonic hypersensitivity to distension by the volume load in mice (Figs. 1C, D).

Effects of Bepridil and Pimozide on the Bladder Pain Accompanying Cystitis in the Mice Treated with CPA
CPA at 400 mg/kg caused bladder pain-like nociceptive behavior and referred hyperalgesia on the skin between the anus and urethral openings accompanying bladder swelling (increased wet weight of the bladder) (Fig. 2), in agreement with our previous report.⁷⁾ Bepridil at 20 mg/kg, administered 3 h after CPA treatment, significantly attenuated the bladder pain-like nociceptive behavior and referred hyperalgesia, but not bladder swelling (Figs. 2A–C). Pimozide at 0.5 and 1 mg/kg, administered in the same manner, significantly reduced the CPA-induced bladder nociception and referred hyperalgesia, but not bladder swelling (Figs. 2D–F).

DISCUSSION

Our data clearly demonstrate that bepridil and pimozide, existing medicines capable of blocking T-channels, sup-

press colitis-related and cystitis-related visceral pain in mice, which involve functional upregulation of $\text{Ca}_v3.2$ channels,^{6,7)} although the extent of contribution of T-channel blockade to their effects on visceral pain is unknown.

A number of preclinical studies have shown that selective T-channel blockers are effective in suppressing somatic and visceral pain including inflammatory or neuropathic components.^{1–3,6,7)} Nonetheless, clinical trials have not sufficiently demonstrated the effectiveness of selective T-channel blockers including Z944 and ABT-639 in reducing somatic pain in humans.^{13–15)} Therefore, selective inhibition of T-channels itself might not be sufficient to effectively suppress pathological somatic pain in humans. Many of voltage-sensitive ion channels, such as N-type Ca^{2+} channels as well as T-channels, Na^+ channels and also hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, regulate neuronal excitability and/or neurotransmitter release in the central and peripheral nervous systems including nociceptors, and participate in pain processing.¹⁶⁾ Thus, bepridil, capable of blocking those multiple voltage-sensitive cation channels including $\text{Ca}_v3.2$,^{4,17,18)} may be beneficial to treat human pain, because multi-channel inhibition is expected to cause synergistic suppression of nociceptor excitation. Nonetheless, the T-channel blockade is considered to play a major role in the induction of the antinociceptive effect of bepridil at least in laboratory animals, considering the IC_{50} values, 0.4–10.6 μM in blocking T-channels at holding potentials between -70 and -100 mV,⁴⁾ and 80 and 4.9 μM in blocking voltage-sensitive Na^+ channels and HCN channels, respectively.^{17,18)} On the other hand, the alleviation of colonic and bladder pain by pimozide, a neuroleptic, in the

present study may be attributable predominantly to T-channel inhibition, but not D₂ receptor blockade, considering the reports showing that typical antipsychotic drugs, haloperidol and sulpiride, or a selective D₂ receptor antagonist, L-741626, did not attenuate visceral hypersensitivity to colorectal distention in rats that had received a colonic instillation of butyrate or acetic acid in the neonatal period.¹⁹⁾ On the other hand, chlorpromazine, another typical antipsychotic drug, ameliorated the colonic hypersensitivity in the same rat models.¹⁹⁾ Of interest is that chlorpromazine as well as pimozide, but not haloperidol, blocks T-channel currents.^{5,20)} There is also evidence for the involvement of serotonin 5-HT_{2A} receptor blockade in the effect of chlorpromazine on visceral pain.¹⁹⁾ It has been shown that the affinity of pimozide and chlorpromazine to 5-HT_{2A} receptors is one-sixth and a half of clozapine, respectively,^{21,22)} indicating that pimozide has one-third affinity of chlorpromazine to 5-HT_{2A}. Therefore, the possibility of the contribution of 5-HT_{2A} blockade to the effect of pimozide on visceral pain cannot be ruled out. Thus, pharmacological blockade of T-channels is considered useful for treatment of visceral pain, and additional actions on other ion channels or receptors might be beneficial in accelerating the therapeutic efficacy, particularly in clinical application. Actually, the role of Ca_v3.2 T-channels may be more critical in processing of visceral pain than somatic pain, considering the previous findings that deletion of Ca_v3.2 gene in mice abolished pathological colonic and bladder pain in mice,^{6,7)} while it did not reduce somatic neuropathic pain, most probably due to compensation.²³⁾

In conclusion, bepridil and pimozide, existing medicines capable of blocking T-channels, alleviate colonic and bladder pain, and may serve as seeds for the development of new medicines for visceral pain treatment.

Conflict of Interest The authors declare no conflict of interest.

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