NSAIDs modulate GABA-activated currents via Ca^{2+}-activated Cl^- channels in rat dorsal root ganglion neurons

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Abstract. The ability of non-steroidal anti-inflammatory drugs (NSAIDs) to modulate γ-aminobutyrate (GABA)-activated currents via Ca^{2+}-activated Cl^- channels in rat dorsal root ganglion neurons (DRG), was examined in the present study. During the preparation of DRG neurons harvested from Sprague-Dawley rats, the whole-cell recording technique was used to record the effect of NSAIDs on GABA-activated inward currents, and the expression levels of the TMEM16A and TMEM16B subunits were revealed. In the event that DRG neurons were pre-incubated for 20 sec with niflumic acid (NFA) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) prior to the administration of GABA, the GABA-induced inward currents were diminished markedly in the majority of neurons examined (96.3%). The inward currents induced by 100 μmol/l GABA were attenuated by (0±0.09%; neurons = 4), (5.32±3.51%; neurons = 6), (21.3±4.00%; neurons = 5), (33.8±5.20%; neurons = 17), (52.2±5.10%; neurons = 4) and (61.1±4.12%; neurons = 12) by 0.1, 1, 3, 10, 30 and 100 μmol/l NFA, respectively. The inward currents induced by 100 μmol/l GABA were attenuated by (13.8±6%; neurons = 6), (23.2±14.7%; neurons = 6) and (29.7±9.1%; neurons = 9) by 3, 10 and 30 μmol/l NPPB, respectively. NFA and NPPB dose-dependently inhibited GABA-activated currents with half maximal inhibitory concentration (IC_{50}) values of 6.7 and 11 μmol/l, respectively. The inhibitory effect of 100 μmol/l NFA on the GABA-evoked inward current were also strongly inhibited by nitrendipine (NTDP; an L-type calcium channel blocker), 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (a highly selective calcium chelating reagent), caffeine (a widely available Ca^{2+} consuming drug) and calcium-free extracellular fluid, in a concentration-dependent manner. Immunofluorescent staining indicated that TMEM16A and TMEM16B expression was widely distributed in DRG neurons. The results suggest that NSAIDs may be able to regulate Ca^{2+}-activated chloride channels to reduce $GABA_A$ receptor-mediated inward currents in DRGs.

Introduction

γ-aminobutyrate (GABA) is a crucial inhibitory neurotransmitter in the mammalian peripheral nervous system (PNS) and central nervous system (CNS) (1). $GABA_A$ receptor is a pentamer comprised of multiple subunits (α_{1-6}, β_{1-3}, γ_{1-3}, π, ε, δ and θ) with an absolute chloride ion channel and diversiform allosteric binding sites through which rapid inhibitory synaptic neurotransmission may be modulated (1). The $GABA_A$ receptor is a favorable target for therapeutic agents including steroids, barbiturates, benzodiazepines, anesthetics and convulsants (2). Recently, it has also been proposed that the β-subunit has an important role in determining the chloride ion selectivity of $GABA_A$ receptors (3,4).

$GABA_A$ receptor antagonists niflumic acid (NFA) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), are the only chloride ion channel blockers able to protect cells from excitotoxicity (5). Accumulating evidence suggests that non-steroidal anti-inflammatory drugs (NSAIDs) modulate $GABA_A$ receptor function in heterologous expression systems (6). NSAIDS at clinically relevant concentrations (low micromolar) are sufficient to potentiate $\beta_{2/3}$-containing $GABA_A$ receptors (7). The NSAID-sensitive $\alpha_1\beta_2\gamma_2$ receptor

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Abbreviations: GABA, γ-aminobutyrate; PNS, periphery nervous system; CNS, central nervous system; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; NSAIDs, non-steroidal anti-inflammatory drugs; CaCCs, Ca^{2+}-activated Cl^- channels; DRG, dorsal root ganglion; SDRs, Sprague-Dawley rats; NTDP, nitrendipine

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subtype is the predominant and the largest $GABA_A$ receptor population in mammalian PNS and CNS (7). In addition to their effect on $GABA_A$ receptors, fenamate NSAIDs also affect a variety of other ion channels (8,9). Several drugs that have an effect on $GABA_A$ receptor function have been revealed to depend on subtypes (subunits combinations), and on specific amino acids situs of specific subunits (10).

Halliwell *et al* (11) revealed that the regulation of $GABA_A$ receptors by one particular anti-inflammatory agent, mefenamic acid, was dependent on the β-subunit. Conversely, Sinkkonen *et al* (12) reported that the potentiation of $\alpha_1\beta_2\gamma_2$ receptors by NFA was dependent on the presence of a γ_2 subunit, which also effects mefenamic acid modulation (11). Antagonism of the $\alpha_6\beta_2\gamma_2$ receptor subtype by NFA has also been reported (12), and the substitution of an α_4 subunit reduced the mefenamic potentiation of $\alpha_1\beta_2\gamma_2$ receptors by 50% (13). Similar observations have been observed in electrophysiological studies regarding the actions of mefenamic acid, pentobarbital and etomidate (11,14,15).

The aim of the present study is to use conventional whole-cell patch-clamp recordings, immunofluorescence and NSAIDs, including NFA and NPPB, to investigate the effect of Ca^{2+}-activated Cl^- channels (CaCCs) on GABA-induced currents in the dorsal root ganglion (DRG) of rats. The present study intended to elucidate the diversity of the modulatory effect route of NSAIDs on GABA-activated currents via CaCCs.

Materials and methods

Isolation of DRG neurons. A total of 120 Sprague-Dawley rats (SDRs) were provided by the Experimental Animal Center of Xinjiang Medical University, Urumqi, China (certificate no. SCXK 2003-0001; age, 8-10 weeks; weight, 250-280 g) irrespective of gender. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Shihezi University (Shihezi, China) and were consistent with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (16). SDRs were bred in separate specific pathogen-free cages at a relative humidity of 40-70%, (24±3˚C), 100-120 lx/12-h dark:light illumination and free access to food and water. The DRG neuron selection and the separation process are described in our previous studies (17,18). Rats were sacrificed by decapitation.

Electrophysiological recordings. A gap-free recording with a sampling interval of 50 msec (17,18) was performed in the present study. Briefly, with the aid of a whole-cell patch clamp amplifier, perforated patch-clamp recordings in the whole-cell mode were performed. Using an Axon 700B amplifier (Axon, San Jose, CA, USA) and pCLAMP version 0.2 hardware and software (Axon), currents were recorded from the DRG neurons *in vitro*. The room temperature was set at 22-24˚C. The resistance of the recording pipette ranged from 3 to 5 MΩ. The experimental procedures were performed according to the Regulations for the Administration of Affairs. Concerning Experimental Animals, formulated by the Ministry of Science and Technology of the People's Republic of China (The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals 2011).

Immunofluorescent staining of TMEM16A and TMEM16B to determine expression in the DRG. Rats were anesthetized with 0.3% (w/v) sodium pentobarbital [Sangon Biotech (Shanghai,) Co., Ltd., Shanghai, China], and perfused through the aorta with 0.9% (w/v) normal saline, followed by fresh 4% (w/v) paraformaldehyde in phosphate-buffered saline [both purchased from Sangon Biotech (Shanghai,) Co., Ltd.] for 10 min for tissue fixation. The lumbar DRG at level L_{4-6} to the nerve injury was removed rapidly and placed in 4% (w/v) paraformaldehyde in PBS for 24 h. The L_{4-6} DRG were cut into 5-μm sections with a freezing microtome (CM1510S; Leica Biosystems, Wetzlar, Germany). Immunofluorescent staining was performed using rabbit anti-TMEM16A polyclonal antibody (1:20; sc-135235) and goat anti-TMEM16B polyclonal antibody (1:20; sc-169622) (both purchased from Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. The sections were incubated for 1 h in a solution containing donkey anti-rabbit IgG-fluorescein isothiocyanate (FITC; 1:50; 711-095-152) and donkey anti-goat IgG-tetramethylrhodamine (TRITC; 1:50; 705-025-003) (both purchased from Jackson ImmunoResearch, West Grove, PA, USA) at 37˚C. Several tissue sections were selected for double-labeling of TNEM16A and TMEM16B and were incubated in a mixture of primary antibodies against TNEM16A and TMEM16B, followed by donkey anti-rabbit IgG conjugated with FITC and donkey anti-goat IgG conjugated with TRITC. Slides were then examined by confocal microscopy (LSM710; Carl Zeiss AG, Oberkochen, Germany). Quantitative analysis of TMEM16A and TMEM16B expression in the DRG was performed by measuring the mean absorbance at 488 and 550 nm (Zeiss LSM 510 System; Carl Zeiss, Jena, Germany) following laser confocal microscopy and using analysis software (ZEN 2009 Light Edition; Carl Zeiss).

Drug application. GABA, muscimol, bicuculline, NFA, NPPB, caffeine and NTDP were purchased from Sigma-Aldrich, (St. Louis, MO, USA). 1,2-Bis(2-aminophenoxy)ethane-N, N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester; BAPTA-AM) was from Merck Millipore (Darmstadt, Germany). Rabbit anti-TMEM16A polyclonal antibody and donkey anti-rabbit IgG-FITC were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). All drugs used in electrophysiological recordings were dissolved in extracellular fluid and applied by gravity flow from a home-made perfusion system consisting a row of tubules connected with a series of individual reservoirs (17). This rapid solution exchange system was manipulated by shifting the tubules horizontally with a micromanipulator (17,18). The time of pre-perfusion of antagonists was 0.5-5 min, and the time of pre-perfusion of GABA was 5-10 sec.

Statistical analysis. Statistical analysis of the data was performed using SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA) and the values of GABA-activated currents are presented as mean ± standard error of the mean and a Student's t test was used to assess the significance. $P<0.05$ was considered to indicate a statistically significant difference.

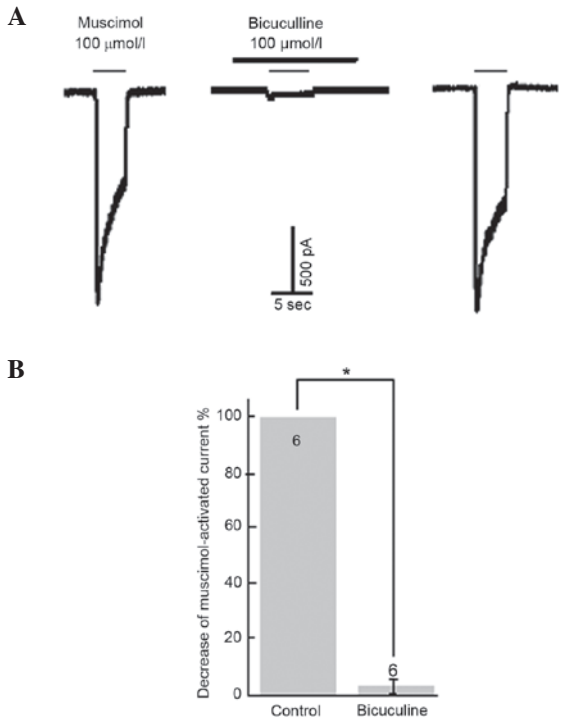

Figure 1. Inhibition of $GABA_A$ receptor antagonist on muscimol-induced depolarization. (A) Blockade of 100 μmol/l muscimol-induced inward current by $GABA_A$ receptor antagonist bicuculline (100 μmol/l). (B) Statistical results indicating the ability of bicuculline to block muscimol-activated currents. *P<0.05 vs. control. Numbers on bars=number of neurons.

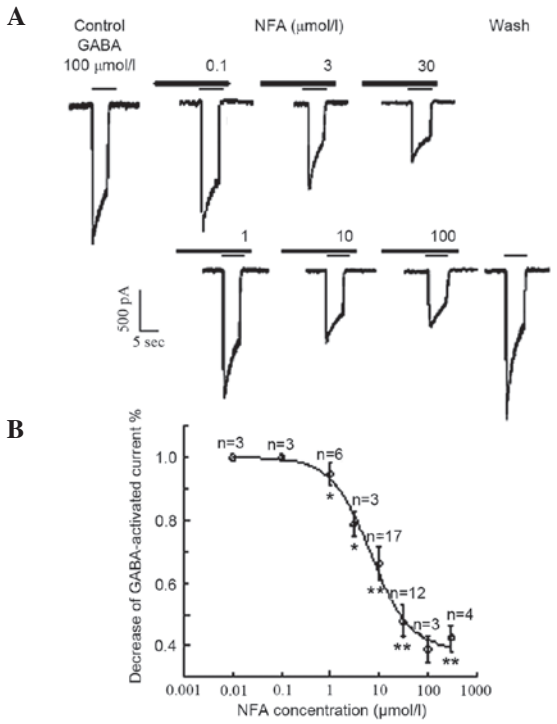

Figure 2. Effects of different concentrations of NFA on GABA-activated inward currents. (A) Results of 100 μmol/l GABA-activated inward currents in the different-concentration of NFA (0.1-100 μmol/l). (B) Inward current induced by 100 μmol/l GABA was suppressed by 0±0.09% (neurons=4), 5.32±3.51% (neurons=6), 21.3±4.00% (neurons=5), 33.8±5.20% (neurons=17), 52.2±5.10% (neurons=4), 61.1±4.12% (neurons=12) and 57.6±4.20% (neurons=4) by 0.1, 1, 3, 10, 30, 100 and 300 μmol/l NFA (neurons=3-17), respectively. *P<0.05 and **P<0.01 vs. control. Data are results of a paired t test.

Results

GABA-induced inward currents. Treatment with different concentrations of GABA (1-1,000 μmol/l) activated an inward current in the majority of cells (94.32%, 150/159) examined. The GABA-induced response was concentration-dependent, and displayed evidence of desensitization at high concentrations (Fig. 1). The activation threshold was ~1 μmol/l and the maximal response was achieved at 300 μmol/l GABA. The value of the dissociation constant was ~30 μmol/l, derived from a concentration-response curve (17,18). The averaged amplitude of 100 μmol/l GABA-evoked inward current was (1.29±0.72 nA; neurons = 52). The selective $GABA_A$ receptor agonist, muscimol (100 μmol/l) mimicked the GABA-evoked response (neurons = 8). A selective $GABA_A$ receptor antagonist, bicuculline (100 μmol/l), suppressed GABA (neurons = 9) and muscimol-evoked (neurons = 8) inward currents (Fig. 1).

Inhibition of GABA-induced inward currents by NFA and NPPB. NFA and NPPB were pre-incubated for 20 sec prior to application of GABA, resulting in the marked attenuation of the GABA-induced inward current in the majority of the neurons examined (96.3%, 52/54). Inhibition of GABA-induced responses by NFA and NPPB were concentration-dependent. The inward currents induced by 100 μmol/l GABA were suppressed by (0±0.09%; neurons=4), (5.32±3.51%; neurons=6), (21.3±4.00%; neurons=5), (33.8±5.20%; neurons=17), (52.2±5.10%; neurons=4), (61.1±4.12%; neurons=12) and (57.6±4.20%; neurons=4) by 0.1, 1, 3, 10, 30, 100 and 300 μmol/l NFA, respectively (Fig. 2A). The inhibition threshold was ~0.1 μmol/l and the maximal inhibition was achieved at 300 μmol/l NFA. The IC_{50} value was ~6.7 μmol/l derived from the concentration-inhibition curve (Fig. 2B). NFA did not alter the half maximal effective concentration EC_{50} value for GABA (~30 μmol/l) (17,19), but reduced the maximal GABA currents by ~60%. The inward current induced by 100 μmol/l GABA was suppressed by 13.8±6.7%, neurons=6, P<0.05; 23.2±14.7%, neurons=6, P<0.01 and (29.7±9.1%, neurons=9, P<0.01, by 3, 10 and 30 μmol/l NPPB, respectively. The inhibition threshold was ~1 μmol/l and the maximal inhibition was achieved by 30 μmol/l NPPB. The IC_{50} value was ~11 μmol/l (Fig. 3).

Effects of NTDP and extracellular calcium on GABA-induced inward currents. The L-type calcium channel blocker, NTDP (0.1-30 μmol/l), inverted the inhibitory effect of 100 μmol/l NFA on 100 μmol/l GABA-induced inward current (Fig. 4). The inhibitory ratio of NFA on inward current induced by GABA were (59.6±8.70%, neurons=4, P>0.05; 43.6±5.10%, neurons=3, P<0.05; 32.3±6.62%, neurons=8, P<0.01; 8.7±7.6%, neurons=6, P<0.01 and 8.6±7.4%, neurons=4, P<0.01, in the presence of 0.1, 1, 3, 10 and 30 μmol/l NTDP, respectively. To investigate the effect of extracellular free calcium on NFA, calcium-free extracellular fluid was utilized. The inhibitory effect of NFA on GABA-evoked inward currents was strongly suppressed by calcium-free extracellular fluid (P<0.01; Fig. 4). The 100 μmol/l GABA-activated current

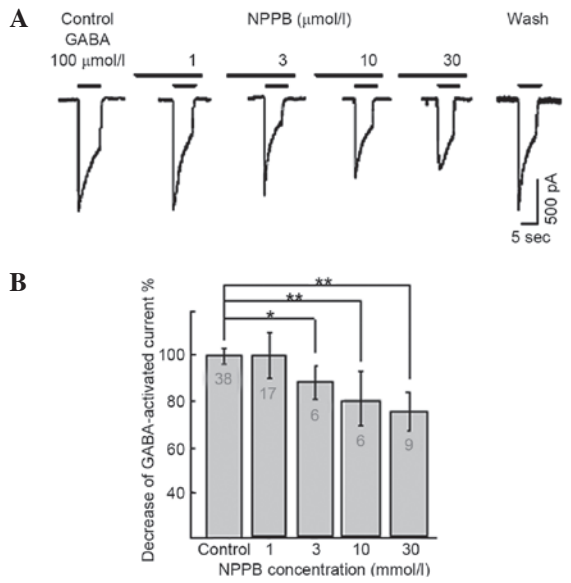

Figure 3. Effects of different-concentration of NPPB on GABA-activated inward currents. (A) Results of 100 μmol/l GABA-activated inward currents with different concentrations of NPPB (1-30 μmol/l). (B) Inward current induced by GABA (100 μmol/l) was suppressed by 13.8±6.7, 23.2±14.7 and 29.7±9.1% by 3, 10 and 30 μmol/l NPPB, respectively. *P<0.05, **P<0.01 vs. control. Results as determined by paired t test. Numbers on bars = number of neurons. NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; GABA, γ-aminobutyrate.

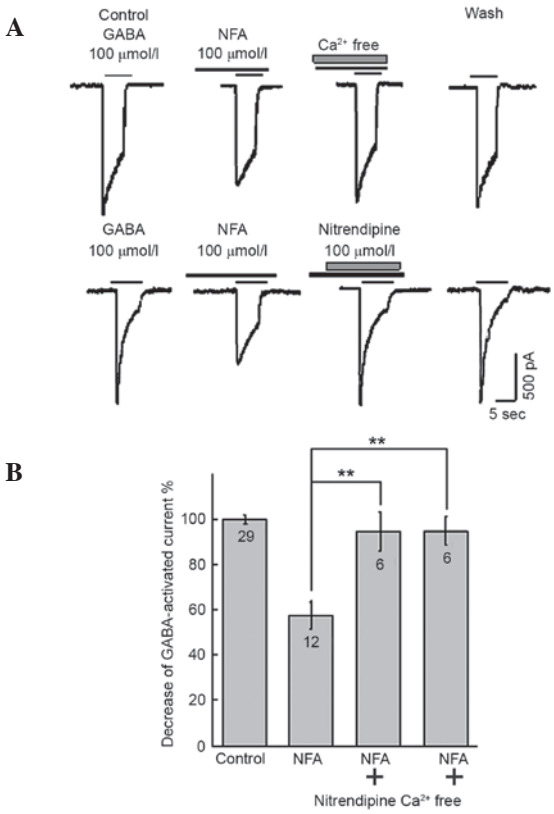

Figure 4. Effects of nitrendipine (NTDP) and exreacellular Ca^{2+} on GABA-activated inward currents. (A) Results of 100 μmol/l GABA-activated inward currents in the presence of 100 μmol/l NFA, 100 μmol/l NFA + calcium-free extracellular solution and 100 μmol/l NFA + 10 μmol/l NTDP. (B) Inward current induced by 100 μmol/l GABA was suppressed by 62.2±12.6, 8.7±7.6 and 8.4±7.2% by NFA, NFA + NTDP and NFA + calcium-free extracellular solution, respectively. **P<0.01. Paired t test. Numbers on bars=number of neurons. NFA, niflumic acid; GABA, γ-aminobutyrate.

with 100 μmol/l NFA were 1,298.8±124.4 pA; neurons = 22 and 775.9±104.9 pA; neurons = 6; P<0.01, in the presence and absence of calcium-free extracellular solution, respectively. The inhibition ratio of NFA on the GABA-evoked inward current was 8.4±7.2% (neurons = 6, P<0.01) in the calcium-free extracellular solution.

Effect of intracellular calcium on the GABA-induced inward current by intracellular calcium. To investigate the effect of intracellular free calcium on the GABA-induced inward current, BAPTA-AM was utilized and caffeine. The inhibitory effect of NFA on GABA-evoked inward current was strongly suppressed by BAPTA-AM and caffeine. The inhibition ratio of NFA on the GABA-evoked inward current was decreased by 48.2±15.7% (neurons=9, P<0.01) and 38.7±13.2% (neurons=7, P<0.05) with BAPTA-AM (100 μmol/l) and caffeine (30 μmol/l), respectively (Fig. 5).

Distribution of TMEM16A and TMEM16B subunits expressed in DRG neurons. Immunofluorescence staining revealed that TMEM16A and TMEM16B expression was widely distributed in DRG neurons, and was predominately located in the cell membranes of various diameters (Fig. 6A and B). Thus, TMEM16A and TMEM16B were co-expressed in the membranes of DRG neurons (Fig. 6C).

Discussion

$GABA_A$ receptor mediates GABA-evoked membrane depolarization responses, or inward current, since the selective $GABA_A$ receptor agonist muscimol mimicked GABA-evoked responses, and the selective $GABA_A$ receptor antagonist bicuculline blocked GABA-activated membrane responses in rat DRG neurons (17,19). The results of the present study indicated that NFA and NPPB, non-steroid anti-inflammatory agents, reduced GABA-activated inward currents. The present study also revealed that NTDP, an L-type calcium channel blocker, a calcium-free extracellular solution, BAPTA-AM, which is a membrane permeable Ca^{2+} chelator and caffeine, a Ca^{2+} consuming drug, also reduced the inhibitory effect of NFA on the GABA-activated inward current. Furthermore, the current study demonstrated that the TMEM16A and TMEM16B subunits were expressed in rat DRG neurons.

A number of cells express CaCCs, which have several physiological functions, including their being developmentally adjusted with maximum peak expression in the period of peripheral synaptogenesis in DRG neurons (20). An association was observed between the expression of CaCCs and the growth competence of sensory neurons (21). CaCCs activation augments after depolarization following spike firing of action potential in neonatal rat DRG neurons (22,23). Axotomy upregulates the expression of CaCCs in adult sensory, nodose and sympathetic ganglion neurons (21,24,25). Additionally, with regard to electrical activity, CaCCs have an important role in other basic cellular functions such as cell adhesion, apoptosis and potentially in volume regulation (26,27). In 2008, the

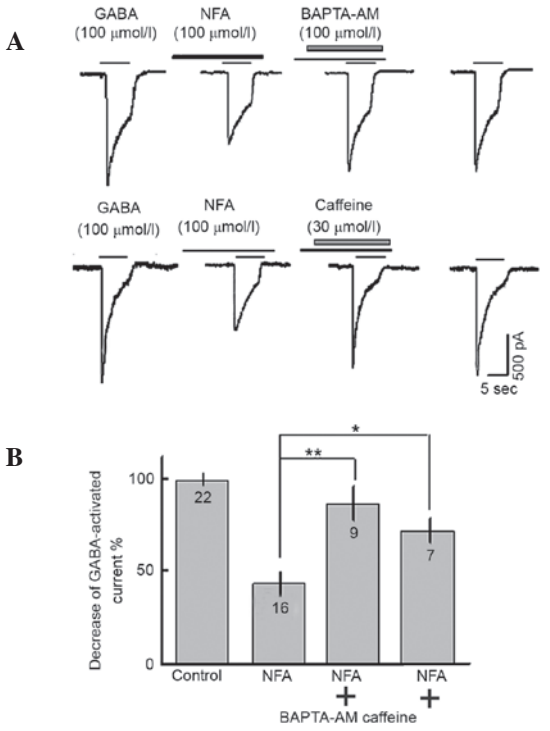

Figure 5. Effects of intracellular Ca^{2+} on GABA-activated inward currents. (A) Results of 100 μmol/l GABA-activated inward currents in the presence of 100 μmol/l NFA, 100 μmol/l NFA + 100 μmol/l BAPTA-AM and 100 μmol/l NFA + 30 μmol/l caffeine. (B) Inward current induced by GABA was suppressed by 61.8±13.4, 18.9±17.6 and 28.4±13.2% NFA, NFA + BAPTA-AM and NFA + caffeine, respectively. *P<0.05, **P<0.01. Results as determined by paired t test. Numbers on bars = number of neurons. NFA, niflumic acid; GABA, γ-aminobutyrate; BAPTA, 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid.

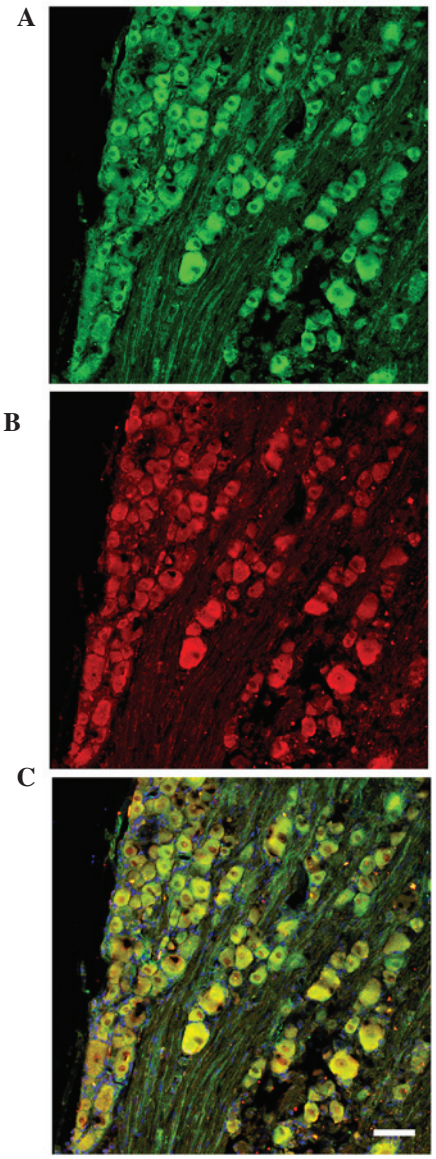

Figure 6. Confocal images of TMEM16A and TMEM16B subunits expressed in the L_{4-6} dorsal root ganglion (DRG). Immunofluorescent staining indicated that (A) TMEM16A and (B) TMEM16B expression were widely distributed in DRG neurons, and were mainly located in cell membranes of various diameters. (C) A and B overlaid (scale bars, 100 μm).

announcements that three labs had cloned genes that encoded classical CaCCs generated considerable interest (28-30); the 2 genes that have been definitively shown to encode CaCCs termed TMEM16A and TMEM16B. CaCCs are activated by an increase in intracellular free calcium concentration following either internal calcium release from Ca^{2+} stores, or external calcium entry through Ca^{2+} channel (31). NFA and NPPB have been indicated to suppress the activity of CaCCs, and NPPB inhibits chloride ion flux through other anionic channels (32). The results of the present study show that NFA and NPPB are able to significantly inhibit the GABA-activated inward current, and NTDP, calcium-free extracellular fluid and BAPTA-AM may significantly reduce the inhibitory effect of NFA on GABA-activated inward current. The present results also suggest that NSAIDs have an important role in the GABA-activated inward current via CaCCs in DRG neurons in rats, and support the hypothesis proposed by the present study. GABA activates the $GABA_A$ receptor to open chloride ion channels, the chloride ion efflux induces the depolarization response of the membrane of DRG neurons (17). Conversely, voltage dependent L-type Ca^{2+} channels may be activated by depolarization, and lead to increased intracellular Ca^{2+}. Furthermore, the NFA-induced increase in intracellular Ca^{2+} is likely due to Ca^{2+} release from an intracellular store (33-35). CaCCs are activated by an increase in intracellular calcium concentration, which in turn increases the driving force for chloride ion efflux (28). Finally, the synergistic action of chloride ion efflux via $GABA_A$ receptors and NFA-sensitive CaCCs results in GABA-activated currents or depolarization responses in rat DRG neurons. The depolarization arising from Cl^- efflux through CaCCs indicates a mechanism of electrical amplification of the GABA-activated currents (Fig. 7).

The binding of intracellular signaling molecules or extracellular ligands activates a conformational change that opens or closes the pores of ligand-gated ion channels such as GABA, glutamate, serotonin and acetylcholine, as well as the cyclic nucleotide-gated ion channels that have important roles in sensory biology (17). However, the structure and function of ligand-gated ion channels and their integral receptors have yet to be elucidated. Therefore, it is important to develop novel tools to investigate the interactions between various receptor subunits, which may benefit the future designing of receptor subtype-selective therapies.

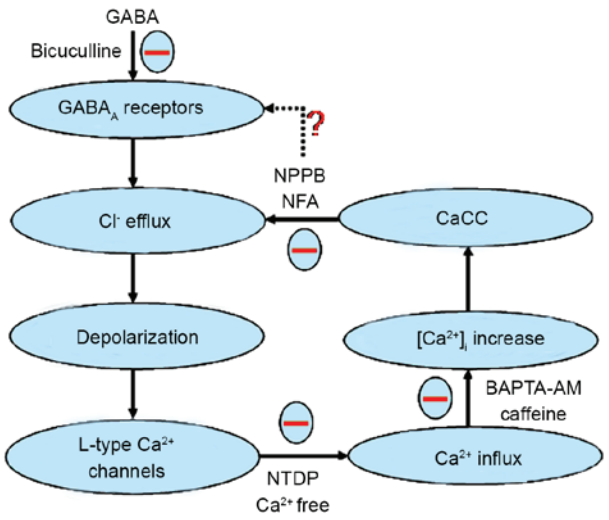

Figure 7. Schematic displaying the effects of CaCCs on GABA-activated inward currents and depolarization. GABA activates the $GABA_A$ receptor to open the Cl^- channel and the Cl^- efflux induces the depolarization response (inward current) of the membrane of dorsal root ganglion (DRG) neurons. Then, voltage dependent L-type Ca^{2+} channels are activated by the depolarization, and give rise to an increase in intracellular Ca^{2+}. CaCCs are activated by an increase in intracellular Ca^{2+} concentration which, in turn, increases the driving force for Cl^- efflux. Finally, the synergistic action of the chloride ion efflux through $GABA_A$ receptors and NFA-sensitive CaCCs causes GABA-activated currents or depolarization response in rat DRG neurons. '-' expressed function was inhibitory, '?' expressed function was not clear. GABA, γ-aminobutyrate; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; NFA, niflumic acid; CaCC, Ca^{2+}-activated Cl^- channels; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; NTDP, nitrendipine.

Ion channel gating is influenced by several factors, including the binding of another ligand and intracellular Ca^{2+} (36). $GABA_A$ integrates the actions of a wide range of therapeutic agents, including steroids, benzodiazepines, barbiturates, convulsants and anesthetics (2). Previous studies have indicated that NFA is able to directly act on $GABA_A$ and NMDA receptors (5,12,37). Notably, another chloride blocker, mefenamic acid, is also able to directly activate $GABA_A$ receptors (38). NFA functions as a positive allosteric modulator of $\alpha_1\beta_2\gamma_2$, and a negative modulator of $\alpha_6\beta_2$ and $\alpha_6\beta_2\gamma_2$ (and $\alpha_1\beta_2$) $GABA_A$ receptors. Despite the knowledge that NFA shares the same site as furosemide to mediate its inhibitory effect, the site for the positive regulation remains elusive, and is dependant on the presence of the γ_2 subunit, yet separable from the benzodiazepine binding site (11,12).

It has been suggested that the niflumate potentiation of $GABA_A$ function is through a pure direct allosteric mechanism (12). Conversely, it has been reported that the activation of $GABA_A$ opens NFA-sensitive anion channels (39,40). Furthermore, $GABA_A$-mediated chloride ion influx lowers the magnitudes of NFA- and NPPB-sensitive chloride currents in motorneurons. NFA and other fenamate blockers of CaCCs (41,42), and recombinant $GABA_A$ (43). The expression levels of rat homopentamer $GABA_A$ receptors display GABA-independent activation (44). In addition, NFA activates single chloride ion channels, likely to be an isoform of the $GABA_A$ receptors in mouse sperm (45). Therefore, it is possible that alternative mechanisms exists for NFA action on the $GABA_A$ receptor.

In the present study, NFA did not alter the EC_{50} value but reduced the maximal response of GABA currents, which is consistent with noncompetitive antagonism. Similar results have been reported for furosemide (46) and NFA (12) previously. Changes in the expression and function of α_2, but not α_6 subunits of $GABA_A$, were observed in L_4-L_6 DRG neurons by whole-cell patch-clamp and immunofluorescence.

Further experiments are needed to determine whether NFA and NPPB may directly act upon the $GABA_A$ α_2 subunit in DRG neurons, and the physiological activator of CaCCs may also provide further elucidation regarding the contribution of CaCCs to electrical activity. As NSAIDS are highly subtype-selective, further studies to investigate its behavioral and cognitive effects are warranted (11). We propose that expression of CaCCs in DRG should increase in response to peripheral nerve injury, and enhance the responses of $GABA_A$ receptors. Therefore, the upregulation of CaCCs may prominently enhance 'the presynaptic inhibition' of GABA in the primary afferent endings, and have involvement in pain modulation.

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