

The inhibitory effects of bupivacaine, levobupivacaine, and ropivacaine on K_{2P} (two-pore domain potassium) channel TREK-1

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Abstract

Purpose Bupivacaine, levobupivacaine, and ropivacaine are amide local anesthetics. Levobupivacaine and ropivacaine are stereoisomers of bupivacaine and were developed to circumvent the bupivacaine's severe toxicity. The recently characterized background potassium channel, K_{2P} TREK-1, is a well-known target for various local anesthetics. The purpose of study is to investigate the differences in inhibitory potency and stereoselectivity among bupivacaine, levobupivacaine, and ropivacaine on K_{2P} TREK-1 channels overexpressed in COS-7 cells.

Methods We investigated the effects of bupivacaine, levobupivacaine, and ropivacaine (10, 50, 100, 200, and 400 μ M) on TREK-1 channels expressed in COS-7 cells by using the whole cell patch clamp technique with a voltage ramp protocol ranging from -100 to 100 mV for 200 ms from a holding potential of -70 mV.

Results Bupivacaine, levobupivacaine, and ropivacaine showed reversible inhibition of TREK-1 channels in a concentration-dependent manner. The half-maximal inhibitory concentrations (IC_{50}) of bupivacaine, levobupivacaine, and ropivacaine were 95.4 ± 14.6 , 126.1 ± 24.5 , and 402.7 ± 31.8 μ M, respectively. IC_{50} values indicated a rank order of potency (bupivacaine > levobupivacaine > ropivacaine) with stereoselectivity. Hill coefficients were 0.84, 0.93, and 0.89 for bupivacaine, levobupivacaine, and ropivacaine, respectively.

Conclusion Inhibitory effects on TREK-1 channels by bupivacaine, levobupivacaine, and ropivacaine demonstrated stereoselectivity: bupivacaine was more potent than levobupivacaine and ropivacaine. Inhibition of TREK-1 channels and consecutive depolarization of the cell membrane by bupivacaine, levobupivacaine, and ropivacaine may contribute to the blockade of neuronal conduction and side effects.

Keywords Bupivacaine · Levobupivacaine · Ropivacaine · TREK-1 · Two-pore domain potassium channel

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Introduction

Bupivacaine, levobupivacaine, and ropivacaine are the most commonly used amino-amide local anesthetics belonging to the family of *n*-alkyl-substituted pipercolyl xylydines [1]. Ropivacaine and levobupivacaine are less toxic than bupivacaine to the central nervous system and heart in human and animal studies [2, 3]. Small changes of the side chain in chemical structure may cause different effects such as a stereoselectivity of local anesthetics. Several studies have shown that the stereoselective effects of local anesthetics on ion channels play a central role in the blockade of neuronal conduction and toxicity [4–6].

Generally, the major mechanism of local anesthetics is the inhibition of voltage-gated Na^+ channels, resulting in blockade of generation and conduction of the action potential [7]. In addition, increasing evidence suggests that local anesthetics also potently block K^+ channels to alter the resting potential or repolarization in neurons [8]. The studies using voltage clamp recordings from *Xenopus Oocytes* reported that local anesthetics inhibit K^+ channels, and may thereby enhance the conduction blockade [8, 9]. $\text{K}_{2\text{P}}$ The TREK-1 channel is a subtype of the two-pore domain potassium ($\text{K}_{2\text{P}}$) channels characterized by 2 pore domains and 4 transmembrane segments [10, 11]. TREK-1 is highly expressed in the nervous system, and stabilizes the resting membrane potential and membrane excitability [12]. Kindler and Yost [11] reported $\text{K}_{2\text{P}}$ channels as a new target of local anesthetic action. Recently, lidocaine has been demonstrated to have an inhibitory effect on the human $\text{K}_{2\text{P}}$ TREK-1 channel by the phosphorylation of Ser348 in the C-terminal domain of human $\text{K}_{2\text{P}}$ TREK-1 [13].

Here, we investigate the differences of inhibitory potency and stereoselectivity among the analogs of lidocaine, such as bupivacaine, levobupivacaine, and ropivacaine on $\text{K}_{2\text{P}}$ TREK-1 channels expressed in COS-7 cell by using whole cell patch clamp recordings.

Materials and methods

Construction of rat TREK-1 plasmid

cDNA encoding full-length rat TREK-1 (GenBank Accession No. AY727922) was obtained by an RT-PCR-based gateway cloning method as described previously [14]. The resulting PCR product cloned into the pDONR207 vector (Invitrogen) and was confirmed by sequencing. For electrophysiological experiments, this plasmid combined into the pDEST-GFP-C vector [14] to make a N-terminal EGFP-tagged rat TREK-1 plasmid (pEGFP-rTREK-1).

Cell culture and DNA transfection

COS-7 cells were cultured in a 5% CO_2 humidified incubator at 37 °C in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Invitrogen), and 1% penicillin/streptomycin (100 U/ml and 100 $\mu\text{g}/\text{ml}$, Invitrogen). COS-7 cells were subcultured in 35-mm culture dishes at 40–60% density 16 h before DNA transfection. Subsequently, the cells were transfected with the pEGFP-rTREK-1 plasmid DNA using Effectene transfection

reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Whole cell patch clamp recordings

Transfected cells were identified by detecting green fluorescent protein under a fluorescence microscope and were chosen for electrophysiological recordings. Whole cell recordings were obtained at room temperature using 3–5 $\text{M}\Omega$ patch pipettes and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in a bath solution consisting of (in mM): 150 NaCl, 3 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, 7.5 mg/ml sucrose, and 1.0 mg/ml D(+)-glucose with the pH 7.4 adjusted by NaOH. The different concentrations (10, 50, 100, 200, and 400 μM) of bupivacaine (Sigma-Aldrich, St. Louis, MO, USA), levobupivacaine (Chirocaine[®], Abbott, Nycomed Pharma AS, Solbaer Veien, Norway), and ropivacaine (Naropin[®]; AstraZeneca, Alma Road, North Ryde, Australia) were determined according to the reference with other previous studies [11], and were made with the perfusate. An internal solution contained (in mM): 150 KCl, 4 NaCl, 1 MgCl_2 , 0.5 CaCl_2 , 10 HEPES, 10 EGTA, 2 ATP, and 0.3 GTP with the pH 7.4 adjusted by KOH. Patch pipettes were pulled by an electrode puller (P-97 Flaming/Brown Micropipette Puller, Shutter Instrument Co., USA).

Recordings were usually digitized at 2 kHz, and the output of the patch clamp amplifier (Axopatch ID; Axon Instruments, Union City, CA, USA) was usually filtered at 1 kHz using pCLAMP software. All recordings were stored on a computer hard disk. All electrophysiological

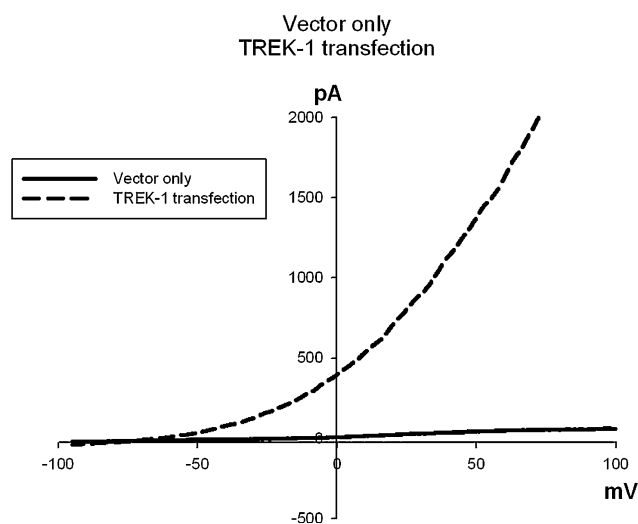


Fig. 1 Expression of $\text{K}_{2\text{P}}$ channel TREK-1 in COS-7 cell. Original recordings were obtained with a holding potential of -70 mV by ramp protocol from -100 to $+100$ mV with a duration of 200 ms in COS-7 cell with vector only ($n = 6$) and COS-7 cell with TREK-1 transfection ($n = 6$)

measurements were carried out at room temperature (21–23 °C). Pulse generation and data acquisition were done using a personal computer, pCLAMP 10.0, and Digitata 1322 interface (Axon Instruments, Union City, CA, USA). Data were analyzed using pCLAMP 10.0 (Clampfit) and SigmaPlot 10.0 (SPSS Inc., Chicago, IL, USA) software. Voltage commands were applied and currents recorded and analyzed with pCLAMP software (Molecular Devices, Sunnyvale, CA, USA). Before seal formation, the voltage offset between the patch electrode

and the bath solution was adjusted to produce zero current. Cells were held at -70 mV and the depolarizing ramp (200 ms, from -100 to $+100$ mV) was applied in 5 s intervals (Fig. 1).

Local anesthetics were dissolved in the extracellular medium to the desired concentration just before use. Immediately after establishing whole cell clamps, control recordings were taken and then drug solutions were applied rapidly to the cell by the bath application system with pinch valves (Warner instrument, Hamden, CT, USA). Cells were washed after measuring the current amplitudes during application of local anesthetics.

Statistics

SigmaPlot 10.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. For dose–response experiments, current amplitudes according to different concentrations of local anesthetics were measured by evoking the current with a ramp pulse protocol from -100 to $+100$ mV over 200 ms. To obtain concentration–response curves, the percent inhibition of currents by local anesthetics was quantified at various test concentrations according to the following equation: percent inhibition = $100 (I - I_{\text{drug}}/I_{\text{control}})$. The concentration–response data were fitted by the Hill equation in the form $y = 1/1 + ([D]/K_i)^{nH}$; where y = inhibition, D = concentration of the drug, nH = Hill coefficient, and K_i = half-maximal inhibitory concentration or IC_{50} value. All data are expressed as mean \pm SD. The Kruskal–Wallis test and the Mann–Whitney test were used for statistical comparisons. A P value <0.05 was considered statistically significant.

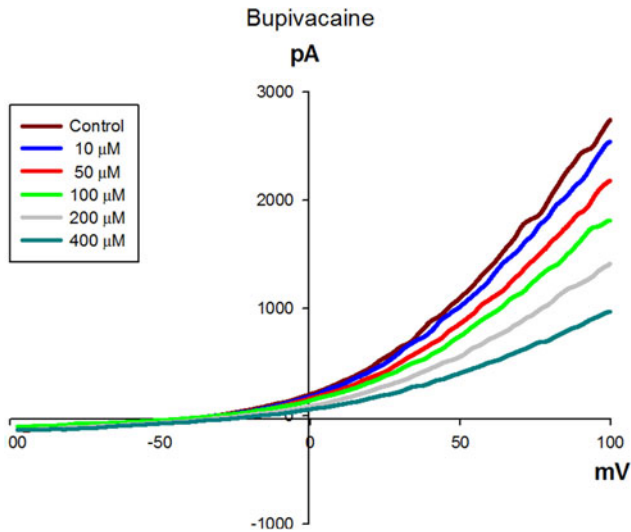


Fig. 2 Inhibitory effects of bupivacaine (10, 50, 100, 200, and 400 μM) on K_{2P} channel TREK-1. Original recordings were obtained with a holding potential of -70 mV by ramp protocol from -100 to $+100$ mV for 200 ms

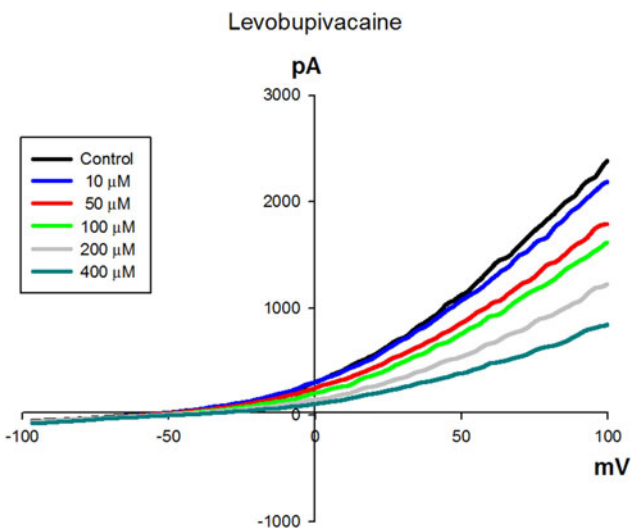


Fig. 3 Inhibitory effects of levobupivacaine (10, 50, 100, 200, and 400 μM) on K_{2P} channel TREK-1. Original recordings were obtained with a holding potential of -70 mV by ramp protocol from -100 to $+100$ mV for 200 ms

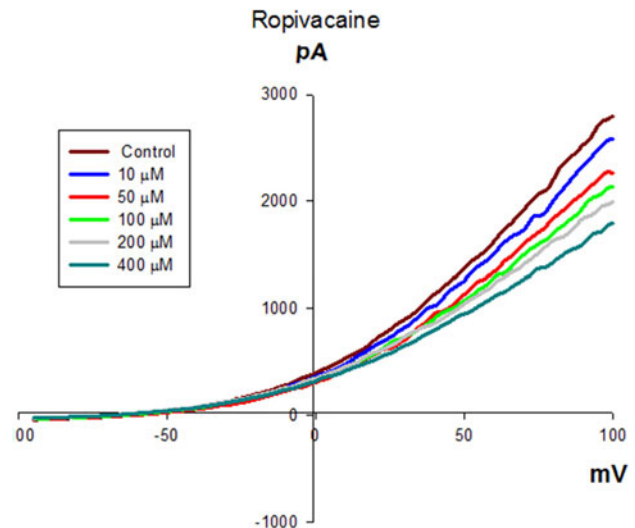
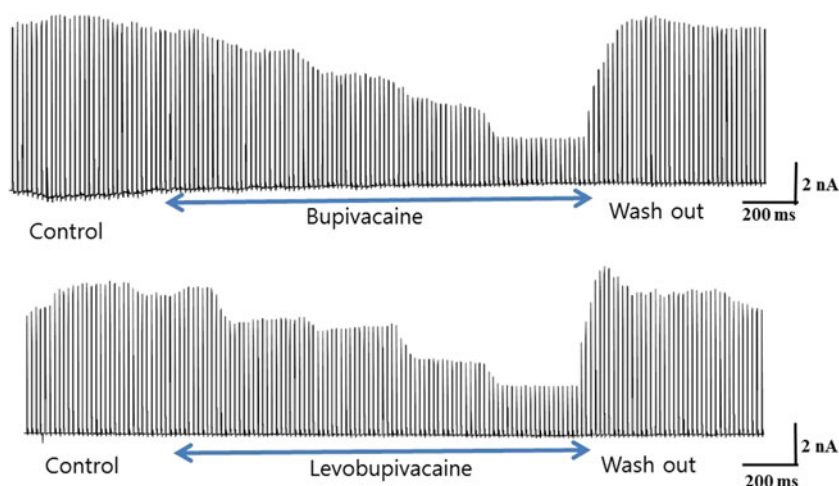


Fig. 4 Inhibitory effects of ropivacaine (10, 50, 100, 200, and 400 μM) on K_{2P} channel TREK-1. Original recordings were obtained with a holding potential of -70 mV by ramp protocol from -100 to $+100$ mV for 200 ms

Fig. 5 The channel currents were recovered after washout; therefore, the inhibitory effects of bupivacaine, levobupivacaine, and ropivacaine on K_{2P} channel TREK-1 channels, were fully reversible



Results

As a test for the presence of TREK-1 channels, COS-7 cells with TREK-1 transfection had current amplitudes of 348.63 ± 31.27 pA at a test potential of 0 mV ($n = 6$), which were greater than those recorded from the COS-7 cell with vector only, 9.45 ± 14.31 pA ($n = 6$) ($p < 0.05$). COS-7 cells with TREK-1 transfection displayed larger outward currents with an instantaneous and a time-dependent component upon depolarization (Fig. 1).

TREK-1 currents were elicited by depolarizing voltage steps from a holding potential of -70 mV before and after the application of bupivacaine, levobupivacaine, and ropivacaine (Figs. 2, 3, 4).

Application of bupivacaine ($n = 6$), levobupivacaine ($n = 6$), or ropivacaine ($n = 6$) inhibited TREK-1 currents in all test potentials in a concentration-dependent manner (Figs. 2, 3, 4). The channel currents were recovered after washout; therefore, the inhibitory effects of bupivacaine, levobupivacaine, and ropivacaine on TREK-1 channels, were fully reversible (Fig. 5).

The half-maximal inhibitory concentrations (IC_{50}) for bupivacaine, levobupivacaine, and ropivacaine were 95.4 ± 14.6 , 126.1 ± 24.5 , and 402.7 ± 31.8 μ M, respectively, ($p < 0.05$). IC_{50} values indicated a rank order of potency (bupivacaine > levobupivacaine > ropivacaine); thus, showing that the inhibitory effect of these local anesthetics on TREK-1 channels was stereoselective. Hill coefficients were 0.84, 0.93, and 0.89 for bupivacaine, levobupivacaine, and ropivacaine, respectively (Fig. 6).

Discussion

In this study, we showed that bupivacaine, levobupivacaine, and ropivacaine inhibited TREK-1 channels in a reversible and concentration-dependent manner. Bupivacaine was

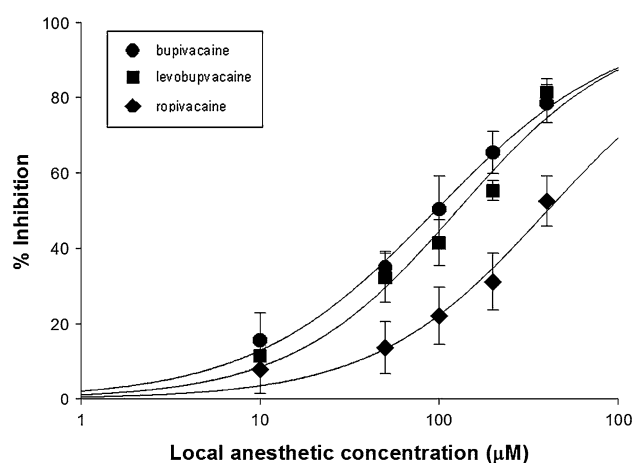


Fig. 6 Percentage inhibition of K_{2P} channel TREK-1 currents by bupivacaine, levobupivacaine, and ropivacaine (10, 50, 100, 200, and 400 μ M). Each point represents the mean \pm SD ($n = 6$)

approximately 1.3- to 4.2-fold more potent than levobupivacaine and ropivacaine in blocking TREK-1 channels. These results suggested that bupivacaine and bupivacaine enantiomers such as levobupivacaine and ropivacaine acted in a stereoselective-specific way to inhibit TREK-1 channels.

Generally, bupivacaine, levobupivacaine, and ropivacaine are amino-amide local anesthetics used for regional anesthesia. Classically, bupivacaine is marketed as a racemic mixture with equimolar amounts of both dextro-rotatory [R(+)] and levorotatory [S(-)] enantiomers. Both levobupivacaine and ropivacaine are the pure S(-) enantiomers of *n*-butyl-pipecoloxylidide and *n*-propyl-pipecoloxylidide, and they only differ in the length of the N-substituted side chain [15]. The biophysical properties (molecular weight, Pka, and protein binding) of the three agents are similar, while the chemical structure and lipophilicity are different [4, 15]. The R(+) and S(-) enantiomers of local anesthetics have been demonstrated to have

different affinities for Na⁺ or K⁺ channels, which results in a significant reduction in the toxicity of the S(−) enantiomer as compared with the R(+) enantiomer [16, 17].

Nowadays, both Na⁺ and K⁺ currents are known as having involvement for the action of local anesthetics, disrupting ionic flow and propagation of the action potential [11]. Under physiological conditions, the blockade of K⁺ channels might lead to a pronounced block of conduction in nerve fibers via membrane depolarization and resulting Na⁺ channel inactivation [18].

The K⁺ channel gene family includes voltage-gated (K_V), calcium-activated (K_{Ca}), inwardly rectifying (K_{ir}), and tandem two-pore (K_{2P}) channels [19]. Previous studies have suggested that the major effects of general or local anesthetics might result from K⁺ channel activation, specifically K_{2P} channels [20, 21]. Background or leak conductances are major determinants of membrane resting potential and input resistance, two key components of neuronal excitability. The main background K⁺ channel in the nervous system are the TASK and TREK channels. Open at rest, they are involved in the maintenance of resting membrane potential in the nervous system [22, 23]. K_{2P} channels are not activated by changes in transmembrane potential or by changes in intracellular Ca²⁺, they have high open probabilities in the background. When these K⁺ channels pass current, they tend to draw the membrane potential closer to K⁺ equilibrium potential. By so doing, the tendency of voltage-gated Na⁺ and Ca²⁺ channels to open is resisted, thus inhibiting action potential propagation [11, 24].

The interaction of K_{2P} TREK-1 with local anesthetics may contribute to conduction blockade and unwanted side effects such as CNS excitability and cardiac arrhythmia [11] or desired effects such as neuroprotection [25]. K_{2P} channels exert control over neuronal excitability through their influence on the resting membrane potential. Local anesthetic inhibition of K_{2P} channels can block repolarization currents, thereby delaying recovery of membrane potential after a depolarization. This effect contributes to action potential prolongation and leads to side effects of local anesthetics [23, 24, 26].

González et al. [17] reported that bupivacaine and ropivacaine block the K_V1.5 channel in a stereoselective manner, and then the longer the side chain represents the higher the potency of the drug. In our study using TREK-1 channels, IC₅₀ values indicated a rank order of potency (bupivacaine > levobupivacaine > ropivacaine) and represent that levobupivacaine and ropivacaine [both S(−) enantiomers] were less toxic than bupivacaine [R(+) enantiomer].

Punke et al. [27] reported that bupivacaine depolarizes the cell membrane of Chinese hamster ovary cells expressing TREK-1 in an immediate, concentration-

dependent, and reversible manner similar to the results of our study using COS-7 cells expressing TREK-1.

Previous studies have shown that TREK-1 activity was inhibited by the cAMP/PKA pathway and phosphorylation of S333 located at the distal end of the C-terminal domain is responsible for cAMP-mediated inhibition of TREK-1 [28]. Nayak et al. [13] also revealed on their TREK-1 study using human embryonic kidney cells that lidocaine has an inhibitory effect on the TREK-1 channel via the mechanisms of phosphorylation of Ser348 in the C-terminal domain of TREK-1, and lidocaine produced reversible concentration-dependent inhibition of TREK-1 current, with an IC₅₀ value of 180 μM. The inhibition of TREK-1 channels by amide local anesthetics such as bupivacaine, levobupivacaine, ropivacaine may be activated by the same mechanism.

The potency ratios among these agents are still subjects of investigation, numerous clinical studies suggest a rank order of potency (ropivacaine < levobupivacaine < bupivacaine) [4, 15, 29]. A relative potency ratio of bupivacaine:levobupivacaine:ropivacaine are 1.00:0.83:0.77 in a recent human epidural study [30] and 1.00:0.75:0.23 in our COS-7 cell study. The disparities between studies were attributed to the difference of methods or cell lines. Despite the large range of IC₅₀ values, the Hill coefficient for each dose response relationship was similar among drugs (Fig. 6), suggesting those three compounds target the same motif of TREK-1 channel. The different IC₅₀ among the three compounds could be due to stereoselective interaction between each compound and TREK-1. To our knowledge, this is the first evidence demonstrating a stereoselective interaction between indicated 3 drugs such as bupivacaine, levobupivacaine, and ropivacaine and TREK-1 channels using whole cell patch clamp recordings.

In conclusion, the K_{2P} channel TREK-1 may contribute to the stereoselective blockade of neuronal conduction and toxic effects of local anesthetics such as bupivacaine, levobupivacaine, and ropivacaine.

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Conflict of interest All authors declare no financial interests.

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