

Bupivacaine inhibits a small-conductance calcium-activated potassium type 2 channel (SK2) in HEK293 cells

Hongfei Chen

Shandong University School of Medicine: Shandong University Cheeloo College of Medicine

Fangfang Xia

The First Affiliated Hospital of Wenzhou Medical University

Zhousheng Jin

The First Affiliated Hospital of Wenzhou Medical University

Zhijian Fu (✉ zhijian_fu@163.com)

Shandong Provincial Hospital <https://orcid.org/0000-0002-9387-9768>

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Abstract

Background: Bupivacaine blocks many ion channels in the heart muscle, which could cause severe cardiotoxicity. Small conductance calcium-activated potassium type 2 channels (SK2 channels) are widely distributed in the heart cells and are involved in relevant physiological functions. However, whether bupivacaine can inhibit SK2 channels is still unknown. This study investigated the effect of bupivacaine on SK2 channels.

Methods: The SK2 channel gene was transfected into human embryonic kidney 293 cells (HEK-293 cells) with Lipofectamine 2000. The whole-cell patch clamp technique was used to study the effect of bupivacaine on SK2 channels. Concentration-response relationship of bupivacaine for inhibiting SK2 current (0 mV) was fitted to a Hill equation, and the half-maximal inhibitory concentration (IC₅₀) value was determined.

Results: Bupivacaine inhibited the SK2 channels reversibly in a dose-dependent manner. The IC₅₀ value of bupivacaine, ropivacaine and lidocaine on the SK2 current was 16.5, 46.5, and 77.8 μ M, respectively. The degree of SK2 current inhibition by bupivacaine was dependent on the intracellular concentration of free calcium.

Conclusions: The results of this study suggested a new inhibitory effect of bupivacaine on SK2 channels. Future studies should be concerned with the effects of SK2 on bupivacaine cardiotoxicity.

Background

Local anesthetics (LAs), such as bupivacaine, ropivacaine, and lidocaine, are often used for regional anesthesia and analgesia. Due to their different chemical structures, their cardiotoxicity also differs: bupivacaine > ropivacaine > lidocaine. Bupivacaine is one of the long-acting, lipophilic LAs; it is used for analgesia perioperatively due to its high analgesic efficacy and long-lasting effect. However, accidental delivery or excessive absorption of bupivacaine into blood circulation may cause severe arrhythmia or even cardiac arrest.¹⁻³ According to the statistics, the incidence of LA-induced toxicity in peripheral nerve block was 7.5–20/10000.^{4,5} The mechanism of bupivacaine cardiotoxicity has not been fully elucidated. It is currently believed that bupivacaine could block sodium^{6,7}, L-calcium^{8,9}, and potassium channels^{10,11}, which may be involved in bupivacaine cardiotoxicity.

Calcium-activated potassium channels are calcium-dependent channels triggered by intracellular calcium.¹² In humans, calcium-activated potassium channels can be divided into three categories: large-conductance channels, intermediate-conductance channels, and small-conductance channels. Small-conductance calcium-activated potassium type 2 channels (SK2 channels) are involved in hyperpolarization after the action potential. These channels function in the atria,¹³ ventricles,¹³ atrioventricular nodes,¹⁴ and Purkinje cells,¹⁵ which play important roles in cardiac conduction. Due to the important role of SK2 channels in regulating the action potential, its dysfunction may lead to atrial or

ventricular arrhythmia.^{3,16} For example, studies have shown that the expression level of the SK2 gene in cardiomyocytes of patients is closely related to atrial fibrillation.¹⁶ To our knowledge, there have been no reports on the effect of bupivacaine on SK2 channels thus far. We hypothesized that bupivacaine directly suppresses SK2 currents.

In this study, we transfected HEK 293 cells with the SK2 gene. The whole-cell patch-clamp technique was applied to demonstrate that bupivacaine can inhibit SK2 currents. Our aim was to demonstrate the capacity of bupivacaine to inhibit SK2 channels and the effect of calcium concentration on its blockade.

Methods

Cell line culture and gene transfection

HEK293 cells were all purchased from the institute of Life Sciences of Chinese Academy of Sciences (China). After being harvested by 0.25% trypsin, the cell lines were grown at 37 °C in 5% CO₂ and 95% air conditions and cultured in Dulbecco's minimal essential medium (DMEM) mixed with 10% fetal bovine serum (FBS), 75-µg/mL streptomycin, and 75-U/mL penicillin. Before transfection, cells were added to a plate with a density of about 2×10^5 cells/cm². Transfection was performed when 85% confluence was reached. The plasmids (pCDNA3/rSK Ca₂) used in this study were obtained from OriGene (USA). We performed all the transfections with Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. As described previously, we established stable expression the SK2 gene in HEK293 cells (the cells are herein referred to as SK2 cells).¹⁷ Before the patch-clamp experiment, SK2 cells were seeded for about 24 h in the glass cover.

Drugs and solutions

Trypsin, FBS, penicillin, streptomycin, and DMEM were all obtained from Gibco Invitrogen Corp. (USA); Bupivacaine, ropivacaine, and lidocaine were purchased from Sigma-Aldrich (USA). The Tyrode's solution comprised the following: NaCl, 137 mM; KCl, 5.4 mM, MgCl₂, 1.8 mM; HEPES, 10 mM; and glucose, 10 mM; pH was maintained at 7.4 with NaOH. The pipette solution comprised the following: MgCl₂, 1.15 mM; potassium gluconate, 144 mM; and CaCl₂, 0.25 mM/0.5 mM /1.0 mM); pH was maintained at 7.2 with KOH.

Patch-clamp experiments

All experiments were conducted with the whole-cell patch-clamp technique.¹⁸ The coverslip containing SK2 cells was placed into an inverted Olympus microscope (IX70, Japan) on the cell chamber. The solutions were added into the reservoirs respectively from superfusion system (DADVC-8PP, ALA SCIENTIFIC, USA). The DAD-VC systems go out with a Micromanifold consisting of 8 tubes of polyamide-coated quartz glass of 100 µm ID. The Micromanifold enables up to 8 solutions from the reservoirs to flow into a small common space of less than 1 µL. The Micromanifold with a micromanipulator can easily be moved around the cell preparation and pointed at the target cell.

The EPC-10 amplifier (HEKA, Germany) was applied to the whole-cell patch-clamp technique. A glass electrode with 1.2-mm outer diameter was pulled out by the microelectrode puller (P-97, SUTTER, USA) to achieve a resistance of 1.5 M Ω –3.0 M Ω after adding the pipette solution. Under the microscope, SK2 cells with smooth cell membranes were picked up to record the currents. After gigaseal formation, we introduced a negative pressure to break the SK2 cell membrane. Voltage stimulation and data recording were performed using the Pulse 8.0 software (HEKA, Germany). All experiments were performed at 36 °C. SK2 cells could produce stable currents at 0 mV; therefore, we used currents at 0 mV for comparisons in the following experiments. SK2 cells were recorded for currents at three different phases: baseline, inhibition, and washout. The baseline phase involved the perfusion of SK2 cells with Tyrode's solution. The inhibition phase involved the perfusion of SK2 cells with Tyrode's solution containing LAs. The washout phase involved the replacement of LA-containing Tyrode's solution with normal Tyrode's solution. The currents recorded at the three phases were defined as Current_{baseline}, Current_{inhibition}, and Current_{washout}. Normalization current was represented by Current_{inhibition}/Current_{baseline}. Normalization inhibition was calculated as (Current_{baseline} - Current_{inhibition})/Current_{baseline}.

Statistical analysis

The SPSS software (version 19.0, Chicago, IL, USA) was used to analyze the data. The normality of data was tested using the Shapiro–Wilk test, and the normally distributed data were expressed as the mean \pm standard deviation. Differences between the two groups were assessed by Student's t-test, and ANOVA was used for comparisons of multiple groups. A *P* value < 0.05 indicate statistical significance.

The relationship between local anesthetic concentration and its inhibitory effect on SK2 currents was fitted in a non-linear fashion using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). The equation was $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$, where HillSlope represents the steepness of the family of curves, Top and Bottom represents plateaus in the units of the Y axis, X represents logarithm of concentrations of LAs (0, 0.5, 1, 2, 2.5, and 3), and Y represents normalization current. Normalization current was calculated as Current_{inhibition}/Current_{baseline}.

Results

Concentration–response relationship of bupivacaine, ropivacaine, and lidocaine on the inhibition of SK2 currents

The effect of LAs and apamin on SK2 currents was determined on a SK2 cell (Fig. 1A). The half-maximal inhibitory concentration (IC₅₀) value for bupivacaine was 16.5 $\mu\text{mol/L}$ (95% CI: 12.46–21.83; Fig. 1B). The IC₅₀ value for ropivacaine and lidocaine was 46.5 $\mu\text{mol/L}$ (95% CI: 31.37–69.03) and 77.8 $\mu\text{mol/L}$ (95% CI: 55.66–108.7), respectively (Fig. 1C and 1D).

Inhibition of SK2 currents with LAs was reversible

Next, we explored whether the inhibitory effect of bupivacaine was reversible. In this part, SK2 currents from SK2 cells were recorded while exposed to 1 μM , 10 μM , and 100 μM bupivacaine. Consequently, SK2

currents measured at 0-mV membrane potential were completely reversed to the baseline value after washout ($P > 0.05$; Fig. 2).

Modulation of the inhibitory effect of bupivacaine by calcium concentration

The opening probability of the SK2 channel is related to intracellular calcium concentration. Here, we tested whether the inhibitory effect of bupivacaine on the SK2 current was affected by calcium concentration. Figure 3A shows traces of the SK2 current induced in the presence of intracellular free calcium concentrations of 0.25, 0.50, and 1.0 μM . The results showed increase in the SK2 current as the calcium concentration increased and reached 1.0 μM ($P < 0.05$). Figure 3B shows that bupivacaine inhibited SK2 currents to different extents in the presence of different intrapipette concentrations of free calcium. The results showed that SK2 currents were inhibited the least at a calcium concentration of 1.0 μM ($P < 0.05$).

Discussion

The results revealed the following: 1) Bupivacaine could reversibly inhibit the SK2 channel in a dose-dependent manner; 2) the IC₅₀ values of bupivacaine, ropivacaine, and lidocaine for inhibiting SK2 were 16.5 μM , 46.5 μM , and 77.8 μM , respectively; and 3) the intracellular calcium concentration could affect the inhibitory effect of bupivacaine on SK2 currents.

After transfection with the SK2 gene, SK2 cells produced stable SK2 currents, which could be inhibited by apamin. Therefore, the SK2 current is also called apamin-sensitive current.¹⁹ Since the opening of the SK2 channel was mainly dependent on the calcium concentration, we recorded the SK2 current at 0 mV voltage. The intracellular free calcium concentration was controlled using the electrode solution, and the extracellular buffer did not contain free calcium.

Bupivacaine cardiotoxicity results from the blockade of a wide range of myocardial ion channels, the most important being the sodium channel. In this study, we used the whole-cell patch-clamp technique to investigate the effects of LAs on SK2 currents and measured the IC₅₀ values of bupivacaine, ropivacaine, and lidocaine. Bupivacaine ranked first in its potency of inhibiting SK2 currents, followed by ropivacaine and lidocaine. Interestingly, this order of potency is consistent with the order of LA cardiotoxicity. The maximum recommended clinical dose of bupivacaine is 175 mg. If a patient weighing 70 kg is given 175 mg of bupivacaine (blood volume is about 7% of body weight), bupivacaine plasma concentration could reach 104.2 μM . If 175 mg bupivacaine reaches the heart quickly, it would immediately cause cardiac arrest. The concentration of bupivacaine in the heart would be much higher than 104.2 μM . Therefore, theoretically, the concentration of bupivacaine in the heart can reach an IC₅₀ value of 16.5 μM .

In the past, Martín et al.²⁰ had studied the inhibitory effect of bupivacaine on large-conductance calcium-activated potassium channels in smooth muscle cells of human umbilical artery. In his study, bupivacaine could block these potassium channels. Additionally, Sbarbaro et al.²¹ found that lidocaine could also block SK2 currents in nerve cells. However, lidocaine blocked SK2 currents only when it

exceeded clinical concentrations. It has been suggested that the blockade of SK2 channels by lidocaine is unlikely to cause clinical effects. However, our study found that SK2 channels were very sensitive to bupivacaine and ropivacaine. The specific mechanism of this inhibition is still unclear. Since the inhibitory effect of bupivacaine on the SK2 channel could affect several physiological functions, this inhibitory effect of bupivacaine should be taken into account and considered as bupivacaine cardiotoxicity.

We also found that the intracellular calcium concentration could affect the inhibitory effect of bupivacaine on SK2 currents. SK2 channel proteins are coupled with calmodulin, and the binding of calcium with calmodulin would alter the conformation and function of SK2 channels.²² Since studies have suggested that bupivacaine alters intracellular calcium concentrations, bupivacaine is expected to indirectly regulate the SK2 channel. To eliminate the effects of this process, we controlled the intracellular calcium concentration in the pipette solution in this study. Consequently, this concentration-dependent inhibition of SK2 currents suggests that the concentration of intracellular free calcium contributes to bupivacaine cardiotoxicity.

Bupivacaine reportedly inhibits several ion currents in the heart (e.g., sodium channels, L-calcium channels, and potassium channels), and this study adds SK2 channels to the list of ion channels affected by bupivacaine. SK2 channels could cause arrhythmia depending on their expression levels in cardiomyocytes.^{23, 24} These channels could cause arrhythmia when the gene is expressed too much or too little in cardiomyocytes.²⁵ SK2 channels also participate in mitochondrial function.^{26–29} Therefore, the effects of SK2 channels on the action potential and mitochondrial function suggest that the blockade of SK2 channels is involved in bupivacaine cardiotoxicity. More experiments are needed to demonstrate this hypothesis.

Limitations

Under normal circumstances, the SK2 channel proteins are coupled with calmodulin, and the binding of calcium with calmodulin would affect the conformation and function of these channels.²² However, because only the SK2 gene was transfected into HEK293 cells in our study, this effect of calcium on SK2 channels would be affected.

Conclusions

The results of this study suggest a new inhibitory effect of bupivacaine on SK2 channels. Future studies should be concerned with the effects of SK2 channels on bupivacaine cardiotoxicity.

Abbreviations

SK2 channel: small-conductance calcium-activated potassium type 2 channel; HEK293 cell: human embryonic kidney 293 cell; IC50: half-maximal inhibitory concentration.

Declarations

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Committee of Animal Use and Care, and the Institutional Committee of Human and DNA Research at Wenzhou medical University.

Consent for Publication

Not applicable.

Availability of data and material

The datasets used and analyzed in the current study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HC, FX and ZJ designed the study, analyzed the data and wrote the manuscript. FZ conducted the study and wrote the manuscript. All authors have read and approve the final manuscript.

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Figures

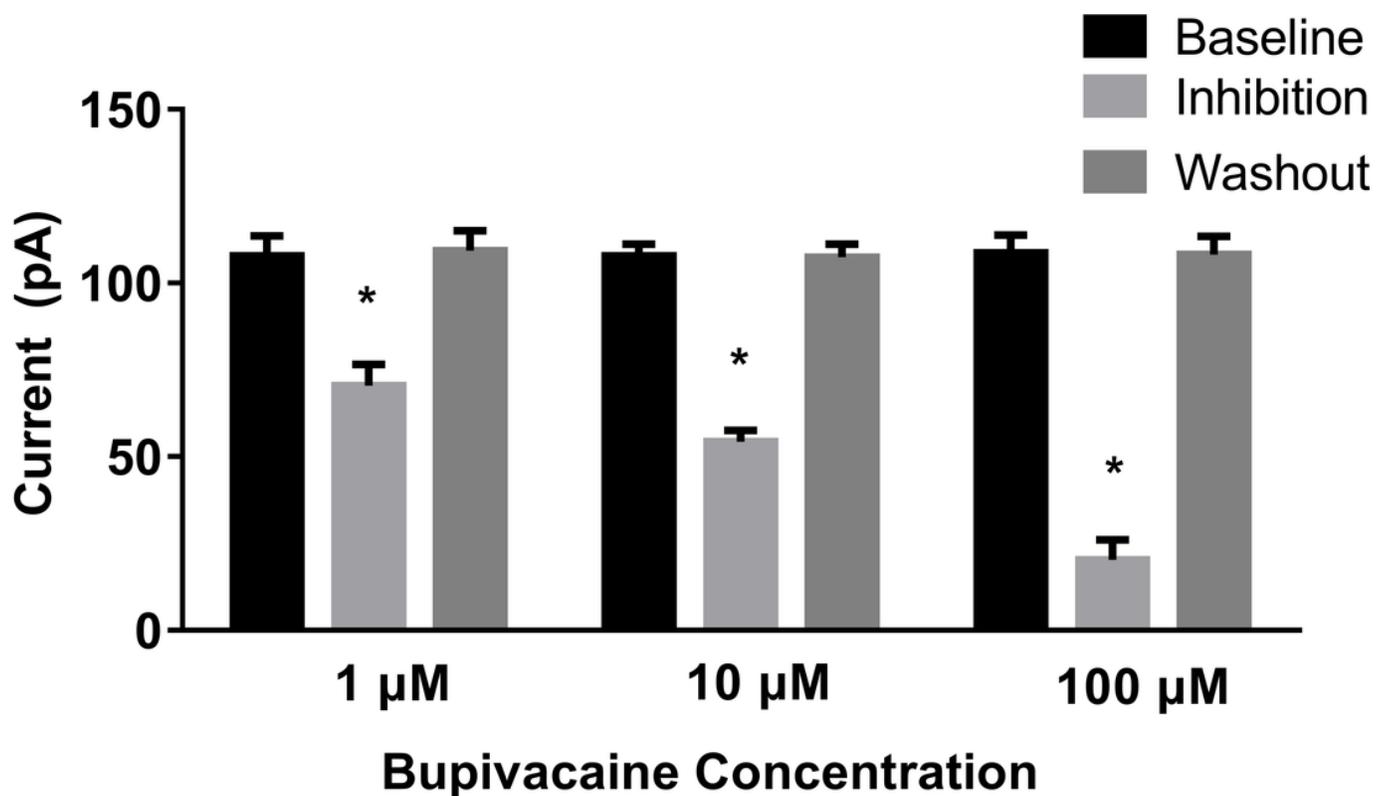


Figure 1

Concentration-dependent inhibitory effects of bupivacaine, ropivacaine, and lidocaine on SK2 currents. (A) Time course of SK2 currents obtained at 0 mV in the presence or absence of lidocaine, ropivacaine, bupivacaine, and apamin. (B, C, and D) Dose-dependent effects of bupivacaine on inhibiting the SK2 current (0 mV) were fitted to the Hill equation to obtain the IC50 value of bupivacaine, ropivacaine, and lidocaine, respectively. The equation was $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$. The pipette solution contained 1 μM free calcium.

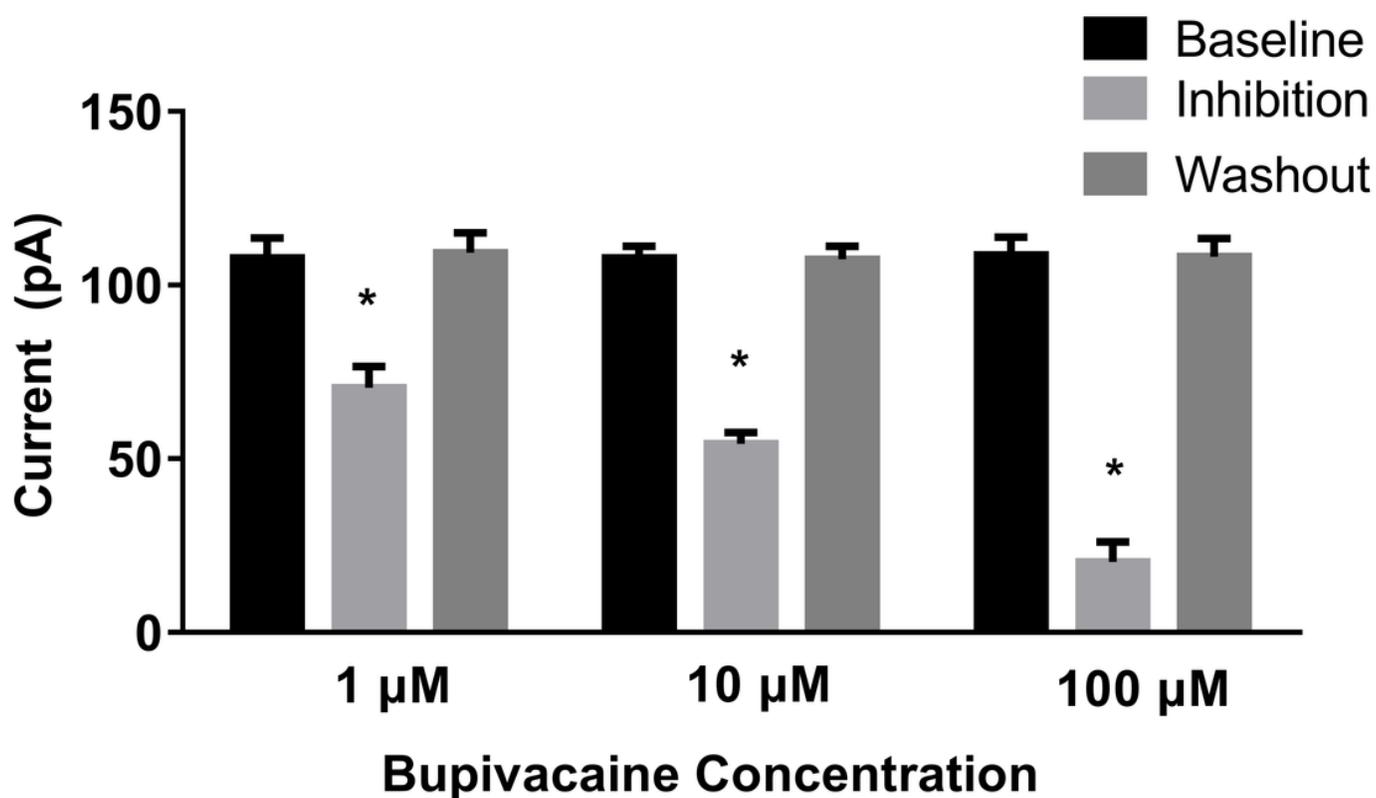


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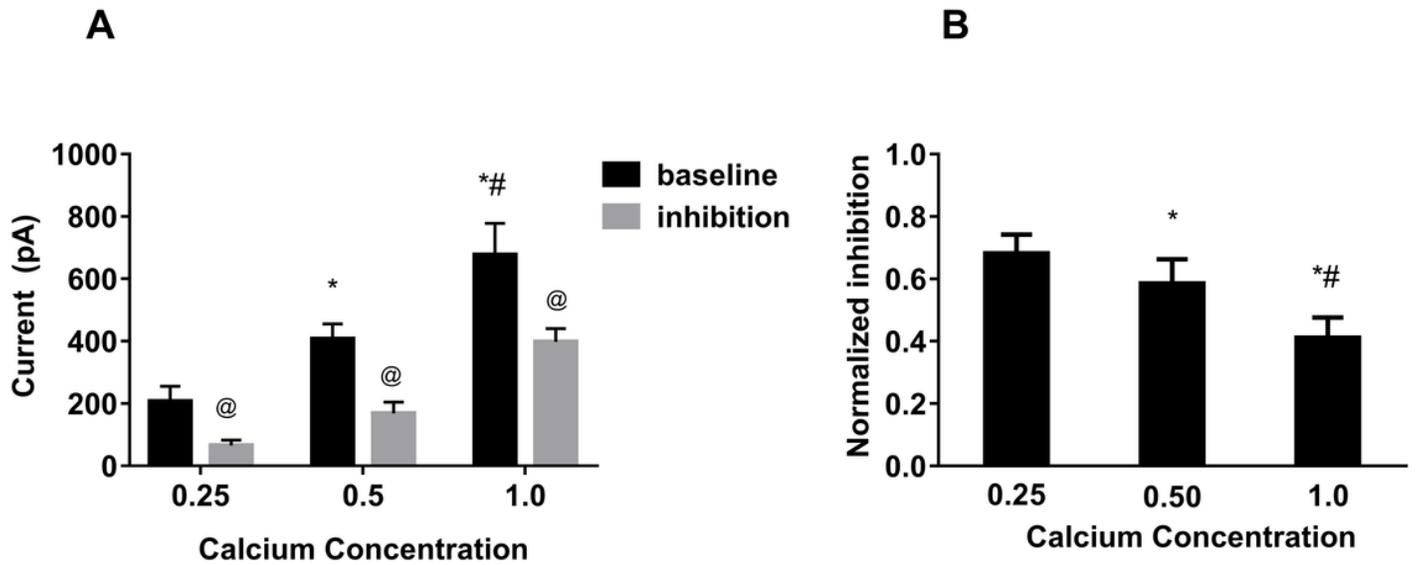


Figure 2

Inhibitory effect of bupivacaine on SK2 current is reversible ($n = 7$ in each concentration). SK2 currents (0 mV) obtained at baseline, inhibition, and washout phases with exposure to 1 μM , 10 μM , and 100 μM bupivacaine. Baseline: perfusion with Tyrodé's solution. Inhibition: perfusion with Tyrodé's solution containing bupivacaine. Washout: replacement of bupivacaine-containing Tyrodé's solution with normal Tyrodé's solution. Normalization inhibition was calculated as $(\text{Current}_{\text{baseline}} - \text{Current}_{\text{inhibition}}) / \text{Current}_{\text{baseline}}$. The intrapipette free calcium concentration was 1 μM . * $P < 0.05$, compared with baseline value.

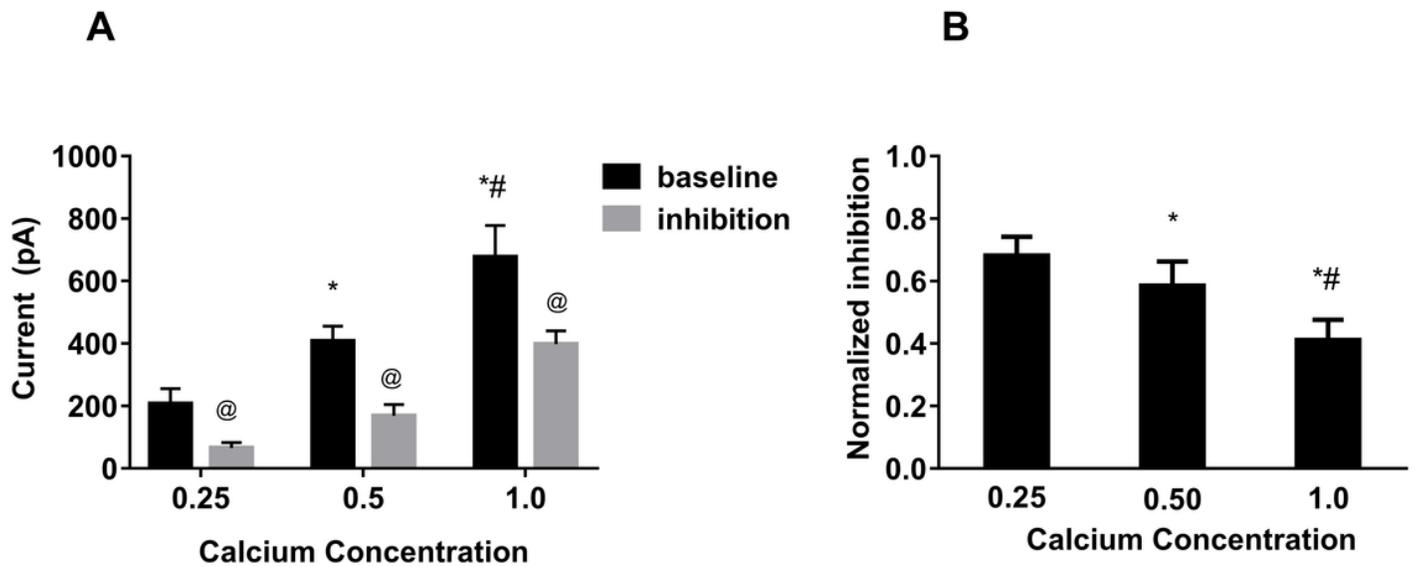


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Inhibitory effect of bupivacaine on SK2 current is reversible (n = 7 in each concentration). SK2 currents (0 mV) obtained at baseline, inhibition, and washout phases with exposure to 1 μ M, 10 μ M, and 100 μ M bupivacaine. Baseline: perfusion with Tyrode's solution. Inhibition: perfusion with Tyrode's solution containing bupivacaine. Washout: replacement of bupivacaine-containing Tyrode's solution with normal Tyrode's solution. Normalization inhibition was calculated as $(\text{Current}_{\text{baseline}} - \text{Current}_{\text{inhibition}}) / \text{Current}_{\text{baseline}}$. The intrapipette free calcium concentration was 1 μ M. *P < 0.05, compared with baseline value.

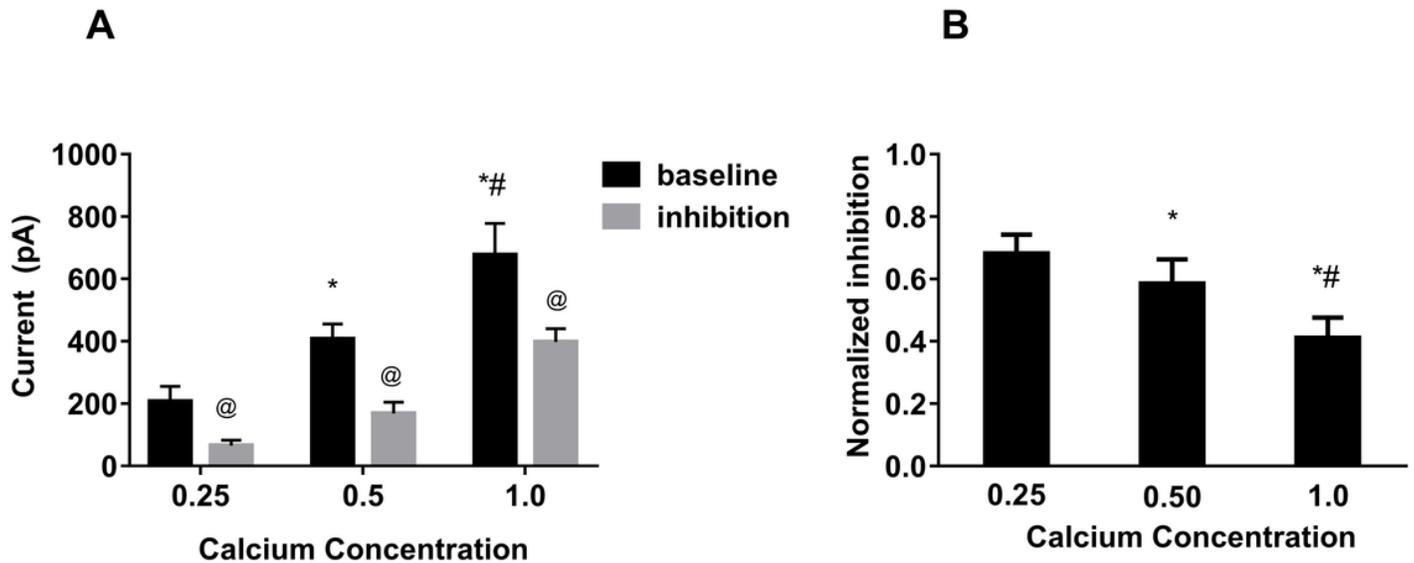


Figure 3

Effect of calcium concentration on the inhibitory effect of bupivacaine. (A) SK2 currents obtained at 0 mV in the presence of different concentrations of free calcium. (B) The degree of inhibition of SK2 currents (0 mV) by 10- μ M bupivacaine when the pipette solution contained different concentrations of free calcium. Baseline: perfusion with Tyrode's solution. Inhibition: perfusion with Tyrode's solution containing bupivacaine. Washout: replacement of bupivacaine-containing Tyrode's solution with normal Tyrode's solution. Normalization inhibition was calculated as $(\text{Current}_{\text{baseline}} - \text{Current}_{\text{inhibition}}) / \text{Current}_{\text{baseline}}$. *P < 0.05, between 0.25 μ M group and 0.5 μ M group, #P < 0.05, between 0.5 μ M group and 1.0 μ M group. @P < 0.05, between inhibition value and baseline value.

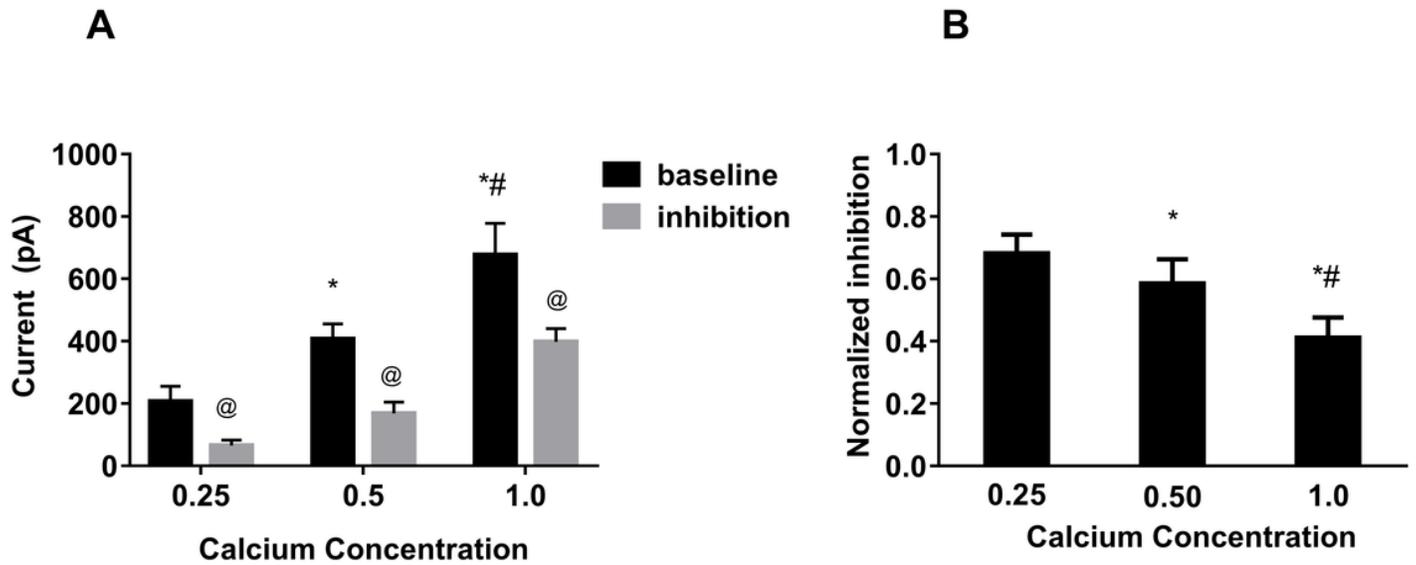


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