# Inhibition of Martentoxin on Neuronal BK Channel Subtype ($\alpha+\beta4$): Implications for a Novel Interaction Model

Jian Shi,* Hui Qiong He,* Rong Zhao,* Yan-Hong Duan,† Jin Chen,* Ying Chen,† Juan Yang,† Jian Wei Zhang,† Xue Qin Shu,† Ping Zheng,‡ and Yong Hua Ji†

*Graduate School of the Chinese Academy of Sciences, Institute of Physiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, People's Republic of China; †School of Life Sciences, Shanghai University, Shanghai, 200444, People's Republic of China; and ‡State Key Laboratory of Medical Neurobiology, Fudan University Shanghai Medical College, Shanghai, 200032, People's Republic of China

ABSTRACT Martentoxin as a 37-residue peptide was capable of blocking large-conductance $Ca^{2+}$-activated $K^+$ (BK) channels in adrenal medulla chromaffin cells. This study investigated the pharmacological discrimination of martentoxin on BK channel subtypes. The results showed that the iberiotoxin-insensitive neuronal BK channels ($\alpha+\beta4$) could be potently blocked by martentoxin ($IC_{50} = \sim80$ nM). In contrast, the iberiotoxin-sensitive BK channel consisting of only $\alpha$-subunit was less sensitive to martentoxin. Distinctively, martentoxin inhibited neuronal BK channels ($\alpha+\beta4$) with a novel interaction mode. Two possible interaction sites of neuronal BK channels ($\alpha+\beta4$) might be responsible for the binding with martentoxin: one for trapping and the other located at the pore region for blocking. In addition, the inhibition of martentoxin on neuronal BK channels ($\alpha+\beta4$) depended on cytoplasmic $Ca^{2+}$ concentration. On the other hand, in vivo experiments from EEG recordings suggested that neuronal BK channels ($\alpha+\beta4$) were the primary target of martentoxin. Therefore, this research not only sheds light on a unique ligand for neuronal BK channels ($\alpha+\beta4$), but also highlights a novel model approach for the interaction between $K^+$ channels and specific-ligands.

## INTRODUCTION

Voltage-dependent large-conductance $Ca^{2+}$-activated $K^+$ channels, often referred to as BK channels, resemble a unique class of ion channels that couple intracellular chemical signaling to electric signaling (1). BK channels have been shown to regulate neuronal firing (2–5), endocrine cell secretion (6,7), and smooth muscle tone (8,9).

Functional BK channels with voltage- and $Ca^{2+}$-dependence are formed by a tetrameric assembly (10) of pore-forming $\alpha$-subunits (Slo$\alpha$). They share close homology with voltage-gated $K^+$ (Kv) channel $\alpha$-subunits, but differ by having additional hydrophobic segments (S0 to S10 in total) resulting in an extracellular N-terminal and a long cytosolic C-terminal where at least one of the regulatory $Ca^{2+}$-binding domains resides (11). Although alternative splicing of Slowpoke-related genes results in channels with altered activation and phosphorylation properties (12,13), tissue-specific expression of accessory $\beta$-subunits is responsible for many of the fundamental diversities between cell types. $\beta$-subunits have two transmembrane segments, connected by an ~120-residue extracellular domain. Four $\beta$-subunits named $\beta1$, $\beta2$, $\beta3$, and $\beta4$ have been cloned so far. BK channels consisting of $\alpha$ and $\beta4$ subunit are proved to distribute widely in neuronal tissues. Heterologous coexpression of the accessory $\beta4$ subunit with $\alpha$-subunit confers some different properties from that of other BK channel subtypes, including very slow gating kinetics and the resistance to iberiotoxin and charybdotoxin (14,15). Specifically, the detailed roles of the BK channel subtype ($\alpha+\beta4$) in the brain or neuronal tissues remain unknown due to the lack of specific tool for this channel.

Martentoxin is a 37-amino acid toxin purified from the venom of the Chinese scorpion (*Buthus martensi* Karsch). Initial studies showed that martentoxin at the applied dose of 100 nM could strongly block BK currents in adrenal medulla chromaffin cells, and BK currents blocked by martentoxin could be recovered fully, and the recovery was much faster than that by charybdotoxin (16). Subsequently, other electrophysiological studies (17,18) showed that martentoxin inhibited the delayed rectifying potassium current (IK) in rat hippocampal neurons, with very low potency (at $\mu$M levels). These results showed that this peptide has a great preference for BK channel over Kv channel (a 1000-fold difference).

So far, BK channels of hippocampal neurons and adrenal medulla chromaffin cells have been investigated to be comprised of many subtypes. Alternate splicing of $\alpha$-subunits and different $\beta$-subunits constitute these BK channels in these cells. However, the absence of specific tool to BK channels ($\alpha+\beta4$) results in an unclear comprehension of the function of this BK channel type in the neuronal system. Hence, the aim of this study is to investigate whether martentoxin can differentiate the BK channel subtypes, especially BK channels ($\alpha+\beta4$), and the interaction mode between martentoxin and the BK channel subtype.

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Address reprint requests to Dr. Yong-Hua Ji, School of Life Sciences, Shanghai University Shang-Da Road 99, Shanghai, 200444, People's Republic of China. Tel. and Fax: 86-21-66135189; E-mail: yhji@staff.shu.edu.cn.

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# METHODS

## Cell culture and transfection

Human embryonic kidney cells (HEK 293) were cultured in DMEM supplemented with 10% FBS. Culture dishes were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$, and subcultured approximately every 5 days. The plasmids containing hSlo$\alpha$ (U23767) and $\beta$4 (KCNMB4; AF207992) are gifts from N.W. Davies (University of Leicester) and J.D. Lippiat (Leeds University). Cells were transiently transfected with Lipofectamine reagent (Invitrogen, USA) at a ratio of 5 $\mu$l reagent with 1 $\mu$g total plasmid per 35-mm well. Cells were used for electrophysiological study in 48–72 h after transfection.

## Electrophysiological recording

Whole-cell voltage-clamp experiments were carried out as described previously (19), using an EPC-9 amplifier (HEKA eletronik, Germany) at room temperature (21°C–25°C). Patch pipettes were fabricated from glass capillary tubes by PP-830 Puller (Narishige, Japan) with the resistance of 4–7 M$\Omega$. Data acquisition and stimulation protocols were controlled by a Pentium III computer (Legend, Beijing, China) equipped with Pulse/PusleFit 8.3 software (HEKA eletronik, Germany). Capacitance transients were canceled and the cells whose value of series resistance higher than 20 M$\Omega$ were omitted. Leak subtraction was carried out using P/6 protocol. Data were low-passed at 10 kHz. The rate of solution exchange was studied using solutions with different KCl concentrations and found to be ~95% complete within 20 s. The holding potential was −70 mV. Unless stated specifically, all the recordings were done with the pulse of +60 mV. Untransfected HEK293 cells used in experiments has an average inherent current density D = 5.53 ± 0.21 pA/pF ($n$ = 15) at +60 mV and 1 $\mu$M martentoxin had no obvious effects on these net currents. These currents were too small compared to that evoked in transfected cells and were therefore ignored in analysis.

## $Ca^{2+}$ fluorescence measurements by Fura-2

The HEK293 cells were incubated with 5 $\mu$M Fura-2/AM (Molecular Probes, Dojindo Laboratories, Kumamoto, Japan) in HEPES buffered solution for 60 min at 37°C. The measurement of changes in cellular $Ca^{2+}$ concentration by Fura-2 was carried out as described previously (20). Fluorescence images were acquired with an inverted microscope (IX-70, Olympus Optical Co., Tokyo, Japan) equipped with a digital CCD camera (C4742-95-12NRB, Hamamatsu Photonics K. K., Hamamatsu, Japan). A high-speed scanning polychromatic light source (C7773, Hamamatsu Photonics K. K.) was used for alternating excitations at wavelengths of 340 nm and 380 nm. Data collection and analyses were carried out using a $Ca^{2+}$ imaging system (Aquacosmos Ver1.2, Hamamatsu Photonics K. K.). The sampling interval of Fura-2 fluorescence measurements was 2 s.

## EEG recordings

Male Sprague-Dawley rats (250–300 g) were used for the experiments (Shanghai experimental animal center, Chinese Academy of Sciences). All animal procedures were approved by the committee of Laboratory Animals, Chinese Academy of Sciences. The animals were anesthetized with sodium pentobarbital (40 mg/kg body weight, intraperitoneally) and were placed in a stereotaxic frame. The guide cannula was implanted into the dorsal hippocampus (AP, −4.3 mm; L, 2.5 mm; V, 2.5 mm) according to the method described (21). As for EEG recording, the recording electrode (AP, −3.5 mm; R, 2.0 mm; V, 1.5 mm) was positioned stereotaxically into the frontal cortex contralateral to the guide cannula implanted, whereas the reference electrode (AP, −10.0 mm) was placed in the cerebellum. The guide cannula and electrodes were fixed with dental cement and thus the wound was closed. The rats were allowed to recover for 3–4 days before the EEG recording.

The EEG activity was recorded by a bioamplifier (Model SMUP-E Bioeletric Signals Processing System, Shanghai Medical College of Fudan University, China) with a range of 100 mV and a passband of 0.1–40 Hz. Analog data were sampled at 1000 Hz. Rats subjected to EEG recordings were divided into two groups: control group without injection and the experimental group injected with various drugs in saline solution (total volume = 1 $\mu$l). For experimental group, after a 0.5 h baseline recording of EEG (control), drugs were injected into the dorsal hippocampus (AP, −4.3 mm; L, 2.5 mm; V, 2.5 mm). EEG recordings were maintained for 0.5 h after the administration of drugs. When testing was completed, the animals were sacrificed with ether and perfused intracardially with 200 ml of sterile saline followed by 400 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was removed and frozen sections of 40-$\mu$m were made in the region of cannula track for cresyl violet staining. Animals only with the accurate placement of cannula without tissue damage were used for data collection.

## Solutions and drugs

In the voltage-clamp mode, the bath solution for patch-clamp recordings contained (in mM): NaCl 135, KCl 5, $MgCl_2$ 1.2, $CdCl_2$ 2.5, HEPES 5, glucose 10 (pH 7.4 with NaOH).The pipette solution was composed of (in mM): NaCl 10, KCl 117, $MgSO_4$ 2, HEPES 10, MgATP 2, EGTA 1. The pH of the solution was adjusted to 7.2 with KOH. The total $Ca^{2+}$ to be added to give the desired free concentration was calculated using the program Maxchelator (http://www.stanford.edu/%7Ecpatton/maxc.html). The HEPES buffered solution for $Ca^{2+}$ imaging had a composition of (in mM): NaCl 137, KCl 5.9, $MgCl_2$ 1, $CaCl_2$ 2.5, HEPES 10, glucose 15 (pH 7.4 with NaOH). The high KCl solution was different from the HEPES buffered solution with 150 mM KCl. The toxin was dissolved in the bath solution, supplemented with 1 mg/ml bovine serum albumin (BSA) to prevent adherence of the toxin to the vials and the perfusion apparatus. Application of 1 mg/ml BSA alone did not alter BK channel function. Iberiotoxin and TEACl was purchased from Sigma (St. Louis, MO).

## Data analysis

Data were analyzed by PulseFit 8.5 (HEKA eletronik, Germany) and Origin 7.0 (Northampton, MA). Results of data analysis were expressed as mean ± SEM and n represents the number of the cells examined. The significant difference was compared using the unpaired Student's $t$-test. The degree of toxin block was calculated by expressing the remaining current after each drug exposure as a fraction of the current magnitude of the patch before the first drug exposure (i.e., fractional current remaining, $I_f$). The plots of $I_f$ against [martentoxin] were fit with a Hill equation: $I_f = A2 + (A1-A2)/(1 + (x/x0)^p)$, where $A1$ and $A2$ are constant, $x$ represents the different concentration of martentoxin, and $x0$ indicates the $IC_{50}$ value.

# RESULTS

## Inhibition of martentoxin on BK channels ($\alpha+\beta$4)

The effect of martentoxin on BK channels ($\alpha+\beta$4) was investigated through whole-cell electrophysiological recordings. The currents evoked by coexpression of $\alpha$ and $\beta$4 subunits are measured at +60 mV, typical of the characteristics of BK channels as reported (13,22), including slow-activation, slow-deactivation kinetics, and pharmacological insensitivity to iberiotoxin (Fig. 1 *A*). Martentoxin at 400 nM dose could almost completely inhibit the current (Fig. 1 *A*) with a quick and reversible manner (Fig. 1 *B*). The inhibitory effect of 400 nM martentoxin on the channels occurred within 90 s, suggesting a possible direct interaction between martentoxin and BK channels. The inhibition can be reversed after washing. The time course of recovery is within 90 s.

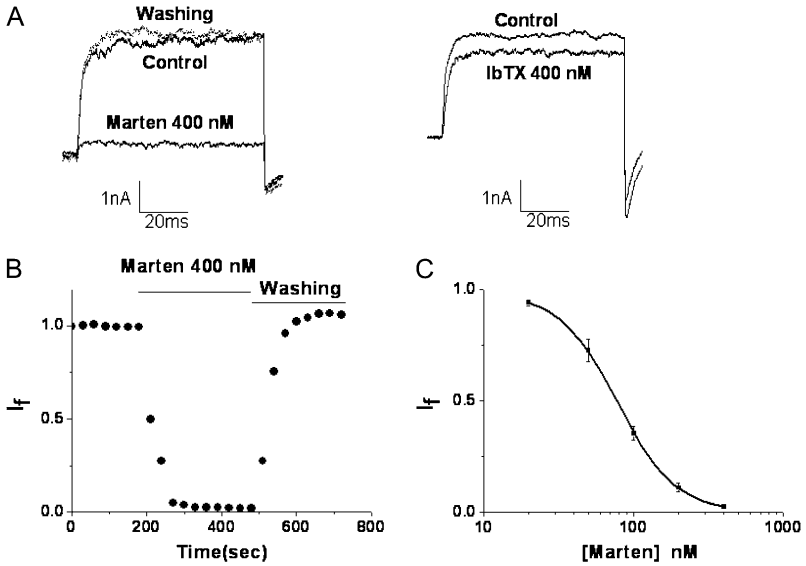


FIGURE 1 Blocking of martentoxin on BK channels ($\alpha+\beta4$). (*A*) Representative whole cell current traces from cells expressing hSlo$\alpha$ and $\beta4$ subunits. The holding voltage was $-70$ mV and the currents were elicited by a pulse of $+60$ mV with 500 nM free $Ca^{2+}$ concentration in the pipette solution. Martentoxin (400 nM) completely inhibited the currents. 400 nM iberiotoxin had no obvious effects on these channels. (*B*) The time course for martentoxin blockage of BK channels ($\alpha+\beta4$). Fast inhibition as well as fast recovery was observed. (*C*) Dose-response curve of martentoxin-induced inhibition of BK channels ($\alpha+\beta4$). Plot of the fraction of unblocked current ($I_f$) versus the martentoxin concentration. Each point presents data from 5–8 cells. The curve was obtained as described in Methods. The current in the presence of martentoxin and the control current were both measured at $+60$ mV with 500 nM free $Ca^{2+}$ in the pipette solution.

Furthermore, the steady-state current after washing were slightly higher than that of control ($1.12 \pm 0.03$, $p < 0.05$; $n = 12$), different from the current after the sham wash (only bath solution wash without pretreatment of toxin) compared to that of control ($1.06 \pm 0.08$, $p > 0.05$; $n = 10$). The dose-response curve was obtained and the percentage of block is shown as a function of martentoxin concentration (Fig. 1 *C*). The $IC_{50}$ of martentoxin on BK channels is assessed to be $78.01 \pm 5.86$ nM. The Hill coefficient is $2.23 \pm 0.36$, suggesting BK channels ($\alpha+\beta4$) may consist of two interaction sites for martentoxin.

## Sensitivity of martentoxin on BK channels (only $\alpha$)

BK channels composed of only $\alpha$-subunits are sensitive to all the classical toxins such as charbdotoxin and iberiotoxin. However, all these toxins cannot block the BK channel subtype composed of $\alpha$ and $\beta4$ subunits. In this study, the sensitivity of the channels (only $\alpha$-subunit) to martentoxin was investigated. The currents measured at $+80$ mV were not significantly affected by 5-min application of 400 nM martentoxin (Fig. 2), but completely abolished by 400 nM iberiotoxin (Fig. 2, *A* and *C*).This shows that martentoxin may be a potential selector to the BK channel subtypes.

## Allosteric modulation of iberiotoxin on the inhibition of martentoxin on BK channels ($\alpha+\beta4$)

Because functional BK channels including $\beta4$ subunits are insensitive to iberiotoxin, it is reasonable to speculate that iberiotoxin could not interact with the channels. As suggested previously (23), iberiotoxin could allosterically modulate the binding of charybdotoxin with bovine aortic sarcolemmal

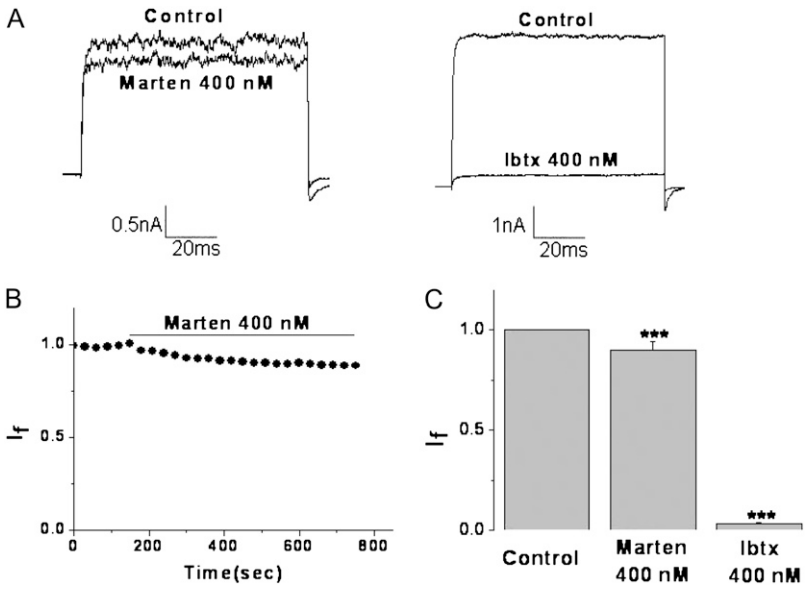


FIGURE 2 Slight effect of martentoxin on BK channels ($\alpha$). (*A*) Representative current traces are shown. The channels were activated by $+80$ mV with a $-70$ mV holding potential. The free $Ca^{2+}$ concentration in the pipette solution was ~500 nM. The currents were hardly inhibited by 400 nM of martentoxin whereas the currents were completely abolished by 400 nM iberiotoxin. (*B*) The time course curve confirmed the lack of sensitivity of this type ($\alpha$) of BK channels to martentoxin. (*C*) Comparison of the effects of martentoxin (n = 5) and iberiotoxin (n = 3) on BK channel ($\alpha$) is shown. The significance was compared between the toxin and the control, respectively. ***$p <$ 0.001.

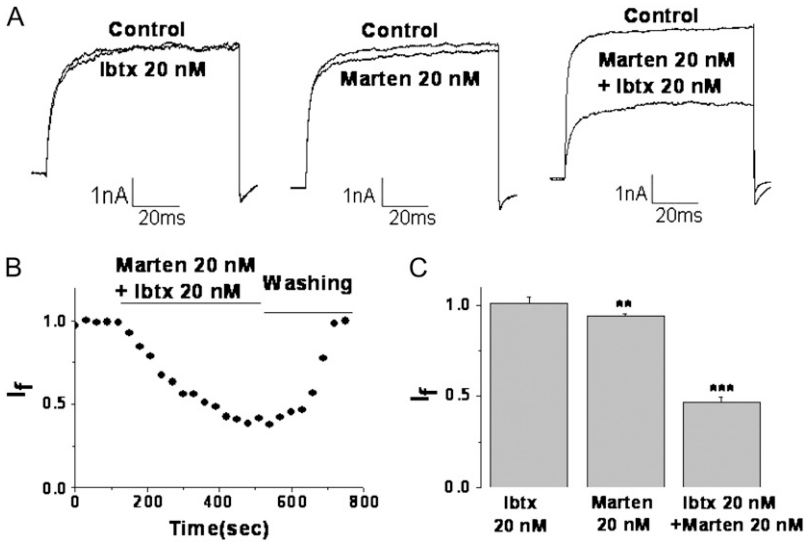


FIGURE 3 Allosteric effect of iberiotoxin on the martentoxin interaction with BK channels ($\alpha+\beta4$). Representative current traces are shown (*A*). Iberiotoxin and martentoxin were applied simultaneously. The currents were inhibited strongly. The time course for inhibition and recovery is shown (*B*). (*C*) The allosteric relationship between iberiotoxin and martentoxin effects on this type of BK channels. The normalized currents after the application of 20 nM iberiotoxin ($n = 4$), 20 nM martentoxin ($n = 5$) and simultaneous application of 20 nM martentoxin + 20 nM iberiotoxin ($n = 5$) are shown. $^{**}p < 0.01$; $^{***}p < 0.001$. The comparison was done between the currents after the application of different toxins and the control currents.

membrane vesicles. We, therefore, investigated whether iberiotoxin can exert similar allosteric modulation on martentoxin's effect.

Iberiotoxin (20 nM) alone hardly blocked the channels, whereas 20 nM martentoxin alone only inhibited the currents slightly ($I_f = 0.94 \pm 0.01$, $n = 5$; Fig. 3 *C*). In contrast, a substantial inhibition of the BK channel currents was recorded in the case of simultaneous administration of 20 nM martentoxin plus 20 nM iberiotoxin (Fig. 3, *A* and *C*). This inhibition occurred within 300 s. $I_f$ by mixture, namely 20 nM martentoxin plus 20 nM iberiotoxin, was ~50% of that by 20 nM martentoxin ($I_f = 0.46 \pm 0.03$, $n = 5$).

Because $\beta4$ subunits of neuronal BK channels ($\alpha+\beta4$) hindered the close of iberiotoxin to the pore region (24), it was speculated whether martentoxin could bind to the pore region. The activities of neuronal BK channels ($\alpha+\beta4$) could be inhibited by TEACl, a classical pore blocker of $K^+$ channel. The currents were obviously reduced ($I_f = 0.60 \pm 0.06$, $n = 4$) by the application of 1 mM TEACl. When an additional 20 nM martentoxin was accompanied with the administration of 1 mM TEACl, the currents seemed to be invariable ($I_f = 0.61 \pm 0.04$, $n = 5$), suggesting that the pharmacological effects of 20 nM martentoxin was covered by 1 mM TEACl. In contrast, when 100 nM martentoxin and 1 mM TEACl were applied simultaneously, the currents were significantly reduced ($I_f = 0.34 \pm 0.01$, $n = 4$) and similar to those after the treatment of 100 nM martentoxin alone ($I_f = 0.35 \pm 0.03$, $n = 6$), suggesting that the pharmacological effects of 1 mM TEACl was covered by 100 nM martentoxin (Fig. 4, *A* and *B*). Thus, the pore region of neuronal BK channels ($\alpha+\beta4$) should be involved in the binding sites for martentoxin absolutely.

The dose-response curve of martentoxin versus BK Channels ($\alpha+\beta4$) suggests that martentoxin probably occupied two interactive sites at BK Channels ($\alpha+\beta4$). Because iberiotoxin reinforced the interaction between BK channels ($\alpha+\beta4$) and martentoxin, it is possible that one of these two sites may be trapping site and the other may be an inhibitory site. Hence, iberiotoxin was used after the application of martentoxin. The results showed that 50 nM martentoxin inhibited the channel currents significantly. When 200 nM iberiotoxin was administrated after the application of 50 nM martentoxin, the channel currents were significantly abolished ($0.14 \pm 0.02$, $n = 5$) and could be recovered after washing (Fig. 5, *A* and *C*).

Contrast to the pretreatment of martentoxin, the pretreatment of iberiotoxin had no similar residual effects on BK channels ($\alpha+\beta4$). The activities of BK channels ($\alpha+\beta4$) were unaffected by 200 nM iberiotoxin but inhibited signif-

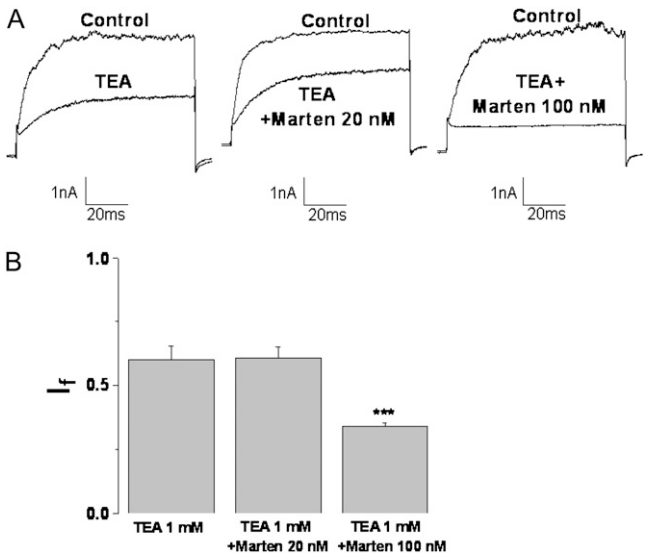


FIGURE 4 Pore region of neuronal BK channels ($\alpha+\beta4$) was involved in the binding with martentoxin. Representative current traces are shown (*A*). The currents were reduced by 1 mM TEACl ($n = 4$), 1 mM TEACl and 20 nM martentoxin or 1 mM TEACl and 100 nM martentoxin. The statistical analysis was presented in *B*. $^{***}p < 0.005$.

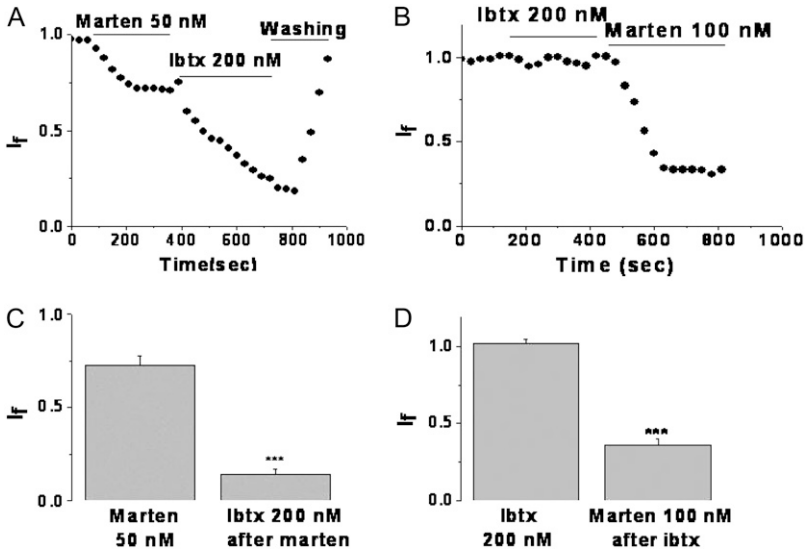


FIGURE 5 Residual effects of martentoxin on the interaction between iberiotoxin and BK channels ($\alpha+\beta4$). The results that martentoxin has a long-lasting modulatory effect on BK channels are shown (*A* and *C*). Iberiotoxin (200 nM) was applied after the application of 50 nM martentoxin. The inhibition evoked by the application of martentoxin was enhanced by the subsequent application of iberiotoxin. This inhibition could be removed by washing very quickly. The inhibition by direct application of 50 nM martentoxin and the subsequent application of 200 nM iberiotoxin was compared in (*C*). On the contrary, no residual effects of iberiotoxin on BK channels ($\alpha+\beta4$) were observed (*B* and *D*). $^{**}p < 0.01$; $^{***}p < 0.001$. The comparison was done between the currents after the application of first toxin and the second toxin.

icantly by the posttreatment of 100 nM martentoxin (Fig. 5, *B* and *D*). $I_f$ for the posttreatment of 100 nM martentoxin ($0.36 \pm 0.04$, $n = 5$)was not significantly different compared to that for the pretreatment of 100 nM martentoxin ($0.35 \pm 0.03$, $n = 8$). Therefore, it is unambiguous that residual effects exist only in the pretreatment of martentoxin.

## Spontaneous electrical activities of rat brain enhanced by martentoxin

In vivo experiments were conducted with pharmacological application of martentoxin on the spontaneous brain electrical activities in rats. Normal discharges of temporal lobe region were not affected by the injection of saline solution or 10 $\mu$M iberiotoxin (1 $\mu$l) into hippocampal region (Fig. 6, *A* and *B*), suggesting that either the abundance of iberiotoxin-sensitive BK channels is rare or this type of BK channels are lack of functions in the generation of the normal nerve

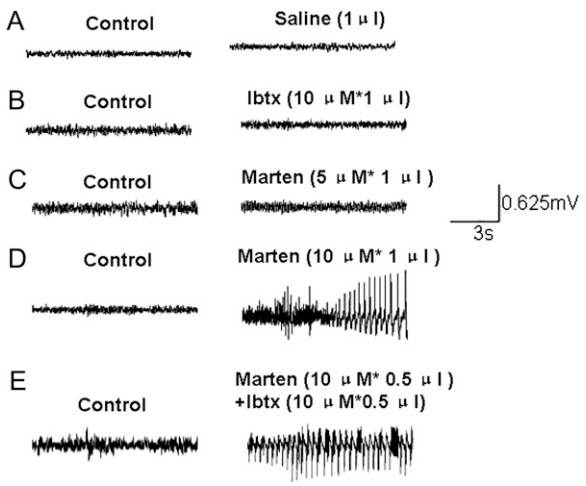


FIGURE 6 Effects of different toxins on spontaneous bursting activities of rat brain. Representative EEG patterns in the temporal lobe evoked by the injection of these drugs into hippocampal region are shown. Saline solution (1 $\mu$l) (*A*) and 1 $\mu$l·10 $\mu$M iberiotoxin (*B*) could not affect the bursting activities. 1 $\mu$l·5 $\mu$M martentoxin (*C*) could not induce abnormal discharges whereas 1 $\mu$l·10 $\mu$M martentoxin (*D*) could. When 0.5 $\mu$l·10 $\mu$M martentoxin and 0.5 $\mu$l·10 $\mu$M iberiotoxin were administrated simultaneously into hippocampus region, the abnormal discharges were more obvious (*E*).

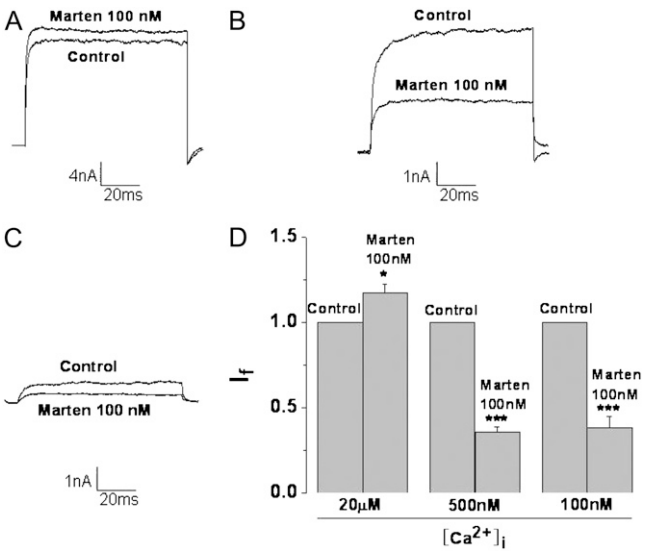


FIGURE 7 Different free $Ca^{2+}$ concentrations in the pipette solutions affected the interaction of martentoxin with the BK channels ($\alpha+\beta4$). When the free $Ca^{2+}$ concentration in the pipette solution was elevated to 20 $\mu$M, the recorded currents were enlarged by martentoxin 100 nM (*A*). However, the currents were normally inhibited by 100 nM martentoxin with 500 nM free $Ca^{2+}$ (*B*) and 100 nM free $Ca^{2+}$ in the pipette solution (*C*). The statistical data are shown (*D*). The normalized current after the application of 100 nM martentoxin with 20 $\mu$M free $Ca^{2+}$ in the pipette solution ($n = 6$), the normalized current after the application of 100 nM martentoxin with 500 nM free $Ca^{2+}$ in the pipette solution ($n = 6$) and that after the application of 100 nM martentoxin with 100 nM free $Ca^{2+}$ in the pipette solution ($n = 5$) are shown. $^{*}p < 0.01$; $^{***}p < 0.001$. The comparison was done between the control current and the currents after the application of martentoxin.

electrical activity in this particular region. The recordings of temporal lobe discharges before and after the injection of martentoxin into hippocampus region showed that 10 $\mu$M martentoxin (1 $\mu$l) evoked an obviously abnormal discharge, whereas 5 $\mu$M martentoxin (1 $\mu$l) was ineffective (Fig. 6, *C* and *D*). To rule out the possible involvement of other K channels on this abnormal discharge, 10 $\mu$M iberiotoxin (0.5 $\mu$l) plus 10 $\mu$M martentoxin (0.5 $\mu$l) injected into the hippocampus regions caused an exacerbated abnormal discharge (Fig. 6 *E*). These results suggest that BK channels ($\alpha+\beta4$) may play a braking role in neural excitability and the inhibition of these channels by martentoxin can induce hyperexcitability in hippocampal spontaneous electrical activities. Allosteric modulation of iberiotoxin on the inhibition of martentoxin on BK channels ($\alpha+\beta4$) happened in these in vivo recordings.

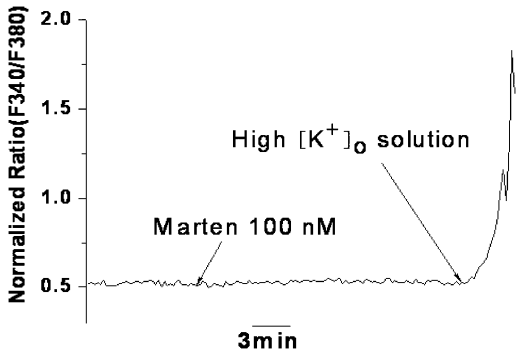


FIGURE 8 Effects of martentoxin on cytoplasmic $Ca^{2+}$. After the application of martentoxin 100 nM, the cytoplasmic $Ca^{2+}$ concentration was unchanged.

## $Ca^{2+}$ dependence of martentoxin modulation

The results showed above were obtained with ~500 nM $Ca^{2+}$ in the pipette solution. The effects of martentoxin on the BK channels ($\alpha+\beta4$) were also investigated with high free $Ca^{2+}$ (20 $\mu$M) in the pipette solution. The channels ($\alpha+\beta4$) could not be blocked by 100 nM martentoxin in the high free $Ca^{2+}$ of pipette solution. Conversely, the channel currents were enlarged to 1.17 ± 0.05 ($n = 6$) of the control (Fig. 7, *A* and *D*). $I_f$ in the presence of 100nM martentoxin were 0.35 ± 0.03 ($n = 6$) and 0.38 ± 0.06 ($n = 5$) at 500 nM (Fig. 7, *B* and *D*) and 100 nM (Fig. 7, *C* and *D*) free $Ca^{2+}$ in the pipette solution, respectively. Furthermore, $Ca^{2+}$ imaging showed that 100 nM martentoxin could not evoke the oscillation of cytoplasmic $Ca^{2+}$ concentration, and only the solution with a high KCl concentration induced a $Ca^{2+}$ rise (Fig. 8). These results showed that the cytoplasmic $Ca^{2+}$ concentration after the establishment of the whole-cell mode might be consistent with that in the pipette solution and remained unchanged during the recording.

## DISCUSSION

This study investigated the pharmacological characterization of martentoxin, a novel BK channel ligand, on neuronal-specific BK channels ($\alpha+\beta4$) and pointed out the possible physiological roles of the BK channels ($\alpha+\beta4$) in excitability. As described previously (15,25–27), neuronal-specific $\beta4$ subunit modulates the voltage dependence, activation kinetics and toxin sensitivity of hSlo channel. Peptides including iberiotoxin, a classical neurotoxin targeting BK channel, charybodotoxin and slotoxin are all selective to the BK channel subtype ($\alpha$). In general, it is very hard to confirm the function of BK channels ($\alpha+\beta4$) in neuronal system with pharmacological approach. Martentoxin may be a unique neurotoxin which can block the BK channels ($\alpha+\beta4$) with a higher preference to the BK channels ($\alpha+\beta4$) than to BK channels ($\alpha$).

As indicated by the data of site mutation (24) and the specificity of extracellular loop of $\beta4$ subunit (15), the polysaccharide chains of the Asn residues located in the extracellular loop of $\beta4$ subunit hindered the close of iberiotoxin

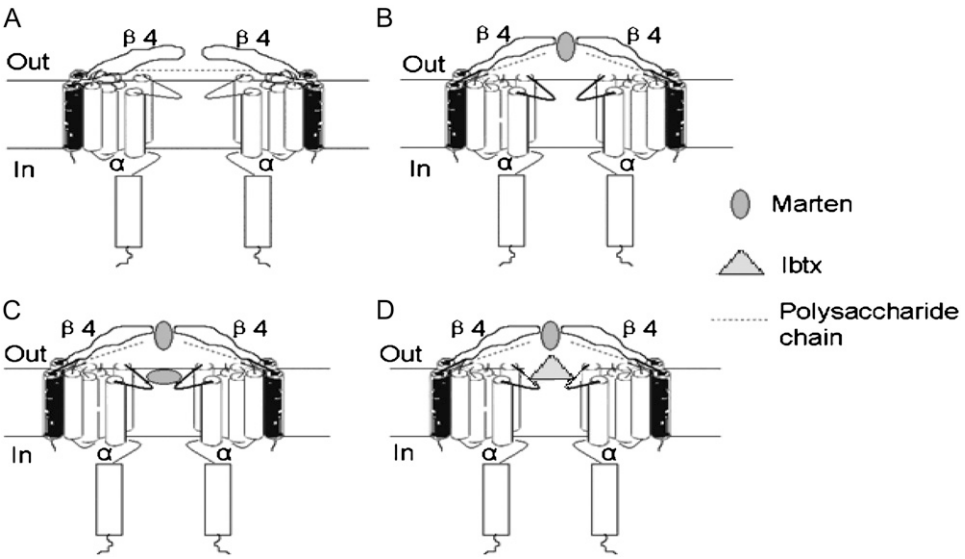


FIGURE 9 Proposed mechanism for the interaction between the BK channels ($\alpha+\beta4$) and martentoxin. The conformation of normal open state (*A*) was changed by the application of martentoxin when the first steady complex was formed (*B*). Then, the low-affinity site was exposed to associate with martentoxin (*C*) or iberiotoxin (*D*). The red represents poly-saccharide chains, green represents the toxins, black represents the two transmembrane segments (TM1–TM2) of $\beta4$ subunit, blue represents the first four transmembrane segments (S0–S4) of $\alpha$-subunit, white represents the fifth and sixth segments (S5–S6) of $\alpha$-subunit. Yellow represents the intracellular helix segments (S7–S10) of $\alpha$-subunit. Only two of the four subunits were shown.

to the pore region and might be mainly responsible for the pharmacological insensitivity.

In this study, it was found that the inhibition of BK channels ($\alpha+\beta4$) by the mixture containing iberiotoxin and martentoxin are far more than that by martentoxion or iberiotoxin alone, and iberiotoxin was enabled to easily block BK channels ($\alpha+\beta4$) in the case of pretreatment of martentoxin, but not in the case of posttreatment of martentoxin. Furthermore, Hill coefficient based on the dose–response curve (Fig. 1 *C*) suggested two binding sites of BK channels ($\alpha+\beta4$) for martentoxin. Taking all results, it may allow us to propose a novel mechanism for the inhibitory effect of $K^+$ channel ligand to its target as shown in Fig. 9. We consider that it is possible that one of two sites for martentoxin is a trapping site and the other is an inhibitory site. First, martentoxin interacts with the trapping site formed by some residues of the extracellular loop of $\beta4$ subunit (Fig. 9 *B*). The interaction between martentoxin and the trapping site may be relatively steady due to a high-affinity even if washing was carried out. This interaction induced the conformational change of the extracellular loop and hence the poly-saccharide chains were diverted from the neighborhood to the suburb of the pore (Fig. 9 *B*). Finally, the pore region was exposed and the inhibitory site was formed to be capable of associating with martentoxin or iberiotoxin (Fig. 9, *C* and *D*). In contrary to the high-affinity of trapping site to martentoxin, the inhibitory site showed a lower affinity to the toxins and the interaction was reversible.

It has been shown that $Ca^{2+}$ usually affects pharmacological characteristics of toxins and chemicals, such as apamin (28), DiCl-DHAA (29), and BMS-204352 (30). The pharmacological diversity dependent on the $Ca^{2+}$ concentrations may come from the discrimination between $Ca^{2+}$ gating- and voltage gating-machinery of BK channel (31). In other words, it is assumed that the tension evoked by $Ca^{2+}$ gating machinery changed the conformation of pore region to some extent, which would indicate that the inhibitory site was disrupted.

Each subtype of BK channels in neuronal tissues might take part in variant physiological function. Considering the dilution of body fluid, we speculate that the concentration of drugs injected was in the nM range when the toxin arrived at the neurons, which was similar to the concentration applied in patch-clamp recordings of single cell. The allosteric inhibition indicated that the BK channels ($\alpha+\beta4$) was the unique target of toxins and may play a braking role in spontaneous neural activity.

The progress made in this study highlights martentoxin as a unique ligand for BK channels ($\alpha+\beta4$) and a novel interaction mode between $K^+$ channels and specific ligands.

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