Anticonvulsant activity of BmK AS, a sodium channel site 4-specific modulator

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\textbf{A B S T R A C T}

The anticonvulsant activity of BmK AS, a sodium channel site 4-selective modulator purified from scorpion venom (Buthus martensi Karsch), was investigated in unanesthetized rats with acute pentylenetetrazole (PTZ)- and pilocarpine-induced seizures. Rats were microinjected in the CA1 region with either saline or BmK AS, followed by epileptogenic doses of PTZ or pilocarpine 30 minutes later. The anticonvulsant efficacy of BmK AS in PTZ- or pilocarpine-evoked seizure-like behavior and cortical epileptiform EEG activity was assessed. Intrahippocampal injections of BmK AS (0.05–1 μg in 1 μL) produced dose-dependent anticonvulsant activity in the PTZ model, suppressing seizure-associated behavior and reducing both the number and duration of high-amplitude, high-frequency discharges (HAFDs) on the EEG. In contrast, BmK AS did not affect the epileptiform EEG in the pilocarpine model over the same dose range, although it did increase the latency to status epilepticus onset and slightly, but significantly, reduced the seizure score. In summary, our results demonstrate that the sodium channel site 4-selective modulator BmK AS is an effective inhibitor of PTZ- but not pilocarpine-induced acute seizures. These results indicate that BmK AS may serve as a novel probe in exploring the role of different sodium channel subtypes in an epileptogenic setting and as a potential lead in developing antiepileptic drugs specifically for the therapy of sodium channel site 4-related epilepsy.

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1. Introduction

Epilepsy is a chronic neurological disorder that affects approximately 1% of the population globally [1]. Although great advances have been made in the development of new antiepileptic drugs, seizures in 20–30% of patients remain refractory to therapies using conventional antiepileptic drugs [2]. Meanwhile, many patients who achieve seizure control with antiepileptic drugs suffer from serious neurotoxicity, sedation, and cognitive side effects [3]. Therefore, despite the beneficial effect of the currently available drugs, there is still a need for effective antiepileptic drugs with decreased adverse effects.

In the past few years, hundreds of polypeptide toxins with multi-pharmacological effects have been purified from the venom of scorpions, spiders, and wasps [4]. Some of these polypeptides have either convulsant or anticonvulsant activity and the latter have been considered to be potential candidates for antiepileptic drugs [5]. On the basis of pharmacological and electrophysiological activities in vivo, several subtypes of α and β scorpion neurotoxins have been identified as possible antiepileptic drugs [6]. In this respect, in traditional Chinese medicine, the body/venom of the Asian scorpion Buthus martensi Karsch is used to treat certain neurological diseases, including apoplexy, epilepsy, facial paralysis, and hemiplegia. The α toxins are sodium channel site 3-selective modulators that prolong the inactivation phase of voltage-gated sodium channels [7]. BmK I, an α-like neurotoxic polypeptide (Karsch BmK) from the venom of the Asian scorpion Buthus martensi, has been shown to have potent nociceptive actions and to prevent epileptic seizures [8–12]. The β toxins, however, alter the activation phase of sodium channels as sodium channel site 4-selective modulators. These β-type neurotoxins from BmK venom have, to date, been identified as BmK IT2, BmK AS, and BmK AS-1. BmK AS is composed of 66 amino acid residues [13], and has been shown to inhibit peak tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium currents in a dose-dependent manner and induce a negative shift in the steadystate inactivation of TTX-R and TTX-S sodium currents in rat dorsal root ganglion neurons [14]. We have previously reported that BmK IT2 functions as an effective anticonvulsant in animal seizure models by modulating sodium channels [15]. Whether BmK AS and BmK AS-1 have similar anticonvulsant properties, however, remains unknown.

Considering that the abnormal functioning of sodium channels is implicated in epilepsy and other disorders and the inhibitory effect of BmK AS on sodium currents observed in previous studies [14,16], we...
aimed to investigate in the present study whether BmK AS, like BmK IT2, also has anticonvulsant activity in experimental seizure models.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (250–300 g body wt), from Shanghai Experimental Animal Center, Chinese Academy of Sciences, were housed four to a cage with water and food available ad libitum and kept in a room with temperature maintained at 22 ± 1 °C, on a 12-hour light/dark cycle. All procedures in animals were approved by the Committee on Laboratory Animals, Chinese Academy of Sciences. An effort was made throughout the experiment to minimize stress in these animals.

2.2. Drugs

BmK AS was purified from BmK venom by RP-HPLC according to the method described previously [17]. The purity of isolated BmK AS was verified by mass spectrometry analysis, and only BmK AS with a purity >99% was used. Pentylenetetrazole (PTZ), pilocarpine hydrochloride, methylscopolamine bromide, and valproic acid sodium salt (VPA) were purchased from Sigma–Aldrich. All drugs were dissolved in sterile isotonic saline for intrahippocampal or intraperitoneal injection.

2.3. Surgery

Each animal was anesthetized with sodium pentobarbital (40 mg/kg body wt, ip) and placed in a stereotaxic frame (Narishige, Japan). Its head was shaved and then prepared for sterile surgery. A midline incision was made to expose the coronal, sagittal, and lambdoid sutures. Then a stainless-steel guide cannula was placed stereotaxically into the right side of the dorsal hippocampus (AP –4.3 mm posterior to bregma, L 2.2 mm lateral to the midline of the skull, and V 2.5 mm ventral to the dura surface) according to the atlas of Paxinos and Watson [18]. EEG recording electrodes (metal with insulation layer, 0.5-mm diameter, homemade) were positioned stereotaxically in the frontal cortex (AP –3.5 mm, R 2.0 mm lateral to the midline, and V 1.5 mm ventral to the dura surface) contralateral to the guide cannula, while the reference electrode was placed on the cerebellum (AP –10.0 mm). The guide cannula and the electrodes were fixed with dental cement to the skull, and the skin was sutured together around this area. The rat was then allowed to recover for at least 3–4 days. At the end of all experiments, animals were sacrificed and brains were removed for cresyl violet staining according to the method described previously [9] to determine placement of the electrode and the guide cannula. Only data from animals with both the electrode and guide cannula in the correct area were used.

2.4. Behavior measurement

2.4.1. Rotarod test

Seven days after cannula implantation, animals were subject to rotarod training trials for 3 consecutive days with three trials a day. The rotating speed of the beam was set at 16 rounds/minute, and each trial lasted 5 minutes. On the testing day, the rotarod test session was started approximately 45 minutes after either saline or BmK AS injection, immediately after the rat completed its open-field test. Latency to falling was recorded and was compared either with the rat’s pre-drug injection value or between the saline- and BmK AS-injected groups.

2.4.2. Open-field test

The open field consisted of an arena (72 × 72 cm) enclosed by walls (36 cm high) (Med Association, Inc., Vermont). On the testing day, rats were habituated to the experimental room for at least 30 minutes before either saline or BmK AS injection. Thirty minutes after injection, at the beginning of the test, rats were placed in the central area of the open field and video recorded for 5 minutes. The total running distance was automatically calculated, and number and duration of rears within the 5-minute testing period were manually counted offline from recorded videos. These data were compared between the saline- and BmK AS-injected groups.

2.5. Seizure behavior measurement

2.5.1. Pentylenetetrazole-induced seizures

Rats were placed in 40 × 30 × 50-cm transparent glass boxes and allowed to acclimate for at least 1 hour before the assay. After anesthetization by ether inhalation as defined by absence of paw withdrawal reflex, BmK AS (1 μl saline) or saline was injected into the hippocampal CA1 area through the guide cannula. The hippocampal CA1 region was chosen to study the anticonvulsant action of BmK AS because of its important role in epileptogenesis of temporal lobe epilepsy and its location in the dorsal hippocampus for easy cannula implantation with minimal tissue damage. Thirty minutes later, in all animals that recovered from the ether, PTZ (70 mg/kg, ip) was administered to induce seizures. After PTZ injection, rats were returned to the boxes and their behavior was continuously monitored for up to 2 hours. Animals were divided randomly into seven groups containing 6–10 animals each (Table 1); group 1 received BmK AS (2 μg in 1 μl saline), alone; group 2 received saline plus PTZ; groups 3–6 received different doses of BmK AS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>0.05 μg BmK AS</th>
<th>0.1 μg BmK AS</th>
<th>0.5 μg BmK AS</th>
<th>1 μg BmK AS</th>
<th>200 mg/kg VPA</th>
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<td>9</td>
<td>10</td>
<td>8</td>
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<td>6</td>
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<td>Mortality (min)</td>
<td>2/9</td>
<td>2/9</td>
<td>3/10</td>
<td>0/8</td>
<td>0/8</td>
<td>0/6</td>
</tr>
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<td>Latency (min)</td>
<td>0.87 ± 0.12</td>
<td>1.27 ± 0.15</td>
<td>1.28 ± 0.10a</td>
<td>1.65 ± 0.18a</td>
<td>1.88 ± 0.26a</td>
<td>1.57 ± 0.15a</td>
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<td>Seizure duration (min)</td>
<td>19.16 ± 1.65</td>
<td>13.26 ± 2.47</td>
<td>16.51 ± 1.88</td>
<td>9.15 ± 1.43c</td>
<td>7.19 ± 0.95c</td>
<td>10.89 ± 1.90c</td>
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<td>Stage 1 and 2 seizures</td>
<td>1.57 ± 0.41</td>
<td>1.07 ± 0.35</td>
<td>0.31 ± 0.09b</td>
<td>0.44 ± 0.15a</td>
<td>0.13 ± 0.05b</td>
<td>0.46 ± 0.20a</td>
</tr>
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<td>Stage 3 seizures</td>
<td>0.91 ± 0.37</td>
<td>0.67 ± 0.45</td>
<td>0.17 ± 0.09</td>
<td>0.04 ± 0.03a</td>
<td>0.03 ± 0.01a</td>
<td>0.05 ± 0.02</td>
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<tr>
<td>Stage 5 seizures</td>
<td>0.17 ± 0.06</td>
<td>0.11 ± 0.1</td>
<td>0.0a</td>
<td>0.07 ± 0.06</td>
<td>0.0a</td>
<td>0.0a</td>
</tr>
<tr>
<td>Total duration</td>
<td>21.80 ± 1.92</td>
<td>15.12 ± 2.49</td>
<td>16.99 ± 1.61</td>
<td>9.71 ± 1.27c</td>
<td>7.19 ± 0.95c</td>
<td>11.40 ± 2.06c</td>
</tr>
</tbody>
</table>

Table 1
Anticonvulsant effect of BmK AS on behavioral seizures induced by pentylenetetrazole (PTZ).

Note: Rats in saline group treated with PTZ died within 120 minutes (n = 2); therefore, the number and duration for behavioral seizures were excluded from statistics. VPA, valproic acid.

*P<0.05, †P<0.01, and ‡P<0.005, compared with saline group (one-way ANOVA, Tukey test).
(0.05, 0.1, 0.5, and 1 μg in 1 μL saline, respectively) plus PTZ; group 7 received VPA (200 mg/kg) plus PTZ.

Experimental procedures were previously published [15]. During experiments, drug administration and behavior observation were performed “double-blinded” by different experimenters. Racine’s 5-point scale [19], as modified by Fathollahi et al. [20], was used to classify behavioral seizures: 1 = facial clonus, 2 = head nodding, 3 = unilateral forelimb clonus, 4 = rearing with bilateral forelimb clonus, 5 = rearing and falling (loss of postural control). The anticonvulsant activity of BmK AS was assessed on the basis of latency to seizures, seizure duration, number of seizures, and severity of seizures within the first 2-hour period after PTZ injection. Latency was defined as the average length of time between PTZ administration and the onset of the first seizure behavior above Racine stage 2. A seizure episode was defined as the time the animal spent in seizure activity. The minimal interval between two countable seizures was 5 seconds. Seizure duration and number were calculated as the sums of multiple seizures for each animal, and seizure severity was calculated based on the maximal seizure score graded 0 to 5 for each animal, as described previously [9]. In some experiments, rats receiving PTZ underwent behavioral observation and electrographic recording simultaneously (see below).

In the lithium–pilocarpine seizure model, all naive rats were injected with lithium chloride (3 mmol/kg, ip). On the following day, methylscopolamine bromide (1 mg/kg, sc) was administered 30 minutes before pilocarpine to reduce the peripheral consequences of the pilocarpine. For intrahippocampal administration, eight rats were administered saline as a control group, and four groups containing six, six, eight, and seven rats were microinjected (1 μL) with 3 mmol/kg, 0.05, 0.1, 0.5, and 1 μg, respectively. Pilocarpine hydrochloride (25 mg/kg, ip) was administered 30 minutes after saline or BmK AS injection. Rats were returned to their boxes and their behavior was continuously monitored for an additional 2 hours. Latency to SE onset was defined as the average length of time between pilocarpine administration and the onset of SE behavior. Seizure severity and classification were evaluated according to Racine [19] as previously indicated.

2.6. EEG recordings

EEG activity was recorded using a Data Acquisition Systems instrument (Powerlab 4/30, AD instruments, Australia) with a range of 2 V and a bandpass of 1.6–100 Hz. Analog data were sampled at 1000 Hz. After a 30-minute period of baseline recording, BmK AS or saline was injected into the hippocampus, and 30 minutes later, PTZ (70 mg/kg, ip) or pilocarpine (25 mg/kg, ip) was administered and the EEG was continuously recorded for an additional 2 hours.

For PTZ-induced seizures, rats were divided into five groups: one group was injected with 1 μL saline plus PTZ (n = 6) as control, and the other four groups were treated with different doses of BmK AS (0.05, 0.1, 0.5, and 1 μg) plus PTZ (n = 6 each). Offline analyses of the recorded EEG data were performed manually as previously reported [15]. An electrographic seizure, defined as a high-frequency, high-amplitude discharge (HADF), was typically associated with behavioral seizures. HAfDs were defined as discharges that lasted at least 5 seconds, had a frequency of at least 8 Hz, and had an amplitude at least two times higher than the baseline EEG [15]. Latency to the first onset of electrographic epileptiform discharges was measured. Electrographic seizure severity was assessed by counting the number and duration of HAfDs. Epileptic spikes were also analyzed. An epileptic spike was defined as a spike with a duration <100 ms or a spike-and-wave with a duration <200 ms [15,21].

In the lithium–pilocarpine SE model, rats were divided into five groups: six rats received saline injection and constituted the control group; the other four groups (n = 6 per group) received different doses of BmK AS (0.05, 0.1, 0.5, and 1 μg, respectively). SE discharge amplitude and spike frequency from each EEG trace were analyzed with the MFlight200 v3.02 program (Shanghai Medical College of Fudan University) [15]. Based on the epileptiform SE discharge progression pattern, EEG data were analyzed and grouped into data from the first 30 minutes and data from the remaining 90 minutes.

2.7. Hippocampal neuron culture

Hippocampi were dissected from P18 embryonic rats, enzymatically treated with 0.125% trypsin (Invitrogen, USA) for about 10–12 minutes, and transferred to complete medium, that is, Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 10% F-12 Nutrient Mixture (F12, Invitrogen, USA). Neurons were mechanically dissociated by triturating with a fire-polished Pasteur pipet and plated at low density (5 × 10^3/mL) into 60-mm culture dishes. After 24 hours, the culture medium was changed to contain 97% neurobasal medium with 2% B27 serum supplement (Invitrogen, USA) and 1% L-glutamine (Invitrogen, USA). Half of the culture medium was replaced with fresh medium every 3 days. Cultures were maintained in a humidified incubator (5% CO2 and 95% O2) at 37 °C for 8–14 days.

2.8. Whole-cell patch clamp recordings

All electrophysiological experiments on cultured cells were performed at room temperature (25 ± 1 °C). The k∞ of cultured hippocampal neurons was recorded with the whole-cell patch-clamp technique. Patch pipets with a resistance between 2 and 5 MΩ were prepared from glass capillaries using a two-step vertical puller (PP-830, Narishige, Japan). In each experiment, the junction potential was set at zero and a holding potential of −70 mV was chosen. Currents were amplified with an Axon 200B patch-clamp amplifier (Axon Instruments, USA) and analyzed with Axon pClamp 8.1 software (Axon Instruments, USA). The external solution contained (in mM): 150 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 Heps, 10 glucose, 2.5 tetraethylammonium chloride (TEA-Cl), and 5 4-aminopyridine, and the pH was adjusted to 7.4 with Tris base. The standard internal solution in the patch pipet contained (in mM): 120 CsCl, 20 TEA-Cl, 2 MgCl2, 10 Heps, 10 glucose, 2.5 tetraethylammonium chloride (TEA-Cl), and 5 4-aminopyridine, and the pH was adjusted to 7.2 with Tris base. The recorded current was filtered at a frequency of 3 kHz and digitized with a sampling rate of 50 kHz. An Ag–AgCl saline bridge was used as the reference electrode. A 50-ms step of depolarization from −70 to 30 mV was applied. The sodium currents were recorded in the absence (control) or presence of either 1 μM TTX or 100 nM BmK AS for 5 minutes in the bathing fluid. Persistent current measured as the mean current in the last 5 ms of each 50-ms step, normalized to the peak transient current.

2.9. Statistical analysis

All data are expressed as means ± SEM. Data for the different groups were compared with a one-way ANOVA plus Tukey test. Values with P < 0.05 were considered significant.

3. Results

3.1. Effect of BmK AS on rat motor activity

The effect of intrahippocampal injection of BmK AS on rat motor activity was studied in open-field and rotarod tests. Before cannula implantation, rats were randomly divided into two groups (n = 5 each). Seven days after cannula implantation, rats were subjected to the trial session on the rotarod three times a day for 3 consecutive days preceding the testing day. On the testing day, after at least 30 minutes of rest in the experimental room, rats were injected with either saline or BmK AS (1 μg) 30 minutes before the open-field test; the rotarod...
3.3. Effects of BmK AS on behavioral seizures induced by pentylentetrazole

Microinjection into the CA1 area of either BmK AS (2 μg in 1 μL) or saline (1 μL) alone did not induce any abnormal behavior including seizures (n = 6). In saline-treated animals, PTZ (70 mg/kg, ip), with an approximately 50-second delay, induced myoclonic jerks followed by generalized clonic seizures and tonic extension in all rats tested (n = 9). BmK AS dose-dependently inhibited PTZ-induced seizure activity as shown by increases in seizure latency (Fig. 2A, Table 1) and reduction in both seizure number and duration (Figs. 2B and C, Table 1). Microinjection of 0.5 and 1 μg BmK AS into the hippocampus prior to PTZ administration dose-dependently reduced seizure duration from 21.80 ± 1.92 minutes in the saline control group (n = 7) to 9.71 ± 1.27 (n = 8, F[1,13] = 39.7, P < 0.005) and 7.19 ± 0.95 (n = 8, F[1,13] = 44.1, P < 0.005) (Fig. 2C) and reduced seizure score from 4.9 ± 0.1 in the saline control group (n = 7) to 3.5 ± 0.4 (n = 8, F[1,13] = 13.8, P < 0.005) and 3.0 ± 0.4 (n = 8, F[1,13] = 34.1, P < 0.005), respectively (Fig. 2D).

3.4. Effects of BmK AS on electrographic seizures induced by pentylentetrazole

Microinjection into the CA1 area of either saline or BmK AS alone did not induce any abnormal activity on the EEG (Figs. 3Aa and Ac). Administration of PTZ (75 mg/kg, ip, n = 6) after saline treatment evoked epileptiform EEG activity with isolated and cluster spikes and burstlike HAFDs (Fig. 3Ab). BmK AS dose-dependently suppressed the epileptiform EEG activity induced by PTZ (Fig. 3Ac). The group EEG data (n = 6 for each group) showed that BmK AS significantly prolonged the latency to epileptic discharges (Fig. 3Ba) and reduced

Fig. 1. Effect of intrahippocampal CA1 injection of BmK AS on rat motor activity. (A) Duration on rotarod of individual rats (a) or rat groups (b) before and 45 minutes after either saline (n = 5) or BmK AS (n = 5) injection. (B) Original individual video traces of the rats in the saline control (a) and BmK AS (b) groups during the 5-minute testing period in the open-field arena. The histogram illustrates the group data for distance traveled (c) and number and duration of rears (d) in the saline control (n = 5) and BmK AS (n = 5) groups during the 5-minute open-field testing period. *P < 0.05, compared with saline group.
both the number and duration of burstlike HAFDs (Figs. 3Bc and Bd), consistent with the observation from behavioral studies. BmK AS had no effect on isolated spikes (Fig. 3Bb).

As a positive control, pretreatment with VPA (200 mg/kg), a widely used antiepileptic drug, also suppressed PTZ-induced seizure activity, reducing the seizure score from 4.9±0.1 (saline control, n=7) to 3.7±0.2 (n=6, F[1,11]=37.8, P<0.005), the number of HAFDs on the epileptic EEG from 6.7±0.6 (saline control, n=6) to 2.8±0.4 (n=6, F[1,10]=26.4, P<0.005), and the duration of HAFDs from 3.70±0.76 (saline control, n=6) to 0.90±0.16 (n=6, F[1,10]=8.8, P<0.005) minutes (Figs. 2 and 3, Table 1).

3.5. Effects of BmK AS on behavioral seizures induced by pilocarpine

Administration of pilocarpine (25 mg/kg, ip) to saline pretreated rats (n=8) induced continuous partial seizures, interrupted by repetitive generalized seizures. Approximately 25 minutes after injection of pilocarpine, all rats pretreated with saline developed SE. Microinjection of BmK AS into the hippocampus prior to pilocarpine administration dose-dependently prolonged latency to onset of SE from 24.63±1.66 minutes in the saline group to 31.24±2.37 (F[1,11]=26.4, P<0.005), and the duration of HAFDs from 3.70±0.76 (saline control, n=6) to 0.90±0.16 (n=6, F[1,10]=8.8, P<0.005) minutes (Figs. 2 and 3, Table 1).

3.6. Effects of BmK AS on electrographic seizures induced by pilocarpine

In the saline control group (n=6), pilocarpine induced continuous high-amplitude and high-frequency spikes or spike-and-wave discharges (ictal discharge) (Figs. 5Ab–c), but not burstlike HAFDs as seen with the PTZ challenge (see Fig. 2A). BmK AS, in the dose range 0.05–1 μg (n=6 for each group), failed to inhibit the pilocarpine-induced cortical EEG discharges. Even at the highest dose (1 μg, n=6) tested, BmK AS, which had effectively suppressed PTZ-induced epileptiform EEG activity, had only a slight effect, without statistical significance, on both amplitude and frequency of pilocarpine-induced ictal discharges, compared with the saline control group (Figs. 5Ad–f and B).

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>0.05 μg BmK AS</th>
<th>0.1 μg BmK AS</th>
<th>0.5 μg BmK AS</th>
<th>1 μg BmK AS</th>
<th>200 mg/kg VPA</th>
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<td>0/8</td>
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<td>Latency to SE (min)</td>
<td>24.63±1.66</td>
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<td>31.24±2.37</td>
<td>31.85±1.59</td>
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<td>Seizure score</td>
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<td>4.3±0.2</td>
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Note. SE, status epilepticus; VPA, valproic acid.

\*P<0.05, \textbf{b}P<0.01, and \textbf{c}P<0.005, compared with saline group (one-way ANOVA, Tukey test).

\*Some of the rats did not reach SE within the 2-hour observation period.

In the saline control group (n=6), pilocarpine induced continuous high-amplitude and high-frequency spikes or spike-and-wave discharges (ictal discharge) (Figs. 5Ab–c), but not burstlike HAFDs as seen with the PTZ challenge (see Fig. 2A). BmK AS, in the dose range 0.05–1 μg (n=6 for each group), failed to inhibit the pilocarpine-induced cortical EEG discharges. Even at the highest dose (1 μg, n=6) tested, BmK AS, which had effectively suppressed PTZ-induced epileptiform EEG activity, had only a slight effect, without statistical significance, on both amplitude and frequency of pilocarpine-induced ictal discharges, compared with the saline control group (Figs. 5Ad–f and B).
As BmK AS had a modest effect on pilocarpine-induced seizure activity, the effect of VPA on the pilocarpine model was investigated. VPA (200 mg/kg) significantly inhibited not only pilocarpine-induced seizure behavior scores, but also pilocarpine-induced epileptic EEG activity. The seizure score of animals administered pilocarpine alone was 4.9±0.1 (n=8) and was significantly suppressed to 3.2±0.3 in animals pretreated with VPA (n=6, F[1,12]=32.4, P<0.005). In addition, pretreatment with VPA reduced the amplitude of pilocarpine-induced epileptic EEG ictal discharges from 0.32±0.02 to 0.19±0.03 mV (F[1,10]=11.5, P<0.01) and reduced EEG spike frequency from 3.90±0.84 to 1.48±0.62 Hz (F[1,10]=4.6, P<0.05) after the first 30 minutes and from 11.23±0.65 to 5.27±0.72 Hz (F[1,10]=28.4, P<0.005).

Fig. 3. Effects of BmK AS on electrographic epileptiform activity induced by PTZ. (A) Representative traces showing normal and epileptiform EEGs recorded before (a, c) and after (b, d) injection of PTZ into rats pretreated with saline (left) or 1 μg BmK AS (right). Expanded traces under the original traces show the detail of the EEG recordings as indicated. (B) Histograms showing the group effects of pretreatment with saline (n=6), BmK AS (0.05, 0.1, 0.5, and 1 μg; n=6 for each dose), and valproic acid (VPA, 200 mg/kg; n=6) on epileptiform EEGs within 2 hours of PTZ injection: (a) latency; (b) spike number; (c) number of high-amplitude, high-frequency discharges (HAFDs); and (d) duration of HAFDs. *P<0.05, **P<0.01, and ***P<0.005, compared with saline group (one-way ANOVA, Tukey test).

Fig. 4. Effects of BmK AS on behavioral seizures induced by pilocarpine. Histograms showing that BmK AS (0.05 μg, n=6; 0.1 μg, n=6; 0.5 μg, n=8; 1 μg, n=7), but not saline (n=8), dose dependently inhibited pilocarpine-induced status epilepticus latency (A) and seizure score (B). Valproic acid (VPA, 200 mg/kg) also significantly reduced behavioral seizures induced by pilocarpine (B). *P<0.05, **P<0.01, and ***P<0.005, compared with saline group (one-way ANOVA, Tukey test).
The present study is the first demonstration, to our knowledge, that BmK AS, a sodium channel receptor site 4-selective modulator, is an effective anticonvulsant able to suppress PTZ-induced acute seizures, though only having a modest effect on pilocarpine-induced seizures. This effect of BmK AS on PTZ-induced seizure behavior and electrographic EEG activity was dose dependent.

4.1. Use of pentylenetetrazole and pilocarpine models

Pentylenetetrazole is a GABAergic receptor antagonist with the capability of inducing generalized tonic–clonic seizures [22]. Therefore, the PTZ acute seizure model has been widely used to assess the efficacy of such antiepileptic drugs [23] as ethosuximide, trimethadione, and valproate [24]. Pilocarpine (an acetylcholine M-receptor agonist) induces partial seizures with secondary generalization at high doses. This is highly isomorphic to human temporal lobe epilepsy, the most common type of partial complex epilepsy in adulthood [25], and has been widely employed to study the efficacy of antiepileptic drugs [26]. Thus, for this study, we chose these two models with different underlying seizure mechanisms to test the efficacy of the anticonvulsant action of BmK AS. Surprisingly, the present experiments showed that BmK AS inhibited PTZ- but not pilocarpine-induced animal seizures, which would suggest involvement of site 4 sodium channels in seizures induced in the PTZ but not pilocarpine seizure model.

4.2. Effects of BmK AS on rotarod performance and locomotor activity of rats

To clarify whether any sedative side effect of BmK AS contributed to the suppressive effect of BmK AS on seizure induction, BmK AS, at the highest dose (1 μg) used in this study to inhibit PTZ-induced seizures, was also tested with respect to its effect on rat locomotor behavior in the rotarod and open-field tests. In the rotarod test, which was designed to assess the ability of animals to balance [27], injection of neither saline nor BmK AS into the hippocampal CA1 area affected the ability of animals to balance on the rotarod beam. In addition, number and duration of animal rears in the open-field test, an index of animal exploratory behavior [27], did not differ between the saline- and BmK AS-injected groups. However, to our surprise, total running distance, which indicates the locomotor activity of the animal, was longer in BmK AS-injected rats than in saline-treated rats. The reason for this increased locomotor activity is unknown and is worth investigating in future studies. Overall, these general behavioral test results indicate that the inhibition of PTZ-induced seizure behavior resulting from

\( P < 0.005 \) for the period 30 to 120 minutes, in the groups treated with pilocarpine alone \( (n = 6) \) and pilocarpine plus VPA \( (n = 6) \), respectively (Fig 5B).

3.7. Effect of BmK AS on \( I_{\text{Na}} \) of rat cultured hippocampal neurons

Currents were elicited by series of 50-ms depolarization steps from –70 to 30 mV in 10-mV increments. At 1 μM TTX completely abolished these currents, indicating that they were generated by the opening of the sodium channels (Fig 6Aa). Similarly, these currents were also significantly suppressed by perfusion of 100 nM BmK AS (Fig 6Ab). The peak sodium currents were significantly reduced from 1.41 ± 0.12 nA in the control to 0.30 ± 0.07 nA in the BmK AS pretreated group \( (n = 7, F[1,12] = 39.4, P < 0.005) \), a 84±9% reduction (Fig. 6Ba). In addition, in three of the seven neurons studied, persistent sodium currents were generated during step depolarization. This persistent current was also reduced by 100 nM BmK AS, with \( I_{\text{peak}} \) decreasing from 0.16 ± 0.03 in the control to 0.05 ± 0.01, a 69±7% \( (n = 3, F[1,4] = 8.4, P < 0.01) \) reduction (Fig. 6b).

4. Discussion

Pilocarpine-induced epileptiform EEG activity was dose dependent.

\( \text{Effects of BmK AS on electrographic epileptiform activity induced by pilocarpine. (A) Representative traces showing normal and epileptiform EEGs recorded before (a, d) and after (b, c and e, f) pilocarpine injection of rats pretreated with saline (left) and BmK AS (1 μg) (right). Expanded traces under the original traces show the detail of the EEG recordings as indicated.} \ (B) \text{Histograms illustrate the group effects of pretreatment with saline} \ (n = 6) \text{or BmK AS (0.05, 0.1, 0.5, 1 μg; n = 6 for each dose) on pilocarpine-induced epileptiform EEG spike amplitude (a) and spike frequency (b) after the first 30 minutes and at 30–120 minutes after} \text{pilocarpine injection. Valproic acid (VPA, 200 mg/kg) significantly suppressed pilocarpine-induced epileptiform EEG activity.} \ (P < 0.05, **P < 0.01, and ***P < 0.005, compared with saline group (one-way ANOVA, Tukey test).} \)
intra-hippocampal injection of BmK AS was not, at least in this experimental condition, due to a sedative side effect of BmK AS.

4.3. Effects of BmK AS on behavioral and electrographic seizures induced by pentylenetetrazole and pilocarpine

In the present study, BmK AS was found to effectively suppress PTZ-induced seizure activity, including latency to seizures, number of seizures, and seizure duration. In addition, BmK AS significantly reduced the number and duration of HAFDs while tested with an EEG marker. These results suggest that BmK AS can modulate PTZ-evoked hyperexcitation. The underlying mechanism is hypothesized to be inhibition of sodium channel activity by BmK AS. Meanwhile, the in vitro patch clamp results in the current study solidify our previous conclusion that BmK AS is a sodium channel site 4-selective modulator on neurons in the central nervous system (see Results and Refs. [14,28,29]). In contrast, in the pilocarpine model, BmK AS had only a modest effect on seizure behavior without any effect on the epileptiform EEG. In addition, even microinjection into the CA1 region of higher doses of BmK AS (2 μg) did not suppress pilocarpine-induced seizures as observed on the EEG (data not shown). Thus, to verify the pilocarpine model, we tested the effect of the clinically used anticonvulsant VPA on pilocarpine-induced seizures under the same experimental conditions. Indeed, at a dose of 200 mg/kg (ip), VPA inhibited PTZ-induced seizures with an efficacy close to that of the 0.5-μg dose of BmK AS and significantly suppressed pilocarpine-induced seizure behavior and epileptiform EEGs. Why BmK AS was effective in the PTZ seizure model but not in the pilocarpine model remains unknown. The explanation may involve the different sodium channel subtypes expressed in different seizure models.

Clinically, the effectiveness of antiepileptic treatment in patients is still inconsistent, with seizures in some patients resistant to current drug treatments. For example, symptoms of some patients may be reduced, but yet their seizures progress [30]. This has been partially attributed to the cause of the seizures. To our knowledge, there are no reports of sodium channel-selective modulators suppressing seizure activity, except for the study on BmK IT2 [15] derived from the same venom studied in our laboratory. Thus, our current study, in combination with our previous study on BmK IT2 [15], provides the only evidence that a site 4-selective sodium channel modulator may have the potential to serve as an anticonvulsant target to develop new antiepileptic drugs. However, whether BmK AS could have anticonvulsant action in other animal models, such as the kainic acid as cyclothiazide seizure models [31], would be worth testing.

4.4. A possible ion channel mechanism for the anticonvulsant action of BmK AS

Voltage-gated sodium channels play a fundamental role in the excitability of neurons. Increasing evidence has linked abnormal sodium channel function to the cause of epilepsy. Accumulated data indicate that the abundance of distinct sodium channel subtypes dynamically expressed in various animal models of temporal lobe epilepsy may make a diverse contribution in the SE setting [32]. Relevant to our current study, induction of seizures with PTZ has been attributed to its property of prolonging sodium transport and to increases in peak sodium current conductance [33]. In addition, the results from our previous studies, which demonstrate that BmK AS inhibits sodium currents of rat DRG neurons [14] as well as partially abolishing sodium currents in neuroblastoma cell lines NG108-15 and B104, which originated from the central nervous system [28,29], strongly suggest that BmK AS is a sodium channel modulator. Indeed, in the current study, BmK AS largely suppressed peak sodium currents, causing an 84% reduction in cultured hippocampal neurons, and dose-dependently inhibited PTZ-induced seizures. In contrast, although sodium channel window currents have been linked to pilocarpine-induced SE [34], no studies have so far demonstrated, to our knowledge, an association between pilocarpine-induced seizures and peak sodium currents. Sodium window currents were augmented in rat dentate gyrus subjected to pilocarpine-induced SE because of a negative shift in voltage-dependent activation and a depolarizing shift in the inactivation current curve [34]. The capability of BmK AS to

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negatively shift both the activation and inactivation current curves of the sodium channel in rat DRG neurons [14] suggests that BmK AS may lack an effect on window currents. Thus, this might explain why BmK AS has only a slight effect on pilocarpine-induced seizure behavior. There is compelling evidence that persistent sodium currents, non-inactivating sodium currents, are particularly instrumental in modulating neuronal excitability and, thus, are regarded to play a key role in determining epileptogenesis [16]. Persistent sodium channel currents have been linked to Nav1.2, Nav1.3, and Nav1.6, where Nav1.2 and Nav1.6 are distributed predominantly in the adult central nervous system [16]. However, it is notable that increases in persistent sodium currents after SE occur only in the chronic period (9–12 weeks after SE in rats) and not in the acute period (2–3 weeks after SE) and are likely mediated by Nav1.6 in the pilocarpine-induced seizure model [35]. Moreover, our previous studies found that BmK AS failed to suppress currents mediated by the Nav1.2 subtype expressed in Xenopus laevis oocytes [36,37], suggesting that BmK AS has a weak effect on Nav1.2-mediated persistent currents. Therefore, in this study, the significant suppression by BmK AS of persistent sodium channel currents of cultured hippocampal neurons can probably be attributed to the preference of BmK AS for other sodium channel subtypes such as Nav1.6, which may not be involved in pilocarpine-induced acute seizure behavior. On the other hand, the finding that sodium currents were completely inhibited by 1 μM TTX as well as BmK AS in this study indicates that TTX-sensitive sodium channels located in hippocampal neurons are potential targets of BmK AS. Of the sodium channel subtypes Nav1.1, Nav1.2, and Nav1.6 in the adult central nervous system [16], Nav1.6 plays a critical role in generation of persistent sodium currents, and persistent sodium currents can be strongly reduced, at least in the cerebellum, if Nav1.6 is not functional [38]. Therefore, the remarkable suppressive action of BmK AS on persistent sodium currents suggests that Nav1.6 is one of the main potential targets of BmK AS. In line with the finding that Nav1.6 may be involved in chronic seizures [35], further studies are needed to verify the different contributions of sodium channel subtypes in acute and chronic seizure settings.

4.5. Functional implications

This study is the first to demonstrate, to our knowledge, that BmK AS, a site 4-selective modulator of sodium channels, could differentially reduce seizures in animal models, that is, prevent PTZ- but not pilocarpine-induced seizure behavior and epileptiform EEG activity. Previous studies have reported that during SE, the mRNAs of sodium channel subtypes Nav1.2 and Nav1.3 change in epileptic hippocampus, both in human and in rat [31,38], and that BmK AS modulates the activity of the Nav1.2 subtype in a U-shaped manner, possibly because of the opposing effect of binding to two distinct sites on voltage-gated sodium channels [37]. Thus, BmK AS seems to be a valuable molecule with distinct pharmacological activity and may have potential either as a tool to investigate sodium channel subtypes involved in seizures or as a lead in anticonvulsant drug development.

In conclusion, the present study demonstrated that BmK AS suppresses PTZ-induced epileptic seizures, but has only modest effects on pilocarpine-induced seizures. The underlying anticonvulsant mechanisms of BmK AS in the PTZ model may be ascribed to the selective modulation of sodium channel subtypes in the hippocampus. The only slight effect of BmK AS on pilocarpine-induced seizures, in comparison to PTZ-induced seizures, suggests that the dynamic abundance of sodium channel subtypes and/or signaling pathways involved in the induction of epilepsy is diverse with respect to the onset mechanisms of these two models. Therefore, BmK AS is probably useful as a novel probe to explore the molecular and pathological mechanism of epilepsy related to sodium channel subtypes and as a potential lead in developing antiepileptic drugs specifically for the therapy of sodium channel site 4-related epilepsy.

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