Alpha-asarone from Acorus gramineus alleviates epilepsy by modulating A-Type GABA receptors

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Abstract

Alpha (α)-asarone is a major effective compound isolated from the Chinese medicinal herb Acorus gramineus, which is widely used in clinical practice as an antiepileptic drug; however, its mechanism of action remains unclear. In this study, we have characterized the action of α-asarone on the excitability of rat hippocampal neurons in culture and on the epileptic activity induced by pentylenetetrazole or kainate injection in vivo. Under cell-attached configuration, the firing rate of spontaneous spiking was inhibited by application of α-asarone, which was maintained in the Mg^{2+}-free solution. Under whole-cell configuration, α-asarone induced inward currents in a concentration-dependent manner with an EC_{50} of 248 ± 33 μM, which was inhibited by a GABA_{A} receptor blocker picrotoxin and a competitive GABA_{A} receptor antagonist bicuculline but not a specific glycine receptor inhibitor strychnine. Measurement of tonic GABA currents and miniature spontaneous inhibitory postsynaptic currents indicated that α-asarone enhanced tonic GABAergic inhibition while left phasic GABAergic inhibition unaffected. In both pentylenetetrazole and kainate seizure models, α-asarone suppressed epileptic activity of mice by prolonging the latency to clonic and tonic seizures and reducing the mortality as well as the susceptibility to seizure in vivo presumably dependent on the activation of GABA_{A} receptors. In summary, our results suggest that α-asarone inhibits the activity of hippocampal neurons and produces antiepileptic effect in central nervous system through enhancing tonic GABAergic inhibition.

1. Introduction

A type γ-aminobutyric acid receptors (GABA_{ARs}) are pentameric proteins that form Cl^{-}-permeable ion channels activated by the inhibitory neurotransmitter GABA in the mammalian central nervous system (CNS) (Stell et al., 2003). GABA_{ARs} are essential for information processing and involved in many pathological processes such as epilepsy, pain, and anxiety (Semyanov et al., 2004). GABA_{ARs} mediate both phasic and tonic inhibition in neurons from hippocampus, cerebellum, thalamus, and sensory cortex (Castro et al., 2011). Whereas synaptic GABA_{ARs} are involved in phasic inhibition responding to GABA release from interneurons, extrasynaptic GABA_{ARs} are responsible for the generation of tonic inhibition (Brickley et al., 1996; Bright and Brickley, 2008; Farrant and Nusser, 2005; Mody, 2001; Nusser and Mody, 2002; Wall and Usowicz, 1997). Accordingly, phasic GABA_{AR}-mediated inhibition represents the rapid and precise transmission of presynaptic activity into a postsynaptic signal, while the tonic inhibition results from random, temporally dispersed activation of receptors that are distributed over the neuronal surface (Bai et al., 2001; Mody, 2001; Semyanov et al., 2004). The tonic inhibition can be modulated by various endogenous and exogenous modulators, developmental, physiological and pathological regulation of GABA uptake and GABA_{AR} expression (Semyanov et al., 2003, 2004). Consequently,
tonic GABAergic currents play a key role in controlling neuronal excitability (Chadderton et al., 2004; Walker and Semyanov, 2008). The tonic inhibition also contributes to the manifestation of disease processes such as anxiety and different epilepsies including chronic epilepsy, typical absence epilepsy, and temporal lobe epilepsy (Clarkson et al., 2010; Cope et al., 2009; Curia et al., 2009; Eichler et al., 2008; Ge et al., 2011; Houser and Esclapez, 2003). Modulation of tonic GABAergic currents and receptors represents a promising strategy for developing new anticonvulsant and anxiolytic drugs as well as therapeutic approaches.

Alpha (α)-asarone (1-propenyl-2,4,5-methoxybenzol, Fig. 1A) is a major effective compound isolated from Acorus gramineus, a Chinese herbal medicine widely used to treat disorders including asthma, bronchitis, and especially epilepsy (Chen, 1984; Gu et al., 2010). Recently, α-asarone was shown to be a potent neuroprotective agent (Limon et al., 2009) and it stimulated glutamate uptake and reduced excitatory synaptic activity (Gu et al., 2010). Furthermore, clinical investigations have shown that α-asarone has a strong sedative and anti-convulsive action in the CNS. However, the therapeutic targets and the underlying mechanisms for the beneficial effect of α-asarone on epilepsy have remained elusive. In this study, we examined the possibility that α-asarone could target at the extrasynaptic GABA<sub>A</sub>Rs and mediate tonic inhibition. The experiments were designed to investigate whether α-asarone suppresses the excitation of hippocampal neurons by enhancing tonic GABA-mediated inhibition, and subsequently exerts an anticonvulsant effect in both pentylenetetrazole (PTZ) and kainate mouse models.

2. Materials and methods

2.1. Cell culture and electrophysiological recording

Animal use in the present study was approved by the Care and Use of Animals Committee of the Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, China. All primary hippocampal neurons were cultured as previously described (Duan et al., 2011). Whole-cell or cell-attached recordings were performed using a patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). The standard extracellular solution contained (mM): 150 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 N-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), and 10 glucose (pH 7.3 with Tris-base, 325-330 milliosmolar with sucrose). The pipette solution with the high Cl<sup>-</sup>/C<sub>O</sub>-concentration was composed of (mM): 120 KCl, 30 NaCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 N-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), and 10 glucose (pH 7.3 with Tris-base). The pipette solution with the low Cl<sup>-</sup>/C<sub>O</sub>-concentration contained (mM): 120 CsOH, 30 NaCl, 0.2 EGTA, 2 Mg-ATP, 10 HEPES, and pH was adjusted to 7.3 with gluconic acid. When the current–voltage (I–V) relationships were constructed under voltage clamp configuration, tetrodotoxin (TTX, 300 nM) and CdCl<sub>2</sub> (100 μM) were added to the standard extracellular solution.
Membrane currents were sampled and analyzed using a Digidata 1320A interface and a personal computer with Clampex and Clampfit software (Version 9.0.1, Axon Instruments). Unless otherwise mentioned, the membrane potential was held at −60 mV under the voltage-clamp mode for whole-cell current recording, and the patch potential was held at 0 mV under cell-attached voltage-clamp mode for firing activity recording, which was evaluated from the mean interspike interval, and analyzed with the MiniAnalysis 6.0.1 program (Synaptosoft, Decatur, GA).

Concentration-response curves were drawn according to a modified Michaelis– Menten equation by the method of least squares (the Newton–Raphson method) after normalizing the amplitude of the response: \( I = I_{max} C/(C_0 + EC_{50}) \), where \( I \) is the normalized value of the current, \( I_{max} \) is the maximal response, \( C \) is the drug concentration, and \( EC_{50} \) is the concentration which induces the half-maximal response and \( h \) is the apparent Hill coefficient.

2.2. Expression of recombinant GABA\(_{\text{ARs}}\)

The rat α1, β2, and γ2 subunit cDNA were obtained from Dr. Yu Tian Wang (University of British Columbia, Vancouver, BC, Canada). The rat α5 subunit cDNA was kindly provided by Dr. David H. Farb (Boston University School of Medicine, Boston, Massachusetts, USA). In brief, HEK-293T cells were cultured at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air. Cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 1 mM l-glutamine, 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Invitrogen). Transient transfection of HEK293T cells was carried out using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen). Cotransfection with a green fluorescent protein expression vector, pEGFP-C3, was used to enable identification of transfected cells for patch clamping by monitoring the fluorescence of green fluorescent protein. Electrophysiological measurements were performed 24–48 h after transfection.

2.3. Brain slice preparation and electrophysiological recordings

Experiments were performed on 400 μm transverse hippocampal slices from 14 to 21-day-old Sprague–Dawley rats as described previously (Zhang et al., 2008a) with minor modifications. Briefly, after decapitation, the rat brains were quickly removed and placed in well-oxygenated (95% O\(_2\)/5% CO\(_2\)) ice-cold artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 2.5 mM kCl, 10 mM d-glucose, 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 1.25 mM NaH\(_2\)PO\(_4\), and 26 mM NaHCO\(_3\). Slices were cut from the dorsal hippocampus with a vibratome (Leica VT 1000S) and incubated at 36 ± 1 °C for 1 h, followed by room temperature (22–25 °C) incubation in oxygenated ACSF before recording. Whole-cell patch clamp recordings were made from hippocampal CA1 pyramidial neurons under control by infrared-differential interference contrast video microscope (Olympus, BX51WI). The holding potential from hippocampal CA1 pyramidal neurons under control by infrared-differential interference contrast video microscope (Olympus, BX51WI). The holding potential from hippocampal CA1 pyramidal neurons under control by infrared-differential interference contrast video microscope (Olympus, BX51WI). The holding potential from hippocampal CA1 pyramidal neurons under control by infrared-differential interference contrast video microscope (Olympus, BX51WI). The holding potential from hippocampal CA1 pyramidal neurons under control by infrared-differential interference contrast video microscope (Olympus, BX51WI).

2.4. Measurement of tonic current

The mean tonic current was measured as described previously (Zhang et al., 2008a) with minor modifications. The baseline was calculated by generating all-point histograms of 10 s epochs at periods B, C, and D (see Fig. 6A), and a Gaussian distribution was fitted to the histogram at periods B, C, and D (see Fig. 6B–D). Grounded the mean of the fitted Gaussians at period D as zero, the means of the fitted Gaussians at periods B and C were then calculated as tonic currents under control or the presence of α-asarone (see Fig. 6E).

2.5. PTZ seizure test

Male C57BL/6 mice (20–25 g) were used to PTZ seizure test. The treated animals received daily administration of 50 mg/kg α-asarone (0.48 ml/kg i.p.) and control animals received daily administration of PEG/DMSO solution (Sigma, St. Louis, MO) for 3 days. Thirty minutes after the last injection, kainate was administered at 2.5 mg/ml i.p.

Ratine seizure scales (Racine, 1972) were used to determine the effects of α-asarone on seizure severity in response to kainate: no response (0), staring and reduced locomotion (1), activation of extensors and rigidity (2), repetitive head and limb movements (3), sustained rearing with clonus (4), loss of posture (5), and status epilepticus and death (6). The score for each 10-min interval during the fixed 60-min trial was evaluated after kainate was injected.

2.7. Chemicals

All chemicals were purchased from Sigma (St. Louis, MO). For the electrophysiological experiments, the tested drugs were initially dissolved as concentrated stock solutions in DMSO and subsequently diluted to the desired concentration in the perfusion solution.

2.8. Statistical analysis

Except where otherwise indicated, the data are presented as means ±S.E.M. Statistical comparisons were made with Student’s t-test or one-way ANOVA. *p < 0.05, **p < 0.01, and ***p < 0.001 were considered statistically significant.

3. Results

3.1. Inhibition of synchronically driven spiking in cultured hippocampal neurons by α-asarone

Under cell-attached voltage-clamp configuration, a mean firing rate of spontaneous spiking recorded in cultured hippocampal neurons was 4.9 ± 2.3 Hz (n = 6), which was completely abolished by a competitive AMPA (non-NMDA glutamate) receptor antagonist 6-cyano–7-nitroquinolinine-2,3-dione (CNQX) at the concentration of 3 μM (Fig. 1B), suggesting that it is synchronically driven. The firing rate of spontaneous spiking was decreased by application of α-asarone in a concentration-dependent manner with an IC\(_{50}\) of 24.4 ± 1.7 μM (Fig. 1E). Removal of external Mg\(^{2+}\) induced hyperexcitation (Fig. 1C); however, the α-asarone-induced inhibition on spontaneous spiking was unaffected by the Mg\(^{2+}\) removal (IC\(_{50}\) = 26.8 ± 4.5 μM) (Fig. 1E). The distribution of interspike intervals of spontaneous spiking showed a significant shift toward longer times with the presence of α-asarone in both the Mg\(^{2+}\)-containing and Mg\(^{2+}\)-free bath solutions (Fig. 1D). These results indicate that α-asarone inhibits both spontaneous spiking and hyperexcitation.

3.2. Activation of GABA\(_{\text{ARs}}\)s in hippocampal neurons by α-asarone

To examine whether α-asarone suppresses neuronal activity through changes in intrinsic membrane excitability of neurons, we tested the effect of α-asarone on evoked firing rate of cultured hippocampal neurons in the presence of a cocktail of transmitter receptor antagonists including CNQX (10 μM), d-APV (20 μM, for NMDA receptors), bicuculline (BMI, 10 μM, for GABA\(_{\text{ARs}}\)s), and strychnine (STR, 1 μM, for glycine receptors). However, no obvious difference was found before and after application of 100 μM α-asarone with respect to firing rates induced by step-depolarization currents (data not shown). Therefore, the intrinsic membrane excitability was not involved in α-asarone induced inhibition of neuronal excitation.

Based on the latest finding that β-asarone potentiated the response of recombinant GABA\(_{\text{ARs}}\)s expressed in Xenopus oocytes (Zaugg et al., 2011), we hypothesized that α-asarone might exert its effect through activation of GABA\(_{\text{ARs}}\)s. As shown in Fig. 2A, β-asarone induced inward currents (I\(_{\text{GABA}}\)s) in hippocampal neurons at high concentrations (EC\(_{50}\) = 248 ± 33 μM). A GABA\(_{\text{A}}\) receptor blocker picrotoxin (PTX, 100 μM) or a competitive GABA\(_{\text{A}}\) receptor antagonist BMI (10 μM), but not a specific glycine receptor inhibitor STR (1 μM), significantly decreased currents induced by 1 mM α-
asarone (Fig. 2C,D), suggesting that the currents occurred through activation of GABAARs. To confirm that the α-asarone-evoked currents were Cl⁻ currents, we measured reversal potentials (Eₜᵣₑᵥ) at two different intracellular Cl⁻ concentrations: 153 and 33 mM. As shown in Fig. 3, Eₜᵣₑᵥ shifted from +1.1 ± 1.9 mV (n = 5) to −28.9 ± 1.4 mV (n = 5) when the Cl⁻ concentration in the pipette solution was changed from 153 to 33 mM, respectively. This change in Eₜᵣₑᵥ was in agreement with the theoretical value for Cl⁻ calculated using the Nernst equation. Collectively, these findings demonstrate that the α-asarone induced currents in hippocampal neurons were mediated by GABAARs.

However, one may argue that α-asarone might affect GABAAR currents indirectly mediated by raising extracellular GABA levels that activates extrasynaptic GABAARs, potentially through the inhibition of GABA uptake and/or GABA transaminase. To test this possibility, we have examined the effects of α-asarone in the presence of inhibitors of GABA uptake and GABA transaminase, which should occlude the α-asarone effects on GABAAR activation if α-asarone were to act on GABA uptake and/or GABA transaminase. As shown in the Fig. 2E,F, when applied alone, either the GABA transporter-1 antagonist, NO-711 (10 μM), or the GABA transaminase inhibitor, vigabatrin (100 μM), was able to induce a negligible inward current. Strikingly, in the presence of NO-711 (10 μM) or vigabatrin (100 μM), α-asarone still evoked a further significantly inward current (Fig. 2E,F), suggesting that α-asarone activates GABAAR largely independent of the inhibition of GABA uptake and/or GABA transaminase. In addition, we further observed α-asarone-induced currents in a cell line that expressed recombinant GABAARs (see below), again strengthening the view that direct activation of GABAAR mediates the suppression of neuronal excitability by α-asarone.

3.3. Synergistic activation of α-asarone and GABA in hippocampal neurons

At lower concentrations, although α-asarone did not activate obvious current in hippocampal neurons, it produced a significant
enhancement of GABA-evoked current (see Fig. 4A,B for a comparison between 100 μM and 1000 μM α-asarone in the absence and presence of 1 μM GABA and Fig. 4C for a summary with more α-asarone concentrations). Comparing the peak amplitudes of the membrane currents evoked by a 20-s pulse of GABA plus α-asarone (IC\textsubscript{GABA,Asa}) and the sum of that evoked by either agonist alone of the same stimulating duration (IC\textsubscript{GABA} + IC\textsubscript{Asa}) obtained from the same cells revealed supra-additive effect only at lower α-asarone concentrations (Fig. 4B,D), indicative of synergistic actions between GABA and α-asarone. However, the finding that the magnitudes of IC\textsubscript{GABA,Asa} at higher concentrations of α-asarone (1 and 3 mM) were equivalent to the arithmetic sum of IC\textsubscript{GABA} and IC\textsubscript{Asa} (Fig. 4B,D) suggested that GABA and α-asarone activated GABA\textsubscript{R}s independently in the hippocampal neurons, i.e. GABA still had additional effect at saturating concentrations of α-asarone.

A previous study has estimated the ambient level of GABA in the cerebral spinal fluid to be 0.8–2.9 μM (Lerma et al., 1986), which may be sufficient to induce tonic inhibition by activating slowly-desensitizing extrasynaptic high-affinity GABA\textsubscript{R}s (Mtchedlishvili and Kapur, 2006). However, the concentration of GABA in the synaptic cleft can reach the millimolar range (Mozrzymas et al., 2006). Thus, we measured the effect of 100 μM α-asarone on currents induced by a wide range (0.1–1000 μM) of GABA concentrations in cultured hippocampal neurons. As shown in Fig. 4E, α-asarone significantly enhanced the peak current amplitudes only at GABA concentrations of ≤3 μM, indicating that α-asarone preferentially acts at GABA concentrations that are more common for extrasynaptic tonic inhibition.

3.5. Enhancement of tonic GABA currents by α-asarone in hippocampal slices

To gain a better understanding of the effect of α-asarone at closer to “in vivo” conditions, we examined the effect of α-asarone on tonic GABA currents in hippocampal slices. Under the condition of high Cl\textsuperscript{−} concentration (153 mM), most CA1 pyramidal neurons displayed a small basal membrane current at −60 mV. As shown in Fig. 6A–D, application of α-asarone (100 μM) further increased the membrane current. Both the basal and α-asarone-enhanced tonic currents were blocked by application of BMI (30 μM), suggesting that the tonic currents were mediated by GABA\textsubscript{R}s. The α-asarone-induced increase in the tonic current was observed in every cell tested (Fig. 6E), indicating that the potentiation of the action of endogenous tonic GABA by α-asarone is common among hippocampal neurons. More interestingly, we found that the same concentration (100 μM) of α-asarone exerted no effect on the peak amplitude (Fig. 7A,B,F), frequency (Fig. 7A,C,G), rise time (Fig. 7A,D), and decay time (Fig. 7A,E) of miniature spontaneous inhibitory
3.6. Inhibition of α-asarone on network hyperexcitability in vivo

It is well known that major causes of seizures may be the loss of inhibitory GABAergic terminals at the site of focal cortical epilepsy or a disturbance in various aspects of GABAergic function (Roberts, 1984). A number of antiepileptic agents exert anticonvulsant effects by inhibiting hyperactivity. To examine whether α-asarone inhibits hyperactivity in vivo, we used the PTZ model of epilepsy, a widely accepted method for evaluation of anticonvulsant drugs targeting at GABA<sub>A</sub>Rs (Huang et al., 2001). As shown in Fig. 8A, the tonic and clonic seizures were observed in mice within 30 min after PTZ injection. Administration of α-asarone (50 mg/kg, i.p.) significantly prolonged the latency to clonic (p < 0.001 compared with Ctrl) and tonic seizures (p < 0.01 compared with Ctrl). Moreover, α-asarone markedly reduced the mortality of the mice (Fig. 8B). Therefore, α-asarone exerted an anticonvulsant effect in the PTZ mouse model, presumably through its potentiation on tonic GABAergic inhibition.

To further evaluate whether α-asarone suppressed hippocampal hyperactivity in situ, kainate was used to establish a more stable mouse epilepsy model (Ben-Ari and Lagowska, 1978; Ben-Ari et al., 1979). We found that treatment with α-asarone significantly reduced the susceptibility of mice to seizure as compared to that of vehicle-injected mice (Ctrl) (p < 0.01 compared with Ctrl, Fig. 8C,D). In summary, α-asarone had a strong effect in regulating network hyperactivity and exerted an anticonvulsant effect in both PTZ and kainate models via facilitating tonic GABAergic inhibition.

4. Discussion

In this study, we demonstrate that α-asarone reduced the neuronal excitability by potentiating the low concentration GABA-induced currents as well as activating native and recombinant postsynaptic currents (mIPSCs). These results further support the idea that α-asarone selectively enhances tonic rather than synaptic (phasic) inhibition of GABA.
GABA\(_\text{AR}\)s in cultured rat hippocampal neurons and HEK-293 cells, respectively. Importantly, \(\alpha\)-asarone enhanced tonic GABAergic inhibition while left phasic GABAergic inhibition unaffected in hippocampal slices. In \textit{in vivo} studies, \(\alpha\)-asarone suppressed the epileptic activity induced by pentylenetetrazole or kainate injection. These results suggest that \(\alpha\)-asarone inhibits the activity of hippocampal neurons and produces antiepileptic effect in the CNS through enhancing tonic GABAergic inhibition.

Previous studies have shown distinct roles of synaptic and extrasynaptic GABA\(_\text{AR}\) subtypes in the control of neuronal excitability (Brickley et al., 1996; Bright and Brickley, 2008; Farrant and Nusser, 2005; Mody, 2001; Nusser and Mody, 2002; Wall and Usowicz, 1997). Activation of extrasynaptic GABA\(_\text{AR}\) receptors by ambient GABA causes tonic inhibition (Bai et al., 2001; Bright and Brickley, 2008; Semyanov et al., 2004; Wall and Usowicz, 1997).

As stated above, the estimated ambient GABA concentrations in the cerebral spinal fluid range from 0.8 to 2.9 \(\mu\text{M}\) (Lerma et al., 1986), which is capable to produce tonic neuronal inhibition by activating extrasynaptic GABA\(_\text{AR}\) subtypes that exhibit high affinity for GABA and slow desensitization (Mtchedlishvili and Kapur, 2006). We found that spontaneous spiking of cultured hippocampal neurons could be suppressed by \(\alpha\)-asarone (Fig. 1). Furthermore, these neurons are capable of forming fully functional synapses and networks such as synaptic currents and Mg\(^{2+}\)-free-induced epileptiform activity (Mangan and Kapur, 2004; Sombati and Delorenzo, 1995). Not surprisingly, the hyperexcitability of neurons perfused with a Mg\(^{2+}\)-free medium was also inhibited with the treatment of \(\alpha\)-asarone.

Alpha-asarone by itself had no effect on the resting membrane current at low concentrations (\(<30\ \mu\text{M}\)) (data not shown), but it dose-dependently induced inward currents (\(I_{\text{Asa}}\)) at higher concentrations with an \(EC_{50}\) of 248 ± 33 \(\mu\text{M}\) (Fig. 2). When co-applied with GABA (1 \(\mu\text{M}\), \(\alpha\)-asarone significantly enhanced GABA-evoked current (Figs. 4A–C and 5A2,B2). Application of \(\alpha\)-asarone induced a downward shift in the membrane current and most of the current was blocked by BMI, PTX, but not STR (Fig. 2).
was noted that α-arosone-induced currents were completely blocked by PTX, but partially by BMI (Fig. 2C–D). Previous studies have shown that the inhibition of GABAAR currents either by the mixed/non-competitive channel blocker PTX or the competitive antagonist BMI acts through distinct mechanisms (Krishek et al., 1996). The less sensitivity of α-arosone-induced currents to BMI suggested the less competitive interaction of BMI with α-arosone when compared to that with GABA, implying that α-arosone might preferentially bind to a site which is different from the BMI-also GABA-binding site in the GABAAR. By contrast, PTX act as a mixed/non-competitive channel blocker which inhibits the GABAAR channel opening induced by GABA as well as α-arosone. Furthermore, the reversal potential for $I_{\text{GABA}}^\text{Asa}$ was not significantly different from that for $I_{\text{GABA}}$, and that for $I_{\text{Asa}}$, which are all consistent with the predicted value for Cl$. Alpha-arosone (100 μM) had no effect on the peak amplitude, rise time, decay time, and frequency of spontaneous mIPSCs (Fig. 7A–E). Taken together, α-arosone selectively enhances tonic GABA-mediated inhibition but does not alter synaptic (phasic) inhibition. Thus, our results suggest that α-arosone suppresses neuronal excitability through enhancement of GABA$\text{A}$R-mediated tonic inhibition in the hippocampus.

Seizures are thought to be a disturbance of the balance between inhibition and excitation. Previous studies have shown that either a loss of inhibitory GABAergic terminals at the site of focal cortical epilepsy or a disturbance in various aspects of GABAergic function can cause seizures (Roberts, 1984). PTZ induced seizure model was widely used to evaluate the effects of anticonvulsants targeting at the GABA system (Huang et al., 2001). PTZ seizure model was initially used to examine the in vivo actions of menthol, which acts selectively at GABAAR-mediated tonic inhibition (Zhang et al., 2008b). A recent study has shown that α-arosone can enter into the brain and reach effective concentrations for modulating GABAergic inhibition, which supports the possibility that α-arosone specifically targets at central nervous system after i.p. administration (Hu et al., 2011). In the present study, we thus tested the anticonvulsant effect of α-arosone with PTZ-induced seizure model through i.p. injection and our results confirmed that α-arosone had obvious anticonvulsant effect and reduced network hyperactivity (Fig. 8A,B) through enhancing GABA tonic inhibition in vivo.

Next, we used the kainate-induced mouse model with acute epilepsy. Previous study showed that kainate induced repetitive seizures that eventually resulted in neuronal damage (Junyent et al., 2009). The overactivation of glutamate receptors induced by kainate results in intracellular calcium overload and thus causes excessive necrosis and apoptosis of intracellular organelles (Fujikawa, 2005). In the present study, we found that α-arosone...
treatment significantly reduced the susceptibility of mice to seizure (Fig. 8C,D). These results together with the anticonvulsant effect of α-asarone in PTZ-treated mice strongly support a role of α-asarone in enhancing tonic inhibition and thus preventing epileptiform hyper-excitability.

There is growing evidence that extrasynaptic GABAARs have subunit compositions different from those of synaptic receptors (Nusser and Mody, 2002; Stell et al., 2003; Tsunashima et al., 1997). The tonic GABAergic inhibition is mediated by α6/β-containing GABAARs in cerebellar granule cells (Stell et al., 2003), by δ-subunit-containing and α5-subunit-containing GABAARs in dentate gyrus granule cells (Stell et al., 2003) and hippocampal pyramidal neurons (Tsunashima et al., 1997), respectively (Farrant and Nusser, 2005). Selective modulators of tonically-activated receptor are used as a valuable tool to investigate the function of tonic inhibition (Atack, 2011). Results from previous studies suggest that the α5 subunit is a specific subunit to form extrasynaptic receptors in hippocampal pyramidal neurons (Tsunashima et al., 1997). In support of this assumption, tonic inhibition was selectively decreased in both CA1 and CA3 pyramidal neurons of hippocampal slices from adult gabar5−/− mice (Glykys and Mody, 2006). Thus, the selective decrease of tonic inhibition resulted in epileptiform hyper-excitability.
hyper-excitability in the CA3 pyramidal layer (Glykys and Mody, 2006). Here, we demonstrate that \(\alpha\)-asarone is a selective enhancer of tonic inhibition of hippocampal pyramidal neurons (Figs. 6 and 7). Therefore, the inhibition of neuronal hyper-excitability and epileptiform activity via selective enhancement of tonic inhibition in pyramidal neurons further confirm that tonic inhibition plays an important role in controlling the network excitability including both physiological oscillations and the pathological propagation of epileptiform activity.

5. Conclusion

In conclusion, the present results suggest that \(\alpha\)-asarone selectively enhances tonic inhibition mediated by GABA\(_A\)Rs that exhibit high affinity for GABA and slow desensitization in CA1 pyramidal neurons of rat hippocampus, leading to suppression of neuronal excitability in vitro and in vivo network hyper-excitability. Our results demonstrate the antiepileptic mechanism of \(\alpha\)-asarone and underscore the importance of tonic inhibition in controlling neuronal excitability.

Acknowledgments

We thank all groups that provided us with GABA\(_A\)R cDNAs. We thank Dr. James Celentano for helpful comments on the manuscript. We thank Ms. Xian Xiao and Mr. Jin Wang for technique assistance. This study was supported by grants from the National Natural Science Foundation of China (91132303, 30970937), Shanghai Municipal Education Commission (Leading Academic Disciple Project, J50201), Shanghai Science and Technology Committee (09JC1408700) and China Postdoctoral Science Foundation (2012M511105).

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