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Rhynchophylline and isorhynchophylline inhibit NMDA receptors expressed in *Xenopus* oocytes

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Abstract

Rhynchophylline and isorhynchophylline are major tetracyclic oxindole alkaloid components of *Uncaira* species, which have been long used as medicinal plants. In this study, the effects of rhynchophylline and isorhynchophylline on the ionotropic and metabotropic glutamate receptor-mediated current responses were examined using *Xenopus* oocytes injected with total RNA prepared from rat cortices or cerebelli. Rhynchophylline and isorhynchophylline (1–100 μM) *per se* failed to induce membrane current, but these alkaloids reversibly reduced *N*-methyl-D-aspartate (NMDA)-induced current in a concentration-dependent but voltage-independent manner. The IC_{50} values of rhynchophylline and isorhynchophylline were 43.2 and 48.3 μM, respectively. Substitution of Ba^{2+} for Ca^{2+} in the recording medium did not alter the extent of rhynchophylline- and isorhynchophylline-induced suppression of NMDA currents. In contrast, neither alkaloid had an effect on the currents mediated by ionotropic kainic acid-type and (±)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors or by the metabotropic glutamate receptor$_{1 \text{ and } 5}$ ($mGlu_{1/5}$). Rhynchophylline and isorhynchophylline (30 μM) significantly reduced the maximal current responses evoked by NMDA and glycine (a co-agonist of NMDA receptor), but had no effect on the EC_{50} values and Hill coefficients of NMDA and glycine for inducing currents. These alkaloids showed no interaction with the polyamine binding site, the Zn^{2+} site, proton site or redox modulatory site on the NMDA receptor. These results suggest that rhynchophylline and isorhynchophylline act as noncompetitive antagonists of the NMDA receptor and that this property may contribute to the neuroprotective and anticonvulsant activity of the *Uncaira* species plant extracts.

Keywords: Rhynchophylline; Isorhynchophylline; NMDA receptor; Glycine site

1. Introduction

Glutamate is a principal excitatory amino acid neurotransmitter in the central nervous system. It activates ionotropic glutamate receptors such as *N*-methyl-D-aspartate (NMDA), kainic acid, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and metabotropic glutamate receptors (Hollmann and Heinemann, 1994). Especially, the NMDA-subtype glutamate receptor is abundant in the cerebral cortex and hippocampus, and plays an important role in learning and memory (Bliss and Collingridge, 1993; Cotman et al., 1989). Besides, excessive activation of NMDA receptors induces the death of central neurons via Ca^{2+} influx and subsequent intracellular Ca^{2+} overload. Such excitotoxic neuronal death appears to contribute to a variety of neurological disorders such as cerebrovascular dementia and chronic neurodegeneration such as Alzheimer's disease (Choi, 1992; Meldrum, 1992; Muller et al., 1995; Parsons et al., 1998). Accordingly, some agents such as NMDA receptor antagonists, calcium channel blockers, and glutamate release inhibitors provide protection against neuronal damage attributed to brain ischemia and chronic neurodegeneration (Block, 1999; del Zoppo et al., 1997).

Rhynchophylline and isorhynchophylline, an epimer of rhynchophylline at the C-7 position of the oxindole alkaloid structure (Fig. 1), are major tetracyclic oxindole alkaloids

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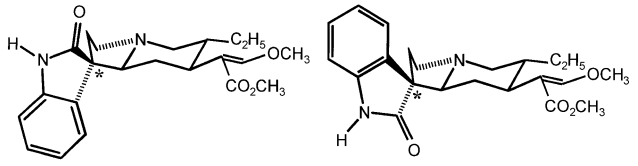

Fig. 1. Stereochemical structures of rhynchophylline and isorhynchophylline used in the present study. The asterisk indicates the spiro C-7 position of a tetracyclic oxindole.

isolated from *Uncaria* species such as *Uncaria rhynchophylla* (MIQ) Jackson and *Uncaria sinensis* (Oliv.) Havil, which have been used as antipyretic, anti-hypertensive and anticonvulsant medications for the treatment of headache, vertigo and epilepsy (Tang and Eisenbrand, 1992). Recent studies demonstrated that the extract of *U. rhynchophylla* has a neuroprotective effect on global cerebral ischemia-induced neuronal damage in rats by reduction of cyclooxygenase-2 mRNA and protein levels in vivo (Suk et al., 2002) and that rhynchophylline and isorhynchophylline reduced glutamate-induced Ca^{2+} influx and protected against glutamate-induced neuronal death in cultured cerebellar granule cells (Shimada et al., 1999). Moreover, a previous report from this laboratory demonstrated that both the extract of *U. rhynchophylla* and rhynchophylline ameliorated transient cerebral ischemia-induced spatial memory deficit in mice (Zhang et al., 2002). Based on these findings, we postulated that the protective effect of rhynchophylline and isorhynchophylline against glutamate-induced excitotoxicity might involve an inhibition of the NMDA receptors. In this study, to clarify the possible molecular mechanism(s) underlying the actions of rhynchophylline and isorhynchophylline, we investigated the effects of these alkaloids on the NMDA receptor function using a receptor expression model employing *Xenopus* oocytes. The present report provides evidence that rhynchophylline and isorhynchophylline exert inhibitory effects on the NMDA subtype of glutamate receptors by acting non-competitively at the NMDA and/or glycine recognition site(s) on the NMDA receptor complex.

2. Materials and methods

2.1. Isolation of total RNA

Male Wistar rats (12–14 weeks old, Japan SLC, Shizuoka, Japan) were used for the experiments. Immediately after the rats were decapitated, the cerebral cortex and cerebellum were dissected and frozen in liquid nitrogen. The frozen tissues were homogenized in Sepasol-RNA I Super® (Nacalai Tesque, Kyoto, Japan) using a glass homogenizer with a Teflon pestle. The total RNA was extracted according to the protocol provided by the manufacturer, and was stored at − 80 °C until use.

2.2. Oocytes preparation and injection

Xenopus oocytes at stage V or VI were prepared by a slight modification of the method described in previous reports (Kang et al., 2002; Leewanich et al., 1998). Briefly, *Xenopus laevis* (Hamamatsu Seibutsu, Shizuoka, Japan) was anesthetized in ice water and a lobe of the ovary was dissected, placed in sterilized modified Barth's solution (MBS: 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 2.5 mM sodium pyruvate and 5 mM HEPES, pH 7.4), and then defolliculated with 1.0 mg/ml collagenase type I (Wako, Osaka, Japan) in Ca^{2+}-free MBS. Oocytes at stage V or VI were injected with 47 nl of 5 mg/ml total RNA prepared from the cerebral cortex or cerebellum and were incubated at 18 °C for 2 days in MBS supplemented with 2.5 unit/ml penicillin and 2.5 μg/ml streptomycin. The MBS was replaced daily. In the experiments, oocytes expressing total RNA from the cerebral cortex were used to examine NMDA-, kainic acid- and AMPA-induced current responses. To induce expression of $mGlu_{1/5}$ receptors, total RNA from the cerebellum was injected into oocytes.

2.3. Electrophysiological recordings

An oocyte was placed in a 50-μl volume chamber and continuously perfused with MBS at 1.5 ml/min at room temperature (22–25 °C) except in some specific cases noted below. The membrane currents were recorded from oocytes at a holding potential of − 60 mV using a two-electrode voltage clamp method (Gene Clamp 500, Axon instruments, Foster City, CA). Electrodes were filled with 3 M KCl and had a resistance of 1–3 MΩ. Only oocytes with resting membrane potential more negative than − 30 mV were used for the experiments. Current responses mediated by ionotropic or metabotropic receptors other than the NMDA receptor were elicited by applying MBS containing agonists for each receptor. To induce NMDA receptor-mediated currents, oocytes were perfused with Mg^{2+}-free and 3 μM glycine-containing MBS or Ba^{2+}-Ringer's solution containing 100 mM NaCl, 2 mM KCl, 2 mM $BaCl_2$, 5 mM HEPES, and 3 μM glycine (pH adjusted to 7.4 with NaOH) to minimize the effects of secondarily activated Ca^{2+}-dependent Cl^- currents. Test drugs were applied to the oocytes for 3 min before and for 30 s simultaneously with each receptor agonist described above. Considering the expected desensitization of current responses evoked by repeated application of each receptor agonist, the currents measured before and after test drug treatment were averaged as control responses. Reversal potentials of NMDA-induced current were measured by inducing the responses at different holding potentials.

2.4. Drugs

Rhynchophylline and isorhynchophylline, tetracyclic oxindole alkaloids (Fig. 1) were isolated from *Uncaria*

species and identified as previously reported (Abdel-Fattah Mohamed et al., 2000). These alkaloids were dissolved in 100% dimethylsulfoxide (DMSO), and then diluted with Mg^{2+}-free MBS to a final concentration of $\leq 0.1\%$ for an electrophysiological study. This concentration of DMSO was tested in oocytes and found not to induce any observable current or not to affect currents evoked by glutamate receptor agonists tested. The following drugs were used: *N*-methyl-D-aspartic acid (NMDA), quisqualic acid, $ZnCl_2$, DL-dithiothreitol, and DMSO (Sigma, St. Louis, MO), glycine, kainic acid, and spermine (Nacalai Tesque), AMPA (Wako), and (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid [(±)-CPP, Research Biochem, Natick, MA].

2.5. Data analysis

The control responses were measured before and after each drug application to take into account possible shifts in the control currents as the recording proceeded. The results are presented as percentages of control responses in order to compensate for variability of the level of receptor in different oocytes. The *n* values are the number of different oocytes studied. Values are expressed as the mean ± S.E.M. except where noted otherwise. Each experiment was carried out with oocytes from at least two different frogs. Statistical analyses were performed with the Student's *t*-test or paired *t*-test. Curve fitting and estimation of EC_{50} values and Hill coefficients from concentration–response curves were performed using PRISM® (GraphPad Software, San Diego, CA).

3. Results

3.1. Inhibitory effects of rhynchophylline and isorhynchophylline on response currents of NMDA receptor in Xenopus oocytes injected with rat cortex total RNA

First, the physiological and pharmacological properties of the expressed NMDA receptors were characterized. Inward current responses to 100 μM NMDA plus 3 μM glycine were recorded in oocytes injected with rat cortex RNA at a clamp potential of −60 mV. The concentration–response relationship of NMDA receptor channels indicated that the EC_{50} values for NMDA in the presence of 3 μM glycine were 73.3 μM (data not shown). (±)-CPP, a competitive NMDA receptor antagonist, at 1 μM blocked the NMDA-induced currents by 76.3 ± 2.1% ($n = 5$). Therefore, the effects of rhynchophylline and isorhynchophylline on currents induced by activation of the NMDA receptor were subsequently examined in the Mg^{2+}-free and 3 μM glycine-containing MBS except where noted otherwise.

As shown in Fig. 2A, when applied alone, rhynchophylline and isorhynchophylline failed to elicit any measurable membrane current. However, when applied to oocytes with 100 μM NMDA, both rhynchophylline and isorhynchophylline reduced the NMDA-evoked current responses by 33 ± 2.3% and 25.5 ± 2.8%, respectively, at 3 μM, and by 60.3 ± 3.1 and 54.9 ± 1.8, respectively, at 100 μM, indicating concentration-dependent inhibition of the NMDA receptor function. Due to limited solubility of these alkaloids, only concentrations as high as 100 μM could be evaluated in this study. The inhibition of NMDA-induced currents by rhynchophylline and isorhynchophylline appeared rapidly and the NMDA response recovered fully following a 5-min washout period. Analysis of the concentration–response curves for rhynchophylline and isorhynchophylline showed that the IC_{50} values of these alkaloids were 43.2 [26.0–72.0] μM (mean [95% CI]) and 48.3 [35.4–65.8] μM (mean [95% CI]), respectively (Fig. 2A).

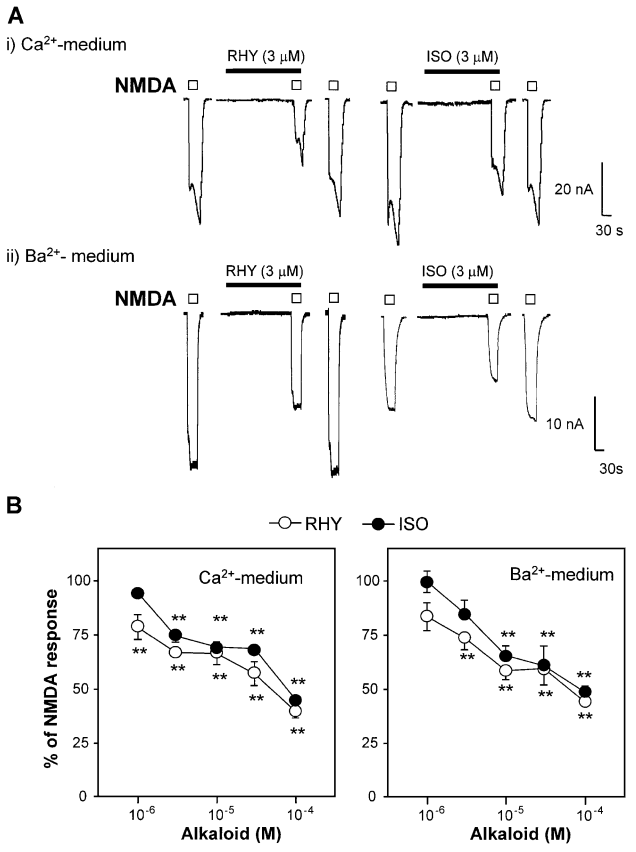

Fig. 2. Rhynchophylline and isorhynchophylline inhibit NMDA current in oocytes injected with rat cortex total RNA. (A) Traces represent NMDA currents recorded in Ca^{2+}-containing Mg^{2+}-free MBS or in Ba^{2+}-containing Mg^{2+}-free MBS. NMDA (□: 100 μM) was applied for 30 s. Horizontal bars above the middle of the traces represent bath application of 3 μM rhynchophylline (RHY) or isorhynchophylline (ISO). (B) Summary of the effects of rhynchophylline (○) and isorhynchophylline (●) on NMDA-induced currents recorded in Ca^{2+} containing Mg^{2+}-free MBS or in Ba^{2+}-containing Mg^{2+}-free MBS. Each data point represents the mean ± S.E.M. from five to seven different oocytes. $^{**}P < 0.01$ versus control response obtained with 100 μM NMDA plus 3 μM glycine (paired *t*-test).

In Mg^{2+}-free MBS containing 0.74 mM Ca^{2+}, Ca^{2+}-dependent Cl^- channels which are intrinsically present in the oocyte membrane are likely activated by the increase in intracellular Ca^{2+} caused by stimulation of exogenously expressed NMDA receptors (Leonard and Kelso, 1990). Thus, in order to test the possibility that rhynchophylline and isorhynchophylline exert inhibitory effects on the Ca^{2+}-activated Cl^- channels and thereby cause an apparent inhibition of the NMDA response in oocytes, we examined the effects of the alkaloids using the Mg^{2+}-free MBS in which Ca^{2+} was replaced by Ba^{2+} (Ba^{2+}-Ringer's solution). As shown in Fig. 2A and B, the extent of rhynchophylline- and isorhynchophylline-induced inhibition of NMDA response was almost the same as that found in Mg^{2+}-free MBS. The IC_{50} values of rhynchophylline and isorhynchophylline in Ba^{2+}-Ringer's solution were 55.1 [24.7–123] μM (mean [95% CI]) and 47.6 [24.5–92.6] μM (mean [95% CI]), respectively, and these values did not differ significantly from the values obtained using Mg^{2+}-free MBS ($P=0.6$ and $P=0.96$, respectively, *t*-test).

3.2. Effects on kainic acid-, AMPA- and quisqualic acid-induced currents in Xenopus oocytes

In order to investigate the receptor specificity of rhynchophylline and isorhynchophylline, we examined the effects of these alkaloids on the current responses elicited by stimulation of ionotropic glutamate receptors of the kainic acid- and AMPA-types and $mGluR_{1/5}$ in *Xenopus* oocytes. Responses were evoked by applying specific agonists for each receptor subtype at concentrations that produced about 50% of the maximal current responses. As summarized in Fig. 3, rhynchophylline and isorhynchophylline at 30 μM, a concentration that caused significant suppression of NMDA currents, had no effect on 100 μM kainic acid- and 10 μM AMPA-induced current responses. We also examined the effects of rhynchophylline and isorhynchophylline on quisqualic acid-induced current response in *Xenopus* oocytes. Consistent with the previous reports (Sugiyama et al., 1987, 1989), application of 1 μM quisqualic acid evoked oscillatory inward currents with a reversal potential of around −20 mV in *Xenopus* oocytes expressing cerebellum total RNA (data not shown), indicating that quisqualic acid-induced currents are mediated by $mGlu_{1/5}$ receptors. These alkaloids did not affect the metabotropic current response (IC_{50}>30 μM; Fig. 3).

3.3. Effects of rhynchophylline and isorhynchophylline are not voltage-dependent or use-dependent

To determine if rhynchophylline- and isorhynchophylline-induced inhibition of NMDA receptors depends on the membrane potential, we analyzed the 100 μM NMDA-evoked current in the Mg^{2+}-free and 3 μM glycine-containing MBS at different holding potentials (−60 to 0 mV). *I–V* plots of NMDA-evoked current were linear and were intercepted at around 0 mV. Rhynchophylline and isorhynchophylline (30 μM) reduced the NMDA-induced current to almost the same extent at all holding potentials tested (Fig. 4A), indicating that the suppressive effects of these alkaloids on NMDA currents are voltage-independent.

To determine whether rhynchophylline and isorhynchophylline block NMDA-activated currents in a use-dependent fashion, we repetitively applied 100 μM NMDA during exposure of the oocytes to the alkaloids (Fig. 4B). The suppression of the amplitude of NMDA currents was unchanged during long-lasting exposure to rhynchophylline or isorhynchophylline, suggesting that the effects of the alkaloids are not use-dependent.

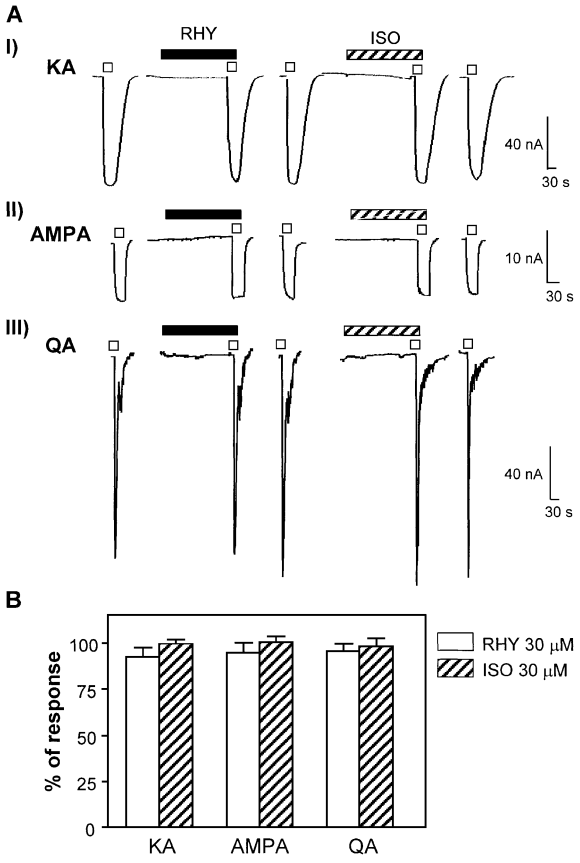

Fig. 3. Effects of rhynchophylline and isorhynchophylline on kainic acid- and (±)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-induced ionotropic currents and quisqualic acid-induced metabotropic currents in *Xenopus* oocytes. (A) Typical current responses caused by 100 μM kainic acid (KA, I), 10 μM (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA, II), and 1 μM quisqualic acid (QA, III) in the presence and absence of rhynchophylline (RHY) and isorhynchophylline (ISO). Open squares in traces represent the application of each receptor agonist for 30 s. Horizontal black and shaded bars above the middle of the traces represent bath application of 30 μM RHY and ISO, respectively. (B) Summary of the effects of 30 μM RHY and ISO on kainic acid-, AMPA- and QA-induced current responses in *Xenopus* oocytes. Each column represents the mean ± S.E.M. from five to eight different oocytes.

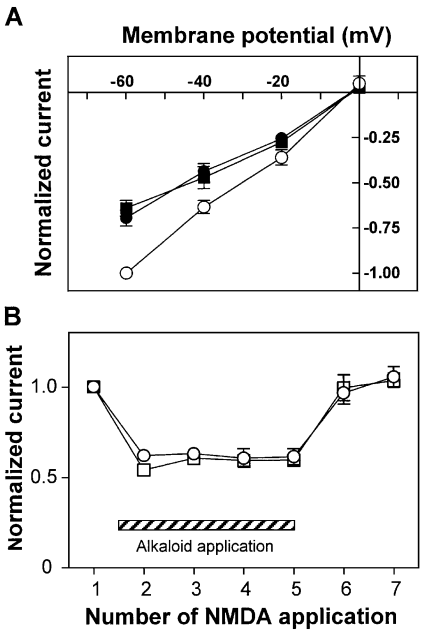

Fig. 4. Rhynchophylline and isorhynchophylline-induced alterations of NMDA currents show no voltage or use dependency. (A) NMDA-induced currents were measured at different holding potentials in the presence and absence of the alkaloids (○: control, ■: 30 μM rhynchophylline, ●: isorhynchophylline). Each data point represents the mean ± S.E.M. of five different oocytes. (B) The amplitude of NMDA currents does not change during long-lasting application of rhynchophylline and isorhynchophylline. NMDA (100 μM: ○, □) was applied for 30 s at 2-min intervals a total of seven times. The horizontal bar indicates the application of rhynchophylline (30 μM) or isorhynchophylline (30 μM) for 7 min. Each data point represents the mean ± S.E.M. of four to five different oocytes.

3.4. Rhynchophylline and isorhynchophylline alteration of the concentration–response curves for NMDA- and the co-agonist glycine-induced currents

We further characterized the inhibitory effects of the alkaloids on the NMDA receptor function by analyzing the concentration–response curves for NMDA and glycine, a co-agonist for the NMDA receptor. Application of NMDA at concentrations ranging from 10 μM to 1 mM revealed that the EC_{50} value and Hill coefficient of NMDA were 61 [46.7–79.6] μM (mean [95% confidence interval (CI)]) and 1.09 ± 0.16, respectively. On the other hand, the EC_{50} values of NMDA recorded in the presence of 30 μM rhynchophylline and 30 μM isorhynchophylline were 44 [30.2–64.2] μM (mean [95% CI], $P = 0.156$, *t*-test) and 49.9 [34.8–71.5] μM (mean [95% CI], $P = 0.363$, *t*-test) with Hill coefficients of 0.97 ± 0.17 and 1.11 ± 0.22, respectively. These values were not significantly different from the values obtained in the absence of the alkaloids (Fig. 5A). In contrast, rhynchophylline (30 μM) and isorhynchophylline (30 μM) significantly reduced the maximal current responses of NMDA receptor channels in *Xenopus* oocytes by 30.6% ($P < 0.01$, *t*-test) and 25.7% ($P < 0.01$, *t*-test), respectively.

Similar results were obtained when the effects of rhynchophylline and isorhynchophylline on the glycine dependency of NMDA-induced currents in *Xenopus* oocytes were analyzed. As depicted in Fig. 5B, perfusion of different concentrations of glycine (0.1–30 μM) with 100 μM NMDA induced increasing inward currents with an EC_{50} value of 1.53 [1.17–2.00] μM (mean [95% CI]) and a Hill coefficient of 1.1 ± 0.12. On the other hand, EC_{50} values for the glycine response recorded in the presence of 30 μM rhynchophylline and 30 μM isorhynchophylline were 2.03 [1.01–4.11] μM (mean [95% CI], $P = 0.44$, *t*-test) and 1.66

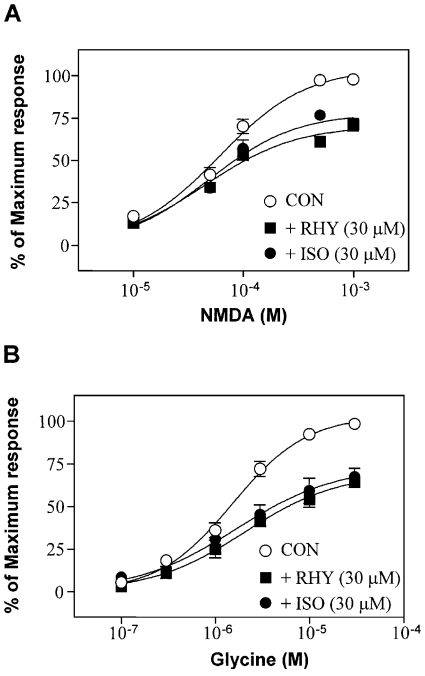

Fig. 5. Noncompetitive inhibition of NMDA- and glycine-induced current response by rhynchophylline and isorhynchophylline in *Xenopus* oocytes expressing rat cortex total RNA. (A) Concentration–response curve for NMDA in the presence and absence of the alkaloids. Glycine concentration in the Mg^{2+}-free medium was fixed at 3 μM and various concentrations of NMDA (10–1000 μM) were applied to *Xenopus* oocytes for 30 s. The alkaloid (30 μM) was applied to the oocytes for 3 min before and for 30 s simultaneously with NMDA. The EC_{50} and Hill coefficient values for NMDA were 61 [46.7–79.6] μM (mean [95% confidence interval (CI)]) and 1.09 ± 0.16 (mean ± S.E.M., $n = 6$), respectively, in the control medium, 44 [30.2–64.2] μM ($P = 0.156$, *t*-test) and 0.97 ± 0.17 ($n = 9$), respectively, in the presence of 30 μM rhynchophylline, and 49.9 [34.8–71.5] μM ($P = 0.363$, *t*-test) and 1.11 ± 0.22 ($n = 7$), respectively, in the presence of 30 μM isorhynchophylline. (B) Concentration–response curve for glycine in the presence and absence of the alkaloids. NMDA (100 μM)-induced currents were recorded in Mg^{2+}-free MBS containing various concentrations of glycine (0.1–30 μM). The alkaloids (30 μM) were applied to the oocytes for 3 min before and for 30 s simultaneously with 100 μM NMDA. The EC_{50} and Hill coefficient values for glycine to enhance NMDA currents were 1.53 [1.17–2.00] μM (mean [95% CI]) and 1.1 ± 0.12 (mean ± S.E.M., $n = 5$), respectively, in the control medium, 2.03 [1.01–4.11] μM ($P = 0.44$, *t*-test) and 1.79 ± 0.36 ($n = 5$), respectively, in the presence of 30 μM rhynchophylline, and 1.66 [0.69–3.96] μM ($P = 0.86$, *t*-test) and 1.36 ± 0.09 ($n = 7$), respectively, in the presence of 30 μM isorhynchophylline.

[0.69–3.96] μM (mean [95% CI], $P=0.86$, *t*-test). The Hill coefficients for glycine in the presence of 30 μM rhynchophylline and 30 μM isorhynchophylline were 1.79 ± 0.36 and 1.36 ± 0.09, respectively. These EC_{50} values and Hill coefficients were not significantly different from the respective control values. However, interestingly, both rhynchophylline and isorhynchophylline at 30 μM significantly reduced E_{max} values of the glycine response by 32.7% ($P<0.01$, *t*-test) and 28.9% ($P<0.01$), respectively.

3.5. NMDA receptor modulators failed to influence the effects of rhynchophylline and isorhynchophylline on NMDA-induced currents

To determine if other modulatory sites on the NMDA receptor channels are involved in the action of rhynchophylline and isorhynchophylline, we examined the inhibitory effects of the alkaloids on NMDA-induced current in the presence and absence of NMDA receptor modulators such as spermine, dithiothreitol, Zn^{2+}, and alterations of pH (Aizenman et al., 1989; Legendre and Westbrook, 1990; McGurk et al., 1990; Tang et al., 1990; Tang and Aizenman, 1993; Vyklicky et al., 1990; Williams, 1994). Spermine (10 μM) and dithiothretiol (1 mM), significantly increased the 50 μM NMDA-induced current response by 27.5 ± 8.6% and 69.8 ± 24% ($n=6$, 10, $P<0.01$), respectively. Moreover, a decrease in protons also significantly enhanced the 100 μM NMDA-induced current by 151.6 ± 32%, while an increase in protons reduced the current response by 57.9 ± 6.1% ($n=8$). On the other hand, $ZnCl_2$ (1 μM) significantly reduced the NMDA-induced current by 27.2 ± 2.5% ($n=6$, $P<0.01$). As summarized in Table 1, these NMDA receptor modulators had no effect on the extent of inhibition of the NMDA response by rhynchophylline and isorhynchophylline.

Table 1
Effects of NMDA receptor modulators on rhynchophylline- and isorhynchophylline-induced inhibition of NMDA currents in *Xenopus* oocytes

Treatment	% Inhibition of NMDA current	
	30 μM Rhynchophylline	30 μM Isorhynchophylline
Exp. 1		
Control	49.4 ± 2.7	41.2 ± 4.9
Spermine (10 μM)	51.6 ± 2.9	40.5 ± 3.6
Control	39.8 ± 3.7	42.8 ± 2.7
Dithiothreitol (1 mM)	46.5 ± 3.9	39.1 ± 2.7
Exp. 2		
Control (pH 7.4)	42.6 ± 2.8	42.9 ± 3.0
pH 6.4	43.6 ± 3.0	45.4 ± 6.4
pH 8.4	39.7 ± 3.6	41.4 ± 4.2
Control	47.4 ± 2.3	46.6 ± 3.6
$ZnCl_2$ (1 μM)	45.2 ± 3.8	43.8 ± 3.1

NMDA (Exp. 1: 50 μM, Exp. 2: 100 μM)-induced current was measured in the 3 μM glycine containing Mg^{2+}-free MBS with or without NMDA receptor modulators (10 μM spermine, 1 mM dithiothreitol, or 1 μM $ZnCl_2$) or in the same MBS with different pH values. The alkaloids were applied to the oocytes for 3 min before and for 30 s simultaneously with NMDA. Spermine (10 μM) and dithiothretiol (1 mM) potentiated NMDA-induced currents by 27.5 ± 8.6% ($P<0.05$, paired *t*-test) and 69.8 ± 24% ($P<0.05$), respectively. Increasing pH of MBS (from 7.4 to 8.4) potentiated the NMDA currents by 151.6 ± 32% ($P<0.05$), whereas reducing pH (from 7.4 to 6.4) suppressed the currents by 57.9 ± 6.1% ($P<0.05$). Each data value represents the mean ± S.E.M. obtained from 5–10 different oocytes.

4. Discussion

In this study, we investigated the effects of tetracyclic oxindole alkaloids, rhynchophylline and isorhynchophylline, on NMDA receptor channels expressed in *Xenopus* oocytes. Our findings clearly demonstrated that these alkaloids produce a concentration-dependent and reversible inhibition of NMDA receptor function in oocytes with similar potency.

Rhynchophylline and isorhynchophylline *per se* at concentrations of 1–100 μM produced no changes of the resting membrane conductance in oocytes injected with or without rat cortex total RNA, indicating that the inhibitory effects of the alkaloids on NMDA-induced currents are not due to activation of ion channels other than the NMDA receptor channel that are intrinsically or exogenously expressed in the oocyte membrane. It has been reported that stimulation of NMDA receptors expressed in *Xenopus* oocytes activates Ca^{2+} channels coupled with the receptors and the oocyte's intrinsic Ca^{2+}-dependent Cl^- channels (Kelso et al., 1992; Leonard and Kelso, 1990). Thus, it was possible that rhynchophylline and isorhynchophylline showed an apparent inhibitory effect on NMDA responses by blocking Ca^{2+}-dependent Cl^- channels. However, this possibility was ruled out by several findings. First, the inhibitory potencies of the alkaloids were not altered by replacement of Ca^{2+} in the recording medium with Ba^{2+}, a poor activator of Ca^{2+}-dependent Cl^- channels in oocytes (Barish, 1983). Second, the analysis of the voltage-and-current relationship in terms of NMDA receptor activation revealed that the NMDA currents in the presence and absence of the alkaloids were reversed at around 0 mV, a potential that is closer to the reversal potential for the NMDA channel in neurons (Mayer and Westbrook, 1987). Third, neither alkaloid had an effect on Ca^{2+}-dependent Cl^- current responses induced by quisqualic acid stimulation of metabotropic glutamate receptors expressed in oocytes.

Rhynchophylline and isorhynchophylline at 30 μM, a concentration that significantly inhibited the NMDA-induced current, had no effect on current responses mediated either by other ionotropic glutamate receptor subtypes such the kainic acid- and AMPA-subtype glutamate receptors or by metabotropic glutamate receptors, as aforementioned. These findings suggest that the alkaloids are able to specifically interact with the NMDA-subtype glutamate receptor. However, considering the fact that the suppressive effects of

rhynchophylline and isorhynchophylline on NMDA responses were independent of membrane potential and agonist-use, it is unlikely that these alkaloids act as direct open channel blockers like MK801, PCP, ketamine and SKF-10,047 (Burnashev et al., 1992; Mori et al., 1992; Yamakura et al., 1993). The present study revealed that rhynchophylline and isorhynchophylline suppress the NMDA response by decreasing the NMDA receptor efficacy (the maximal response) but not the NMDA potency (EC_{50}). Interestingly, this mode of action was also true for the inhibition of the glycine response by the alkaloids: rhynchophylline and isorhynchophylline reduced the potency of glycine to enhance NMDA-activated currents without affecting the efficacy of the glycine site (EC_{50}). Taken together with the fact that neither of the alkaloids showed an effect on the modulation of the NMDA receptor channel activity via the polyamine site, the redox site, the H^+ site or the Zn^{2+} site, our findings raise the possibility that rhynchophylline and isorhynchophylline exert noncompetitive antagonism by allosterically inhibiting NMDA binding to the NMDA recognition site and/or glycine binding to the glycine recognition site on the NMDA receptor channel protein. Nevertheless, further investigation will be required to elucidate the site of action of rhynchophylline and isorhynchophylline on the NMDA receptors.

Both rhynchophylline and isorhynchophylline, an epimer of rhynchophylline at the spiro C7-position of the oxindole moiety, have a tetracyclic skeleton that is composed of oxindole and indolizidine moieties (Tang and Eisenbrand, 1992). In spite of the difference in the stereostructure between these two alkaloids, we found almost the same potency in terms of suppression of the NMDA receptor functions by these alkaloids. On the other hand, recent electrophysiological studies demonstrated that oxindole, a neurodepressant tryptophan metabolite physiologically present in mammalian brain and blood, did not modify the AMPA/kainic acid or NMDA response in rat cortical slices and in mouse cortical wedge preparations at a concentration range of 0.3–3 mM (Mannaioni et al., 1996, 1998). Taken together, the present findings suggest that the indolizidine moiety, but not the oxindole moiety, of the alkaloids plays an important role in inhibition of the NMDA receptor functions in *Xenopus* oocytes. However, we can not exclude the possibility that the presence of the oxindole moiety in both alkaloids is essential to adjust or bend the conformation of the indolizidine moiety to make the alkaloids fit into the putative interaction site at the NMDA receptor complex.

Shimada et al. (1999) reported a protective effect of rhynchophylline and isorhynchophylline on glutamate-induced cell death in cerebellar granule cell culture at a concentration range of 0.3–1 mM. In *Xenopus* oocytes with rat cortex total RNA, we found that these alkaloids exhibited suppressive activity against the NMDA-induced response at a concentration range about 10 times lower than that at which they showed neuroprotection in the cerebellar granule cell culture system. The reason for this discrepancy in the effective concentration range between our and their systems is unclear, but it may be due to the difference in NMDA receptor subunit composition in the systems employed since different compositions of NMDA receptor subunits confer distinct pharmacological and physiological properties on NMDA receptor function (Ishii et al., 1993; Monyer et al., 1992). Nevertheless, in view of the inhibitory effects of rhynchophylline and isorhynchophylline on the NMDA receptor, these alkaloids may serve as useful drugs for treatment and/or prevention of neurodegenerative damage induced by ischemia, trauma, and chronic neurodegeneration such as Alzheimer's disease, since a body of evidence indicates an important role of ionotropic glutamate receptors, particularly the NMDA-subtype glutamate receptor, in neuronal pathogenic processes (Choi, 1992; Meldrum, 1992; Muller et al., 1995; Parsons et al., 1998).

In conclusion, this is the first report demonstrating an inhibitory effect of rhynchophylline and isorhynchophylline on NMDA receptor channels *in vitro*. Rhynchophylline- and isorhynchophylline-induced inhibition of NMDA receptor function may be useful for the treatment or prevention of neuronal diseases that involve excess stimulation of NMDA-subtype glutamate receptors.

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