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Inhibitory effects of corymine, an alkaloidal component from the leaves of *Hunteria zeylanica*, on glycine receptors expressed in *Xenopus* oocytes

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Abstract

We previously reported that corymine, an alkaloidal compound extracted from the leaves of *Hunteria zeylanica* native to Thailand, potentiated convulsions induced by either picrotoxin or strychnine. Therefore, to clarify the mechanism of action of corymine, the effects of corymine on γ-aminobutyric acid (GABA) and glycine receptors were examined. We used *Xenopus* oocytes expressing these receptors and the two-electrode voltage-clamp method. The receptors expressed in oocytes injected with rat brain and spinal cord RNA showed the pharmacological properties of $GABA_A$ and glycine receptors, respectively. Corymine (1–100 μM) partially (20–30%) reduced the GABA responses in oocytes injected with rat brain RNA, while marked (up to 80%) dose-dependent reductions were observed in the glycine responses in oocytes injected with rat spinal cord RNA. These observations suggest that corymine was more effective against the glycine receptors than the GABA receptors. The ED_{50} of corymine on the glycine response was 10.8 μM. Corymine, at 30 μM, caused a shift to the right, with a lower maximal response, of the glycine concentration–response curve. This indicated that the action of corymine on glycine receptors is neither competitive nor purely non-competitive. These observations suggest that a binding site other than the glycine recognition site of the glycine receptors is the site of action of corymine. © 1997 Elsevier Science B.V.

Keywords: Corymine; *Xenopus* oocytes; $GABA_A$ receptors; Glycine receptors

1. Introduction

Hunteria zeylanica GARD. (*H. zeylanica*) is a glabrous tree of the Apocynaceae (Ridley, 1923; Hooker, 1980-1982) which is used as in folk medicines to cure yaws and reduce boils and skin irritations (Whitmore, 1973; Perry and Metzger, 1980). Many indole alkaloids have been reported to be present in the leaves and bark of *H. zeylanica* (Arambewela and Khuong-Huu, 1981; Lavaud et al., 1982; Subhadhirasakul et al., 1994a,b; Takayama et al., 1994). Recently, our laboratory reported that alkaloidal extracts obtained from the bark of this plant exhibited antinociceptive, antipyretic and anti-inflammatory effects (Reanmongkol et al., 1994, 1995). In similar experiments, we found that alkaloidal extracts and a main component, corymine (Fig. 1), obtained from the leaves of this plant potentiated the convulsions induced by either picrotoxin, a γ-aminobutyric acid (GABA) receptor non-competitive antagonist, or strychnine, a selective glycine receptor antagonist, in mice (Leewanich et al., 1996). This led us to study the mechanisms of action of corymine for producing the convulsion potentiation. In this paper we investigated the effects of corymine on GABA and glycine receptors ex-

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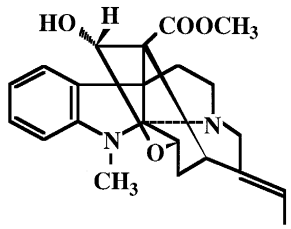

Fig. 1. Chemical structure of corymine.

pressed in *Xenopus* oocytes, using the two-electrode voltage-clamp method.

2. Materials and methods

2.1. Materials

Authentication of the leaves of *H. zeylanica* GARD. was achieved by comparison with herbarium specimens at the Department of Biology, Faculty of Sciences, Prince of Songkla University, Thailand.

2.2. Isolation of corymine from the leaves of H. zeylanica

The dried leaves of *H. zeylanica* GARD. (2.5 kg) were first moistened with 25% ammonium solution and further extracted with various organic solvents (Subhadhirasakul et al., 1994b). Corymine was isolated from portions of crude chloroform extract (10.15 g), using SiO_2 column chromatography and thin-layer chromatography purification as reported previously (Subhadhirasakul et al., 1994b; Takayama et al., 1994).

2.3. Oocytes injection and recording

Defolliculated stage V–VI oocytes were prepared from *Xenopus laevis* (Hamamatsu Seibutsu, Shizuoka, Japan) as described previously (Tohda et al., 1989). Briefly, *Xenopus laevis* were anesthetized in ice-water and a lobe of the ovary was removed and placed in sterile 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 $NaHCO_3$, 7.5 mM Tris-[hydroxymethyl]aminomethane, pH 7.6). Oocytes were then defolliculated with collagenase (Wako, Osaka), 1.5 mg/ml in Ca^{2+}-free MBS at 22°C for 45–60 min. Total RNA was prepared from whole brain or spinal cord of adult male rats (Japan SLC, Shizuoka, Japan), using the guanidium isothiocyanate method (Sambrook et al., 1989). To examine GABA and glycine-induced Cl^- currents, oocytes were injected with 46 nl of total RNA (5 mg/ml) prepared from the brain or spinal cord, respectively. After injection, oocytes were incubated in MBS containing 2.5 u/ml penicillin and 2.5 μg/ml streptomycin at 21°C for 2 days before recording. The MBS was replaced daily.

The transmembrane currents were recorded with a two-electrode voltage-clamp, using a GeneClamp 500 (Axon Instruments, Foster City, CA, USA). The voltage-sensing electrode was filled with 3 M KCl and the current-passing electrode with 3 M ammonium acetate. An oocyte was positioned in a 50 μl chamber and perfused with MBS at room temperature (22–25°C). Vigorous oocytes possessing negative membrane potentials exceeding −20 mV were used for electrical recordings and the membrane potential was maintained at −60 mV. The cells were continuously perfused at 1.5 ml/min and drugs were applied in the perfusate. The drugs were applied until the peak of the response was observed, usually within 30 s or less. Recovery time was 5–30 min, depending on the concentration of drugs applied.

2.4. Drugs

The following drugs were used: γ-aminobutyric acid (GABA) (Nacalai Tesque, Kyoto, Japan), glycine (Wako), midazolam (Yamanouchi, Tokyo, Japan), pentobarbital sodium (Tokyo Kasei Kogyo, Tokyo, Japan), strychnine nitrate (Tokyo Kasei Kogyo), picrotoxin (Nacalai Tesque) and bicuculline (Calbiochem, La Jolla, CA, USA). Corymine was prepared in 100% dimethylsulfoxide (DMSO) (Wako). The final DMSO concentrations ($\leq$ 0.1%) had no pharmacological effect when applied alone. Other chemicals were dissolved in the buffer solution. All drugs were applied via the perfusion system.

2.5. Data analysis

Concentration–response curves were made by applying different concentrations of GABA or glycine in random order and were calculated by using the logistic equation, $E = E_{max} * [drug]^n / (ED_{50}^n + [drug]^n)$, where E_{max} is the maximal effect, n is the Hill coefficient and ED_{50} is the concentration of drug producing 50% of the maximal effect.

3. Results

3.1. Pharmacological properties of the expressed GABA and glycine receptors in Xenopus oocytes injected with total RNA

We first characterized the physiological and pharmacological properties of the expressed receptors. Bath application of GABA or glycine to oocytes injected with rat brain or spinal cord RNA elicited an inward current at a clamp potential of −60 mV (Fig. 2A and Fig. 3A). Concentra-

tion–response curves for GABA and glycine were made and ED_{50} values of 56.3 μM (Fig. 2B) and 464.2 μM (Fig. 3B) were determined, respectively.

With co-application, the responses elicited by GABA at 50 μM were dose dependently reduced by the competitive antagonist bicuculline (0.1–100 μM) (Fig. 2C) and non-competitive antagonist picrotoxin (0.1–100 μM) (Fig. 2D). Pentobarbital (30–300 μM) (Fig. 2E) and midazolam (0.1–1 μM) (Fig. 2F) enhanced the GABA responses at 5 μM. The glycine responses at 500 μM were dose dependently antagonized by the selective antagonist strychnine (0.01–10 μM) (Fig. 3C).

3.2. Efficacy of corymine on the expressed glycine receptors

To clarify the efficacy of corymine on the expressed receptors, corymine at 1–100 μM was co-applied with either 50 μM GABA or 500 μM glycine to oocytes injected with rat brain or spinal cord RNA. Corymine markedly and dose dependently reduced the glycine responses although it had hardly any effect on the GABA responses even at the highest dose applied (Fig. 4). Corymine at the maximum dose (100 μM) inhibited the glycine and GABA responses by 79.6 ± 0.7% and 30.4 ±

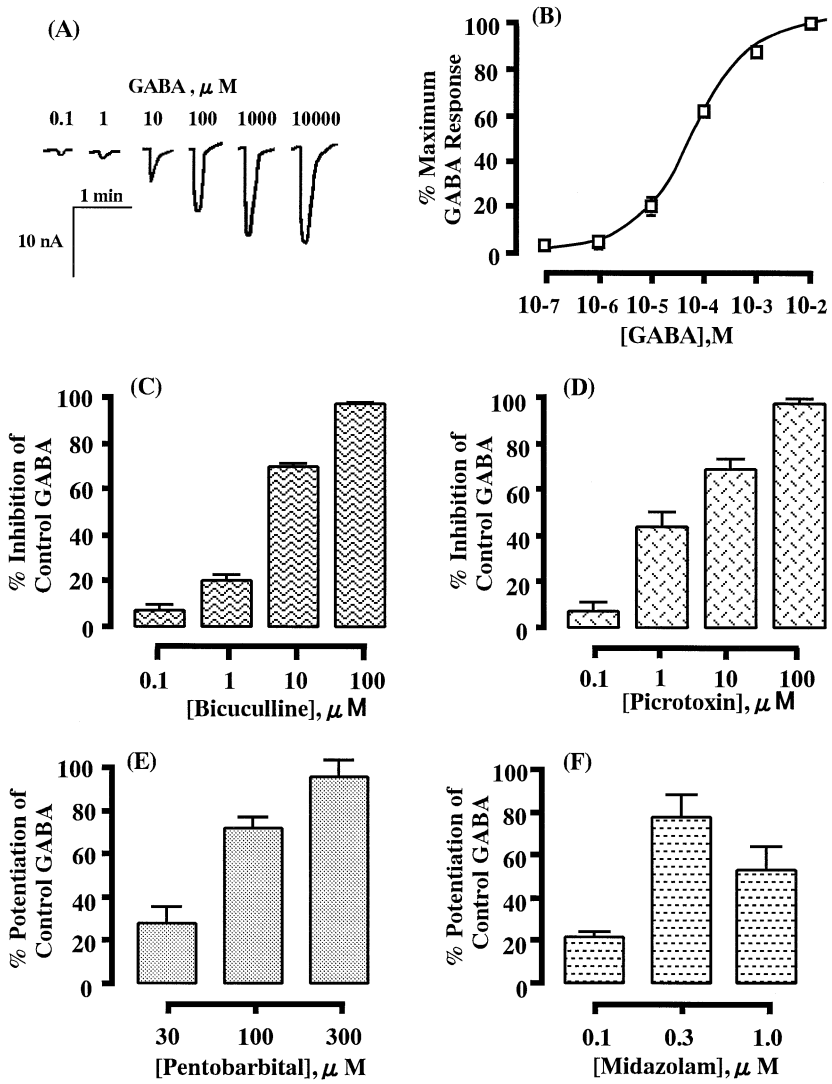

Fig. 2. Pharmacological properties of the GABA receptors expressed in *Xenopus* oocytes injected with rat brain RNA. (A) Different concentrations of GABA applied to an oocyte induced outward currents, denoted by downward deflection of the trace. (B) Responses to various concentrations of GABA, expressed as percentages relative to those elicited by 10 mM GABA as a control. Each point represents the mean ± S.E. for 7 oocytes. The ED_{50} was 56.3 μM. (C) Inhibition of 50 μM GABA-induced currents by bicuculline. Each bar represents the mean ± S.E. for 7 oocytes. (D) Inhibition of 50 μM GABA-induced currents by picrotoxin. Each bar represents the mean ± S.E. for 5 oocytes. (E) Potentiation of 50 μM GABA-induced currents by pentobarbital. Each bar represents the mean ± S.E. for 6 oocytes. (F) Potentiation of 5 μM GABA-induced currents by midazolam. Each bar represents the mean ± S.E. for 5 oocytes. Oocytes were voltage-clamped at −60 mV.

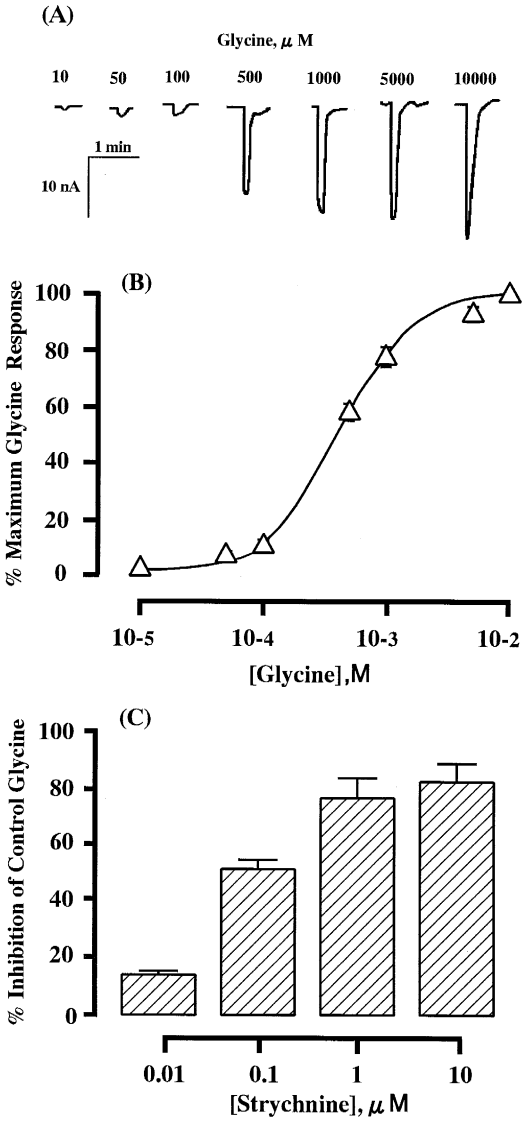

Fig. 3. Pharmacological properties of the glycine receptors expressed in *Xenopus* oocytes injected with rat spinal cord RNA. (A) Different concentrations of glycine applied to an oocyte induced outward currents, denoted by downward deflection of the trace. (B) Responses to various doses of glycine expressed as percentages relative to those elicited by 10 mM glycine as a control. Each point represents the mean ± S.E. for 8 oocytes. The ED_{50} was 464.2 μM. (C) Inhibition of 500 μM glycine-induced currents by strychnine. Each bar represents the mean ± S.E. for 5–10 oocytes. Oocytes were voltage-clamped at −60 mV.

3.3%, respectively. ED_{50} values for corymine at the glycine and GABA receptors were 10.8 and 7.6 μM, respectively.

3.3. Antagonism by corymine of the glycine concentration–response curve

To study the inhibitory mechanism of corymine on the glycine receptors, oocytes were treated with different concentrations of glycine in the absence and presence of 30 μM corymine. Corymine caused a shift of the glycine response curve to the right with a lower maximal response

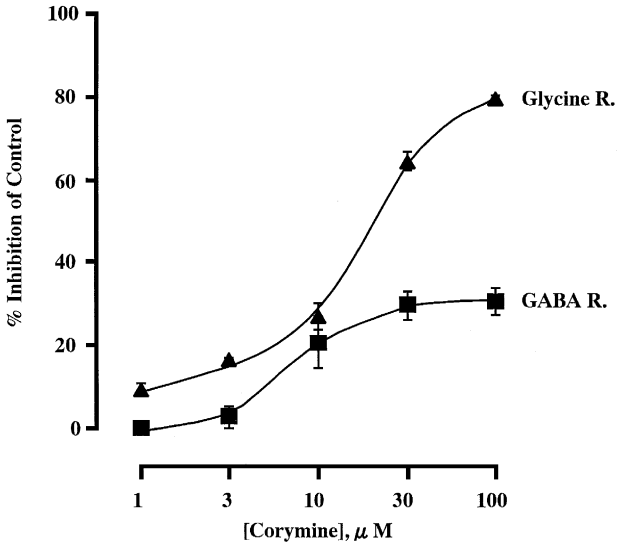

Fig. 4. Efficacy of corymine on the GABA and glycine receptors expressed in *Xenopus* oocytes injected with rat RNA. Corymine (1–100 μM) was co-applied with GABA (50 μM) and glycine (500 μM) to oocytes injected with rat brain (■) and spinal cord (▲) RNA, respectively. Data are expressed as percentages of inhibition relative to control GABA or glycine responses. Corymine caused 30.4 and 79.6% inhibition of GABA and glycine responses and the ED_{50} was 7.6 and 10.8 μM for the GABA and glycine receptors, respectively. Each point represents the mean ± S.E. for 3–5 oocytes.

(Fig. 5). The ED_{50} of glycine was changed from 384.8 to 897.1 μM and the maximal response was decreased to 73.3%.

The glycine responses that were reduced by corymine hardly recovered after washout, in contrast to those reduced by strychnine. The glycine response that were re-

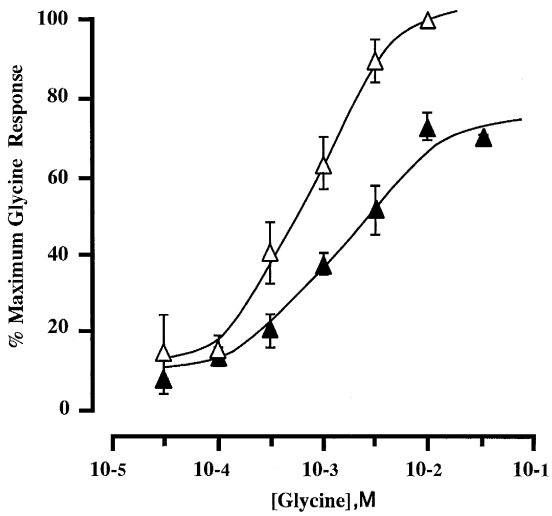

Fig. 5. Antagonism by corymine of glycine concentration–response curve in *Xenopus* oocytes injected with rat spinal cord RNA. Oocytes were treated with glycine (30 μM–30 mM) in the absence (△) or presence (▲) of 30 μM corymine. Data are expressed as percentages of the response elicited by 10 mM glycine as a control. Each point represents data from one oocyte and the mean ± S.E. for 2–5 oocytes.

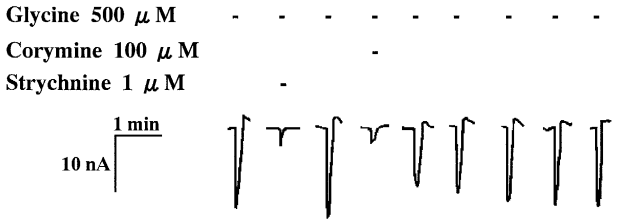

Fig. 6. Recovery of glycine response after corymine or strychnine application in *Xenopus* oocytes injected with rat spinal cord RNA. Oocytes were treated with glycine (500 μM) and either strychnine at 1 μM or corymine at 100 μM. The cells were washed with buffer for 10 min before the next application.

duced by 80% after co-application of 1 μM strychnine recovered to the control level 10 min after washout, but with the same oocyte and an effective dose of corymine (100 μM) the cell required 30 min for recovery (Fig. 6).

4. Discussion

The physiological and pharmacological properties of the GABA and glycine receptors expressed in *Xenopus* oocytes suggest that they represent the native $GABA_A$ and glycine receptors of neurons (Polenzani et al., 1991; Akagi et al., 1991; Wahl et al., 1993; Trombley and Shepherd, 1994; Connolly et al., 1996). The effective concentrations of their agonists and antagonists were in the same range as in previous reports (Hattori et al., 1986; Smart et al., 1987; Parker et al., 1988; Thompson et al., 1996). The potentiating effect of midazolam was reduced at the dose of 1 μM. This result is consistent with previous reports that benzodiazepines potentiate GABA responses at low doses and block them at higher doses (MacDonald and Barker, 1982; Hattori et al., 1986).

Corymine at the maximum dose applied (100 μM) inhibited the glycine responses by 80%, but reduced the GABA responses by only 30%. Thus, corymine more effectively inhibited the glycine responses than the GABA responses. Corymine had little effect on 5-HT and acetylcholine receptors (data not shown). Examination of the concentration–response curves shows that corymine acted neither competitively nor purely non-competitively on the glycine receptors. This phenomenon is in accordance with a mixed type of antagonism previously described to account for the inhibition of the GABA response by picrotoxinin in crustacean muscle (Smart and Constanti, 1986). It was concluded that picrotoxinin probably stabilized the closed form of the GABA-operated ion channel and affected the agonist binding affinity rather than competed with Cl^- for the ion selectivity site. Like GABA receptors, glycine receptors also contain at least two sites for antagonist binding: (i) the glycine recognition site or sites; and (ii) the ion selectivity site in the ionophore (Langosch et al., 1990; Rundstrom et al., 1994). The binding site of strychnine, which is reported to inhibit the response of glycine in a competitive manner (Langosch et al., 1990; Wahl et al., 1993), is assumed to be closely related, but not identical, to that of glycine (Langosch et al., 1990; Betz, 1991; Vandenberg et al., 1992). The effect of corymine on the glycine concentration–response curve suggested that corymine might act by binding to a site that is different from the site for strychnine binding. Further studies are required to elucidate whether this binding site(s) of corymine is an as yet uncharacterized binding site for glycine receptor antagonists, especially on glycine-coupled chloride channels.

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