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A possible mechanism underlying corymine inhibition of glycine-induced Cl^- current in *Xenopus* oocytes

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Abstract

We previously reported that corymine, an alkaloid extracted from the leaves of *Hunteria zeylanica* native to Thailand, inhibited glycine-induced chloride current using a receptor expression model of *Xenopus* oocytes. In this study, we investigated the mechanism underlying the inhibitory action of this alkaloid on glycine current using the same model. Corymine inhibited glycine current in a noncompetitive fashion. Co-application with strychnine, a competitive glycine receptor antagonist, or 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), a Cl^- channel blocker, corymine decreased the ED_{50} value of strychnine, but did not change that of DIDS. Moreover, the inhibitory effects of corymine and either strychnine or DIDS were additive. The desensitization phase of glycine current showed two exponentials and corymine preferentially inhibited the fast component, whereas strychnine affected both of them to the same extent and DIDS preferentially inhibited the slow component. When these drugs were applied repeatedly, the inhibitory effects of corymine and strychnine were not use-dependent and reversible, while the effect of DIDS was use-dependent and irreversible. The inhibitory effect of corymine on γ-aminobutyric acid (GABA) current was less potent than the effect on glycine current, while this alkaloid failed to affect acetylcholine and serotonin currents. These results demonstrate that corymine inhibits glycine-gated Cl^- channels by interacting with the site different from that of DIDS.

Keywords: *Xenopus* oocyte; Cl^- current, glycine-induced; Corymine; Strychnine; DIDS (4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid)

1. Introduction

The glycine receptor is known to contain two binding sites for antagonists (Langosch et al., 1990; Rajendra et al., 1997); the glycine recognition site which can be blocked by the specific competitive glycine receptor antagonist, strychnine (Curtis et al., 1971; Langosch et al., 1990; Wahl et al., 1993; Young and Snyder, 1974) and the ionic site in glycine-receptor Cl^- channel which can be blocked by the putative glycine-channel blocker, cyanotriphenylborate (Rundstrom et al., 1994). Compared to $GABA_A$ receptor-channel blockers, very few glycine receptor-channel blockers are available (Becker, 1992; Rajendra et al., 1997; Smart and Constanti, 1986; Van Renterghem et al., 1987; Yoon et al., 1993).

Corymine is an alkaloid extracted from the leaves of *Hunteria zeylanica* native to Thailand (Subhadhirasakul et al., 1994a,b; Takayama et al., 1994). In a previous behavioral study, we reported that corymine potentiated convulsions induced by picrotoxin (a $GABA_A$ receptor antagonist) and strychnine in mice (Leewanich et al., 1996). Using an electrophysiological technique, we found that corymine more strongly inhibited glycine current than GABA current. The results suggested that a binding site other than the glycine recognition site of the glycine receptors is the site of action of corymine (Leewanich et al., 1997). However, it remains unclear: (i) whether the effect of corymine on glycine current is due to blockage of the glycine binding site, as that of strychnine, or blockage

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of the Cl^- channel, as that of Cl^- channel blockers, and (ii) whether the inhibitory activity of corymine is specific to glycine current. In the present study, to address these issues, we compared the inhibitory effect of corymine on glycine current with those of reference strychnine and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), and examined the effect of corymine on GABA-, acetylcholine- and serotonin-induced currents using a receptor expression model of *Xenopus* oocytes. We used DIDS as a reference drug because it has been shown to block Cl^- transport in erythrocyte (Grinstein et al., 1979; Jennings, 1985), and neuronal membranes (Inoue, 1985) and glycine- and GABA-gated Cl^- channels in *Xenopus* oocytes injected with mRNA from chick and rat brain (Parker et al., 1988).

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 the 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, *X. 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 isolated and defolliculated with collagenase (Wako Pure Chemical Industries, Osaka), 1.5 mg/ml in Ca^{2+}-free MBS at 22°C for 30 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). Poly$(A)^+$ mRNA was then isolated from total RNA by oligo-dT cellulose column chromatography. The mRNA was dissolved in sterile water at a final concentration of approximately 1 mg/ml and injected into the oocytes. The oocytes were incubated in MBS containing 2.5 units/ml penicillin and 2.5 μg/ml streptomycin at 18°C for 2 days before recording. The MBS was replaced daily.

The transmembrane currents were recorded using the two-electrode voltage-clamp method (GeneClamp 500, Axon Instruments, Foster City, CA). The voltage-monitoring 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 continuously perfused with MBS at 1.5 ml/min at room temperature (22–25°C). Vigorous oocytes possessing negative membrane potentials exceeding -20 mV were used for the experiments and the membrane potential was maintained at -60 mV. The drugs were applied until the peak of the response was observed (usually for 30 s or less). The washout period for recovery was 1–15 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), strychnine nitrate (Tokyo Kasei Kogyo, Tokyo, Japan), DIDS (Wako), serotonin (5-HT; Sigma Chemical, MO, USA) and acetylcholine chloride (Wako). Corymine was dissolved in 100% dimethylsulfoxide (DMSO) (Wako). In the experiments, 10 and 30 μM corymine was used. We chose these concentrations according to the IC_{50} (11 μM) value of corymine to inhibit 300 μM glycine-induced current in *Xenopus* oocyte preparation expressing glycine receptors. 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

Peak current amplitudes were measured and used for concentration–response relationships. Concentration–response curves were made by applying different concentrations of glycine in random order and were calculated by using the logistic equation, $E = E_{max} \times [drug]^n / (ED_{50}^n + [drug]^n)$, 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. IC_{50} is the concentration of drug producing 50% of the maximal inhibition.

3. Results

3.1. Antagonism by corymine, strychnine and DIDS of glycine concentration–response relationship

To gain insight into the mechanism underlying corymine inhibition of glycine-induced Cl^- current, we determined

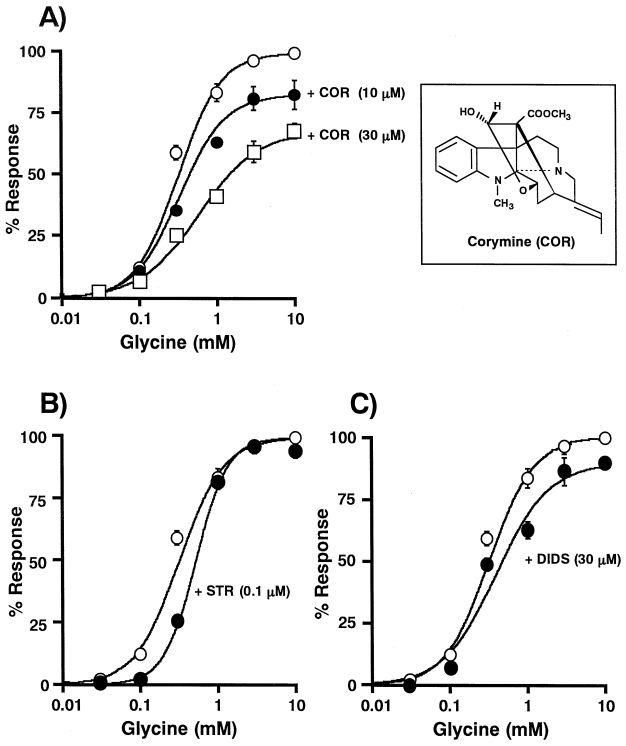

Fig. 1. Effects of corymine (A; COR), strychnine (B; STR) and DIDS (C) on glycine-induced Cl^- current in *Xenopus* oocytes injected with rat spinal cord poly(A)$^+$ mRNA. Oocytes were applied with glycine (0.03–10 mM) in the absence and presence of test drugs. Data are expressed as percentages of the response elicited by 10 mM glycine as the control. Each point represents the mean ± S.E.M. for 2–10 oocytes.

the concentration–response relationships of glycine in the absence and presence of corymine and compared the effects of corymine with those of reference compounds, strychnine and DIDS. In the control experiments, the ED_{50} value of glycine was 317 μM and the Hill coefficient was 1.54, indicating that two glycine molecules bind to one receptor site. Corymine, at 10 and 30 μM, decreased the maximal glycine response by 17 and 32%, respectively (Fig. 1A). The ED_{50} value of glycine in the presence of 10 and 30 μM corymine were 328 and 582 μM, respectively, whereas the Hill coefficients in the presence of 10 and 30 μM corymine were 1.57 and 1.14, respectively. On the other hand, strychnine (0.1 μM) caused a rightward shift of the glycine concentration–response curve without decreasing the maximal glycine response (the ED_{50} value of glycine and the Hill coefficients were 516 μM and 1.86, respectively) (Fig. 1B). DIDS (30 μM) decreased the maximal glycine response by 10% without shifting the glycine concentration–response curve (the ED_{50} value of glycine and Hill coefficient were 391 μM and 1.44, respectively) (Fig. 1C).

3.2. Co-application of corymine with strychnine and DIDS

To further elucidate the mechanism underlying the action of corymine, interactions between corymine and strychnine and between corymine and DIDS, were examined in terms of glycine-induced Cl^- current. Strychnine (0.001–1 μM) and DIDS (1–1000 μM) inhibited the glycine current in a dose-dependent manner. The degree of inhibition caused by these drugs was dose-dependently increased by the co-application with corymine (10 and 30 μM) (Fig. 2). The ED_{50} values of strychnine without and with 10 and 30 μM corymine were 39.0, 22.6 and 12.0 nM, respectively, while the ED_{50} values of DIDS without and with 10 and 30 μM corymine were 39.0, 35.1 and 36.9 μM, respectively.

When applied alone, corymine (30 μM), strychnine (0.1 μM) and DIDS (30 μM) inhibited the glycine current by 70 ± 2.4, 65 ± 6.4 and 49 ± 8.5% (mean ± S.E.M., $n = 4$), respectively, while when two of these compounds (i.e, corymine + strychnine, corymine + DIDS, and strychnine + DIDS) were coapplied, the inhibition of glycine current significantly increased (i.e, 90 ± 1.7, 80 ± 2.7 and 88 ± 1.7% inhibition (mean ± S.E.M., $n = 4$), respectively).

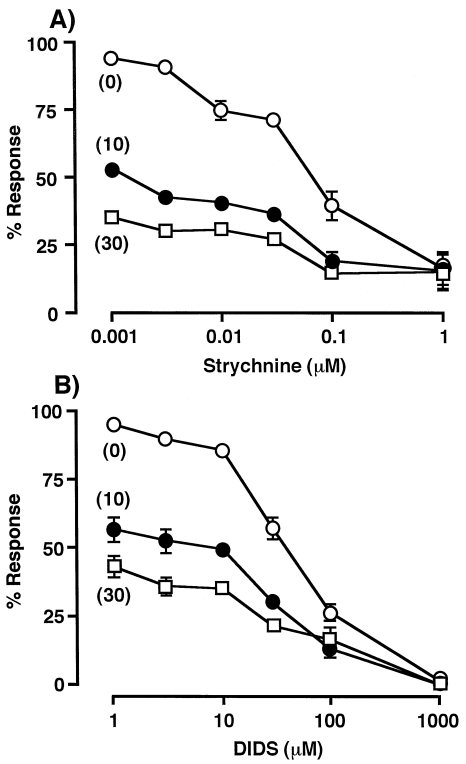

Fig. 2. Effect of corymine on strychnine and DIDS suppression of glycine-induced Cl^- current in *Xenopus* oocytes injected with rat spinal cord poly(A)$^+$ mRNA. Strychnine (A: 0.001–1 μM)- and DIDS (B: 1–1000 μM)-induced suppression of Cl^- current caused by 300 μM glycine was measured in the presence and absence of corymine. Cl^- current caused by 300 μM glycine alone was taken as 100% control response. Data are expressed as percentages of control glycine response. Each point represents the mean ± S.E.M. obtained from 5–8 oocytes (A) or 3–6 oocytes (B). The number in parentheses represents the concentration of corymine added (10 (●) and 30 (□) μM).

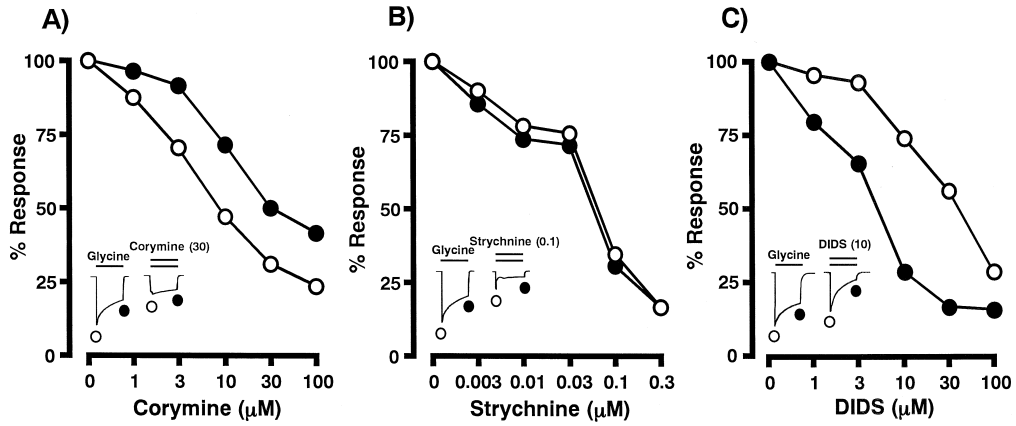

Fig. 3. Effects of corymine, strychnine and DIDS on the desensitization phase of glycine-induced Cl^- current in *Xenopus* oocytes injected with rat spinal cord poly(A)$^+$ mRNA. Glycine (300 μM) was applied to oocytes for 1 min with and without 1–100 μM corymine (A), 0.003–0.3 μM strychnine (B), or 1–100 μM DIDS (C). The peak amplitude (○) and the end current (●) caused by glycine alone were taken as 100%, respectively. Data are expressed as the percentage relative to the control peak amplitude and the control end current (see bars in inset).

3.3. Profile of corymine suppression of glycine current differs from those of strychnine and DIDS

As shown in Fig. 3, the Cl^- current caused by application of 300 μM glycine reached a peak and then slowly declined during a 1-min application period. Coapplication of glycine with corymine, strychnine and DIDS exhibited different desensitization profiles in Cl^- currents. We analyzed the effects of these compounds on the desensitization of glycine-induced Cl^- current using two parameters; peak amplitude and end current at 1 min of glycine response. Corymine (1–100 μM) caused a dose-dependent decrease of the peak amplitude and the end current, but its suppressive effect on the peak amplitude was more potent than the effect on the end current. The IC_{50} value of corymine to inhibit the peak and end current were 12 and 43 μM,

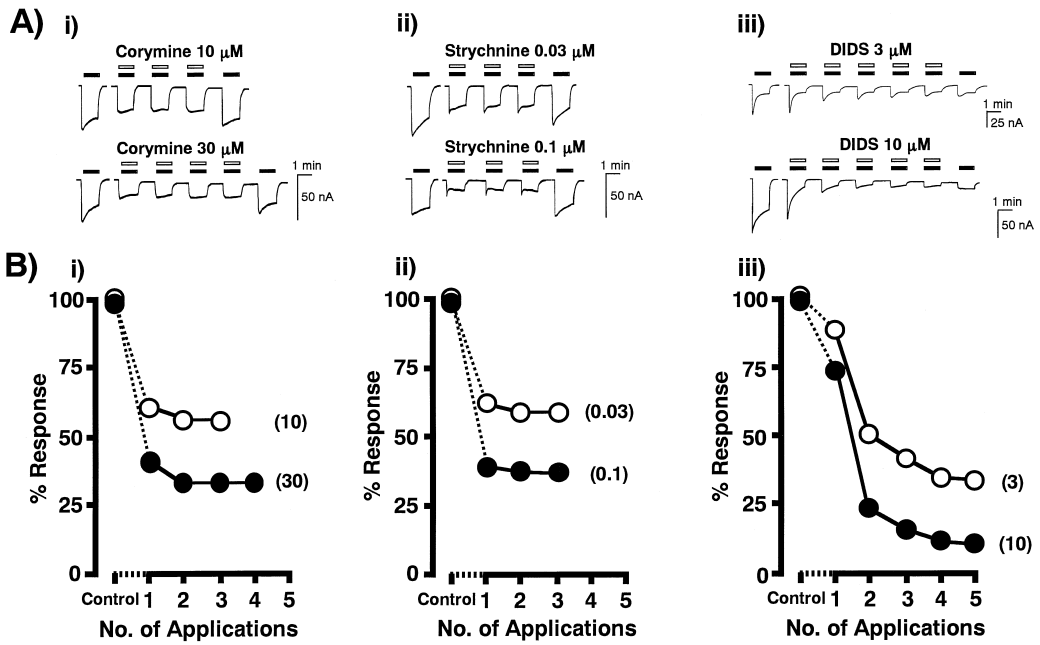

Fig. 4. Effects of repeated application of corymine, strychnine and DIDS on glycine–induced Cl^- current in *Xenopus* oocytes injected with rat spinal cord poly(A)$^+$ mRNA. Glycine (300 μM) was repeatedly applied to oocytes with and without corymine (i:10 and 30 μM), strychnine (ii: 0.03, 0.1 μM) or DIDS (iii: 3 and 10 μM) for 1 min at 2-min intervals. (A) The last current caused by glycine alone shows the recovery of the glycine response. (B) Cl^- current caused by the first application of glycine alone was defined as the control glycine response and expressed as 100%. Percentages of responses relative to the control response were plotted against the number of repeated applications of antagonists. Numbers in parentheses represent concentrations of each antagonist tested.

respectively. Strychnine (0.003–0.3 μM) and DIDS (1–100 μM) also dose-dependently decreased the peak and end current. However, strychnine suppressed the peak amplitude to the same extent as the end current (the IC_{50} values for the peak amplitude and end current were 0.05 and 0.04 μM, respectively). The effect of DIDS on the end current was more potent than the effect on the peak amplitude (the IC_{50} values for the peak amplitude and end current were 37 and 5 μM, respectively).

3.4. The blockage of glycine current by corymine is not use-dependent

We examined the effect of repeated application of corymine on glycine response to test whether corymine inhibition of glycine current is use-dependent. Glycine (300 μM) was repeatedly applied with and without corymine (10 and 30 μM), strychnine (0.03 and 0.1 μM) or DIDS (3 and 10 μM) for 1 min (Fig. 4). The inhibitory activities of corymine and strychnine on glycine currents were not changed by repeated application, while the effect of DIDS was gradually potentiated by repeated application. The inhibitory effect of DIDS was long-lasting, whereas the effects of corymine and strychnine were reversible and rapidly disappeared after washout.

3.5. Antagonism by corymine of GABA-, acetylcholine- and serotonin (5-HT)-induced currents in Xenopus oocytes

To examine the specificity of corymine inhibition of glycine current, we tested the effects of corymine on GABA-, acetylcholine- and 5-HT-induced currents in oocytes injected with rat brain poly(A)$^+$ mRNA. GABA-, acetylcholine- and 5-HT-induced inward current when applied to oocytes clamped at -60 mV (Fig. 5). The ED_{50} values of GABA, acetylcholine and 5-HT were about 20, 5 and 0.03 μM, respectively. Corymine, at 30 and 100 μM, dose-dependently reduced 30 μM GABA-induced current by 14 ± 3.9 (mean $\pm$ S.E.M., $n = 3$) and $31 \pm 0.6\%$ ($n = 3$), respectively. In contrast, corymine had no effect on 10 μM acetylcholine- and 0.1 μM 5-HT-induced currents (decreases of acetylcholine- and 5-HT-induced currents by corymine, 100 μM, were $5 \pm 0.5\%$ (mean $\pm$ S.E.M., $n = 5$) and $3 \pm 1.4\%$ ($n = 5$), respectively).

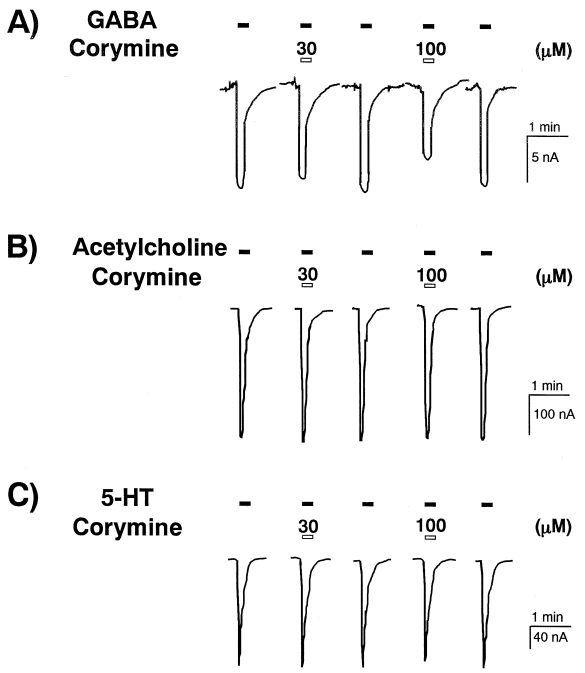

Fig. 5. Effect of corymine on GABA-, acetylcholine- and serotonin (5-HT)-induced Cl^- currents in *Xenopus* oocytes injected with rat brain poly(A)$^+$ mRNA. GABA (A: 30 μM), acetylcholine (B: 10 μM) or 5-HT (C: 0.1 μM) was applied to oocytes with and without corymine (30 or 100 μM).

4. Discussion

The present results demonstrated that corymine acts as a noncompetitive glycine receptor antagonist in *Xenopus* oocytes injected with rat spinal cord RNA and that the mechanism underlying the inhibitory activity of corymine on glycine-induced Cl^- current differs from those of strychnine and DIDS.

Strychnine, a competitive glycine receptor antagonist (Curtis et al., 1971; Langosch et al., 1990; Young and Snyder, 1974), caused a parallel shift of the glycine concentration–response curve to the right without affecting the maximal glycine response, while DIDS, a Cl^- channel blocker (Grinstein et al., 1979; Jennings, 1985), suppressed the glycine current by decreasing the maximal glycine response. These pharmacological profiles of strychnine and DIDS observed in this study are consistent with those reported using cultured or dissociated neurons (Shirasaki et al., 1991; Takahama et al., 1997). Corymine dose-dependently decreased the maximal glycine concentration–response curve, although slight rightward-shift of the curve was observed at a high concentration (30 μM). This finding indicated that corymine inhibited the glycine currents in a noncompetitive fashion (Gaddum, 1957) and raises the possibility that corymine and DIDS may bind the same site at the Cl^- channel of the glycine receptor. However, this possibility seems slight, since the competition experiments showed that corymine failed to change the ED_{50} of DIDS and that the inhibitory effects of corymine and DIDS were additive. Moreover, corymine produced 2–3-fold decrease in the ED_{50} of strychnine and the inhibitory effects of corymine and strychnine were additive. This suggests that the binding of corymine enhance the inhibitory action of strychnine.

When either corymine, strychnine or DIDS was repeatedly applied to oocytes with glycine, the inhibitory effects of corymine and strychnine did not change, while that of DIDS increased gradually. These findings suggest that DIDS-induced, but not corymine- or strychnine-induced,

inhibition of glycine current was use-dependent (Courtney, 1975). It has been reported that use-dependence is one of the characteristics of open channel blockers that require the open state of a channel to inhibit the channel function (Yoon et al., 1993). In addition, a previous report demonstrated that DIDS acted as an open-channel blocker of the cardiac Cl^- channel (Duan and Nattel, 1994). Taken together, it is likely that corymine inhibition is not dependent on an open state of a glycine-gated Cl^- channel.

Consistent with previous reports (Agopyan et al., 1993; Akaike and Kaneda, 1989; Krishtal et al., 1988; Kumamoto and Murata, 1996; Lewis et al., 1991; Walstrom and Hess, 1994), the desensitization phase of glycine currents in *Xenopus* oocyte was fitted with a double-exponential curve which consisted of the fast and slow desensitization components. Van Renterghem et al. (1987) reported that the decrease of the peak glycine current amplitude by an antagonist is associated with the abolition of the fast component. In this study, the inhibitory potency of strychnine on the peak and end currents was almost the same, whereas DIDS more selectively suppressed the end current than it did the peak amplitude. In contrast, the inhibitory effect of corymine on the peak amplitude was more potent than the effect on the end current. These findings indicate different inhibitory profiles of these compounds on the desensitization process of glycine currents and suggest that the fast component of the desensitization process of glycine currents can be more selectively inhibited by corymine than the slow component. These results were partially consistent with the report of Walstrom and Hess (1994) that strychnine affected both the fast and slow components of the desensitization process of glycine current in cultured embryonic mouse spinal cord cells. Schmieden et al. (1989) and Akagi and Miledi (1988) reported that the desensitization rate of glycine receptor depended on the composition of glycine receptor subunits. Thus, it is possible that the different activities of corymine, strychnine and DIDS on the desensitization process of glycine current may be due to the different selectivity of these antagonists for each glycine receptor subunit.

In the present study, corymine also exhibited suppressive activity against GABA-induced Cl^- currents, but it had no effect on 5-HT- or acetylcholine-induced Cl^- currents. Acetylcholine- and 5-HT receptors are known to couple with calcium-dependent Cl^- channels in *Xenopus* oocytes injected with rat brain mRNA (Gundersen et al., 1983; Kaneko et al., 1992; Lübbert et al., 1987; Sakai et al., 1986; Tohda et al., 1989), whereas glycine- and $GABA_A$ receptors form a Cl^- channel inside the receptors (Conley, 1996). This suggests that corymine selectively inhibits the integral Cl^- channel formed by receptor protein complex. Moreover, even though $GABA_A$ receptor has been demonstrated to have a similar molecular structure (Grenningloh et al., 1987; Schofield et al., 1987) and conductance properties (Bormann et al., 1987; Hamill et al., 1983) to the glycine receptor, corymine inhibited glycine current more potently than it did GABA current. The data, taken together, suggest that corymine specifically blocked the glycine-gated Cl^- channels.

It is of interest to note that corymine is a specific Cl^- channel blocker of the glycine receptor and that the inhibitory effect of this alkaloid on the Cl^- channel is not use-dependent, because most channel blockers are thought to suppress channel function by binding to the site within the lumen of an open channel and exert use-dependent inhibition (Inoue and Akaike, 1988; Nagata and Narahashi, 1994; Rundstrom et al., 1994; Yoon et al., 1993). Thus, corymine may be a useful pharmacological tool to investigate the interaction of ligand binding sites at the glycine receptor-Cl^- channel complex.

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