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Open Channel Block of NMDA Receptors by Conformationally Restricted Analogs of Milnacipran and Their Protective Effect Against NMDA-Induced Neurotoxicity

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ABSTRACT We investigated the blocking effect of the conformationally restricted analogs of milnacipran on NMDA receptors by recording the whole-cell currents of *Xenopus* oocytes injected with rat brain mRNA and the single channel currents of cultured hippocampal neurons under voltage-clamp conditions. Their protective effect against excitotoxicity was also investigated on cultured cortex neurons. All conformationally restricted analogs examined blocked activated NMDA receptors, though their structures were quite different from known NMDA receptor blockers. The analogs with a (1S, 2R, 1′S)-configuration such as PPDC ((1S, 2R)-1-phenyl-2[(S)-1-aminopropyl]-N,N-diethylcyclopropanecarboxamide) had lower IC_{50} values than those with other configurations. The empirical Hill coefficients for each compound were close to unity, indicating a 1:1 stoichiometry for the block. PPDC decreased the maximum responses to both N-methyl D-aspartate (NMDA) and glycine without altering their dissociation constants. The blocking effect was enhanced on hyperpolarization. PPDC had no effects on other glutamate receptor subtypes (AMPA, kainate, and metabotropic glutamate receptors) or other neurotransmitter receptors ($GABA_A$, $5HT_{2C}$, and ACh_{M1} receptors) produced by the oocytes. PPDC decreased the mean open time of NMDA receptors without decreasing their elementary conductance. The microscopic blocking rate constant was 2.8×10^7 $M^{-1}s^{-1}$. The macroscopic unblocking rate constant of PPDC was much faster than that of MK-801. Only the analogs with the (1S, 2R, 1′S)-configuration protected the cultures against NMDA-induced neurotoxicity, though they failed to protect against kainate-induced neurotoxicity. These results show that conformationally restricted analogs, at least PPDC, selectively blocked open channels of NMDA receptors. **Synapse 31:87–96, 1999.**

INTRODUCTION

Glutamate has been considered a principal excitatory neurotransmitter which stimulates ionotropic and metabotropic receptors. NMDA receptors named after their selective agonist N-methyl D-aspartate (NMDA) are ionotropic receptor subtypes of glutamate receptors, and are critical in various physiological responses such as the formation of memory (Bear, 1996; Hirsch et al., 1997) and the development of neural systems (Scheetz and Constantine, 1994). However, the excess activation of NMDA receptors causes a wide variety of neurological insults (Choi, 1988).

There are potent blockers against NMDA receptors, such as MK-801 (Ford et al., 1989; Foster et al., 1988; Olney et al., 1987) and memantine (Erdo and Schafer, 1991; Keilhoff and Wolf, 1992). MK-801 leaves the channel only very slowly (Huettner and Bean, 1988) and memantine has fast kinetics of interaction with the NMDA receptors (Muller et al., 1995; Parsons et al., 1995, 1993). Separate genes encode the subunits of NMDA receptors and several combinations of subunits comprising an individual NMDA receptor have a unique pharmacology of antagonism (Bresink et al., 1995; Seeburg, 1993). Although several potent NMDA recep-

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tor blockers have been synthesized, there is the potential for developing different types of blockers against NMDA receptors with different pharmacologies.

Milnacipran ((±)-(Z)-2-(aminomethyl)-1-phenyl-N,N-diethylcyclopropanecarboxamide), a potent inhibitor against the uptake of serotonin (5HT) (Bonnaud et al., 1987; Moret, et al., 1985), was found to suppress the binding of MK-801 to NMDA receptors with an IC_{50} value of ~6 μM (Shuto et al., 1995). Since the structure of milnacipran is quite different from known NMDA blockers such as MK-801 or memantine, as Figure 1 shows, this characteristic structure was considered in the search for novel potent blockers against NMDA receptors. Among the synthesized conformationally restricted analogs of milnacipran (Fig. 1), a 1′-ethyl derivative, PPDC (2b), blocked NMDA receptors but had negligible effects on 5HT uptake (Shuto et al., 1996a,b). PPDC thus appears to be a new pharmacological tool or drag against NMDA receptors. However, its mechanism of blockade remains to be investigated.

Xenopus oocytes injected with mRNA extracted from rat total brain produce functional neurotransmitter receptors of both ionotropic and metabotropic types. Since several metabotropic neurotransmitter receptors, including metabotropic glutamate receptors expressed by the oocytes, are coupled with Ca-activated Cl channels, it is easy to investigate them, as well as ionotropic neurotransmitter receptors, under voltage-clamp conditions. Therefore, in the present experiments we chose the expression system of *Xenopus* oocytes to investigate the effect of PPDC and other conformationally restricted analogs (Fig. 1) on ionotropic and metabotropic glutamate receptors, and on other neurotransmitter receptors such as $GABA_A$, $5HT_{2C}$, and ACh_{M1}. In addition to the macroscopic blocking effect of the conformationally restricted analogs, we investigated the microscopic kinetics of the action of PPDC by recording the elementary currents of NMDA receptors of cultured mouse hippocampal neurons. The protective effect of the conformationally restricted analogs against neurotoxicity induced by NMDA and kainate was also investigated on cultured mouse cortex neurons.

We show that PPDC is a potent and selective open channel blocker against NMDA receptors. We also show that PPDC and the other conformationally restricted analogs with the (1*S*, 2*R*, 1′*S*)-configuration protect cultured hippocampal neurons against NMDA-induced neuronal cell death. Preliminary results were published in abstract form (Yoshii et al., 1996).

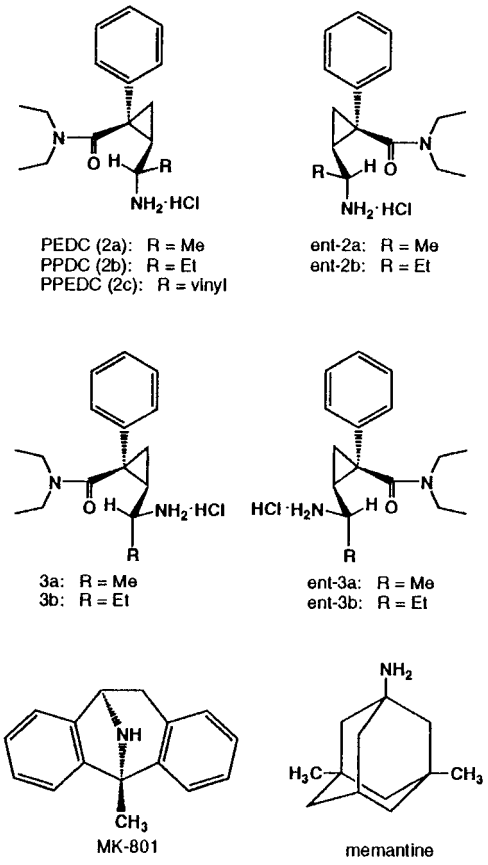

Fig. 1. Structures of conformationally restricted analogs of milnacipran, MK-801, and memantine.

MATERIALS AND METHODS

Whole cell currents from *Xenopus* oocytes

Xenopus oocytes injected with rat total brain mRNA were investigated under two-electrode voltage-clamp conditions as described previously (Kawano et al., 1994; Yoshii and Kurihara, 1989; Yoshii et al., 1987). Briefly, oocytes at stage V and IV (Dumont, 1972) were obtained from anesthetized *Xenopus* and follicle cells surrounding oocytes were removed with collagenase. Rat total brain mRNA was extracted from 10-day-old Wister rats by the guanidine/CsCl method. Each oocyte was injected with 50 μg mRNA and incubated for 2–3 days in Barth's medium. Electrodes (~1 MΩ) were filled with 3 M KCl and the ground electrode was a salt bridge. Oocytes were perfused with a bathing solution (in mM: 96 NaCl, 2 KCl, 1 $CaCl_2$, 10 HEPES/NaOH, pH 7.4) and NMDA, glycine, and conformationally restricted analogs were dissolved in this bathing solution to apply to oocytes. Stimulating NMDA solutions contained 10 μM glycine unless otherwise noted. The

Abbreviations

NMDA	N-methyl D-aspartate
PPDC	(1S, 2R)-1-phenyl-2[(S)-1-aminopropyl]-N,N-diethylcyclopropanecarboxamide
Milnacipran	(±)-(Z)-2-(aminomethyl)-1-phenyl-N,N-diethylcyclopropanecarboxamide
PEDC	(1S, 2R)-1-phenyl-2[(S)-1-aminoethyl]-N,N-diethylcyclopropanecarboxamide
PPEDC	(1S, 2R)-1-phenyl-2[(S)-1-aminovinyl]-N,N-diethylcyclopropanecarboxamide

NMDA-induced currents consisted of a transient current and a following steady state current. In the present experiments, we measured the magnitude of the steady state current as the magnitude of responses at a holding potential of −50 mV unless otherwise noted.

Cell culture

The cultures of mouse cerebral cortex neurons including hippocampal neurons were prepared as described previously (Furue et al., 1997). In brief, hippocampus or cerebral cortex removed from a mouse fetus (16 days gestation) was minced, incubated with trypsin for 15 min at 37°C, then dissociated by trituration. The isolated hippocampal neurons were plated on cover slips (1.8×10^4 cell/cm^2) for elementary current recordings or plated on 24-well plates (1.6×10^5 cell/cm^2) for investigating the protective effect of the conformationally restricted analogs. These isolated neurons were plated in the presence of Eagle's MEM supplemented with glucose bicarbonate, 5% heat-inactivated newborn bovine, and 5% horse serum. One day after plating, the Eagle's medium was replaced by B18 medium, a serum-free medium (Brewer and Cotman, 1989). Then the culture was fed with fresh B18 every 3 days for 10–14 days before making elementary current recordings and for 10 days before exposure to either NMDA or kainate.

Elementary current recordings

The method of Hamill et al. (1981) was used to record elementary currents of NMDA receptors from outside-out patches excised from the cell bodies of cultured hippocampal neurons at room temperature. In brief, a Sylgard-coated and fire-polished glass pipette was filled with an electrode solution (in mM: 150 CsCl, 1 EGTA-Cs, 10 HEPES/CsOH, pH 7.4). The electrode input resistance ranged from 10–20 MΩ. The pipettes were applied to the cell body of cultured neuronal cells to form a giga-ohm seal in a bathing solution (in mM: 145 NaCl, 5 KCl, 2.4 $CaCl_2$, 10 glucose, 10 HEPES/NaOH, pH 7.4). After breaking the patch, we pulled the recording electrode very slowly away from the cell to obtain an outside-out configuration. In the absence and presence of 3 μM PPDC, we applied 100 μM NMDA (no added glycine) dissolved in the bathing solution to the outside-out patch voltage-clamped at −100 mV. Elementary currents were digitized at 20 kHz with an A/D board (Digidata 1200B; Axon Instruments, Inc., Foster City, CA) and then stored in a computer. The elementary currents recorded at least 10 sec after the onset of stimulation with NMDA were analyzed with pCLAMP v. 6.03 software (Axon Instruments). The elementary currents with open times shorter than 0.1 msec were ignored.

Neuronal cell death

The cultures of cerebral cortex neurons were exposed to NMDA or other agonists for glutamate receptors in the absence or presence of the conformationally restricted analogs. Their protective effect against excitotoxicity was estimated by monitoring lactate dehydrogenase (LDH) efflux from damaged neurons during the 24-h exposure (Koh and Choi, 1987). Unless otherwise noted, the mean and SD of LDH efflux were obtained from at least three separate experiments, each of which had three replicate culture plates. The cultured neurons released LDH through the exposure protocol even in the absence of the glutamate agonists. This background LDH efflux was determined on sister cultures within each experiment and used as a control. The mean ± SD of the background LDH activity (IU/l) was 90.8 ± 18.4 (23 separate experiments, each of which had three replicate culture plates).

RESULTS

Suppression curves

The oocytes injected with rat total brain mRNA elicited inward currents in response to the control-stimulating solution (30 μM NMDA supplemented with 10 μM glycine) and the coapplication of PPDC suppressed the responses (inset of Fig. 2). The other conformationally restricted milnacipran analogs (Fig. 1) also suppressed NMDA receptors when they were applied to activated NMDA receptors.

We compared the blocking effect of these compounds (Fig. 1) to the NMDA receptors. As Figure 2 shows, the compounds with a (1*S*, 2*R*, 1′*S*)-configuration such as PPDC, PPEDC (2c), and PEDC (2a) decrease the receptor responses to the control-stimulating solution as concentration increased. In contrast, the compounds with (1*R*, 2*S*, 1′*R*)-, (1*S*, 2*R*, 1′*R*)-, or (1*R*, 2*S*, 1*S*)-configurations were less effective. The responses in the presence of these compounds were well fitted with an equation assuming uncompetitive block by a nonlinear least squares method:

$$R = 1 - [1/(1 + (IC_{50}/[Mil])^n)], \quad (1)$$

where R is the relative response (the responses in the presence of a compound shown in Fig. 1 at a given test concentration of the compound [Mil] calculated relative to those in the absence of the compound). IC_{50} is the concentration of the compound at which the response is reduced to 50%, and n is the empirical Hill coefficient.

As Table I summarizes, the empirical Hill coefficients for each compound are close to unity. The IC_{50} values of the conformationally restricted analogs with a (1*S*, 2*R*, 1′*S*)-configuration ranged from 1.69 μM to 4.75 μM, which is lower than those for the analogs with the other configurations. These results indicate that a (1*S*, 2*R*, 1′*S*)-configuration is important in the blocking action. These IC_{50} values are similar to those obtained from the

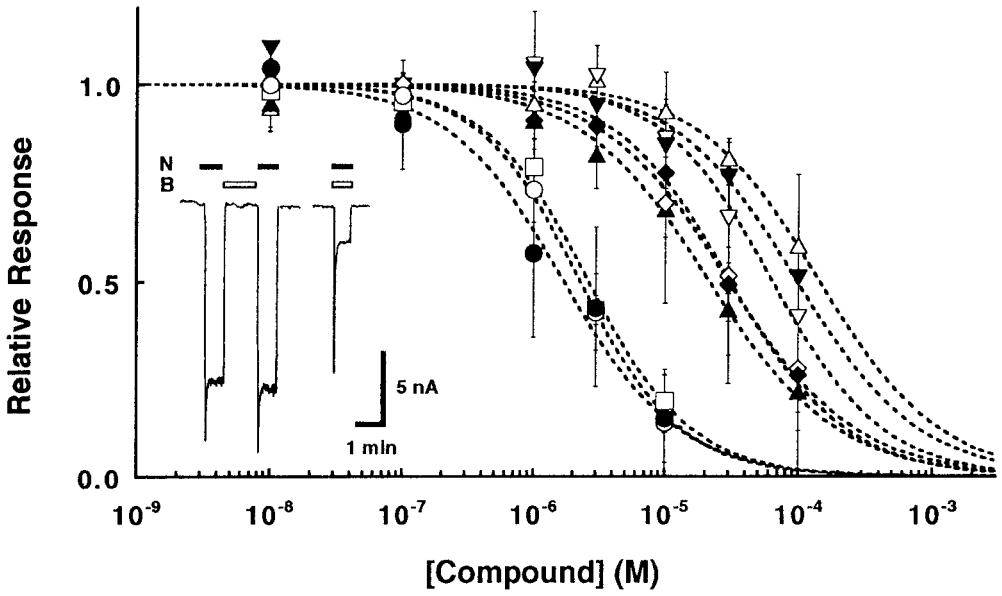

Fig. 2. Suppression curves of conformationally restricted analogs with different stereochemistry for NMDA responses. Whole cell currents evoked by 30 μM NMDA supplemented with 10 μM glycine were recorded in the absence and presence of various concentrations of the compounds. The ratio of residual current was plotted as a function of the concentration of these compounds. PPDC (●), PEDC (○), PPEDC (□), ent-2a (◇), ent-2b (◆) 3a (▽), 3b (▼), ent-3a (△) ent-3b (▲). Inset: the residual currents where horizontal bars show the application of the agonists (N) and 3 μM PPDC (B). Holding potential, −50 mV. In this and following figures, each point represents the mean ± SD obtained from five oocytes unless otherwise noted.

TABLE I. IC_{50}s and Hill coefficients of conformationally restricted analogs of milnacipran

Conformationally restricted analogs	IC_{50} (μM)	Hill coefficient	Correlation coefficient
PPDC (2b)	1.69	0.92	0.99
PEDC (2a)	2.28	1.11	0.99
PPEDC (2c)	2.63	1.09	0.99
ent-2b	31.16	0.99	0.99
ent-2a	30.25	0.90	0.99
3b	98.94	0.94	0.97
3a	64.74	1.04	0.98
ent-3b	21.64	0.89	0.99
ent-3a	145.38	0.95	0.97

These parameters were obtained from suppression curves shown in Figure 2.

experiments on the inhibition of [^{3}H]MK-801 binding in rat synaptic membranes (Shuto et al., 1996b). In the following experiments, we investigated the mode of action of PPDC, because of its potent blocking effect on NMDA receptors and its negligible effect on 5HT uptake.

Selective suppression of NMDA responses

In addition to NMDA receptors, mRNA-injected *Xenopus* oocytes functionally expressed other subtypes of glutamate receptors (AMPA, kainate, and metabotropic glutamate receptors) and other neurotransmitter receptors. We investigated the effect of PPDC on these neurotransmitter receptors. Although 10 μM PPDC significantly blocked the NMDA receptors (two-tailed *t*-test, $P < 0.05$), it neither blocked other glutamate receptor subtypes (kainate, AMPA, and metabotropic glutamate receptors) nor other neurotransmitter receptors ($GABA_A$, ACh_{M1}, and $5HT_{2C}$ receptors) (Fig. 3, Table II). These results show that PPDC is a selective NMDA blocker.

Uncompetitive block on NMDA and glycine sites

Since NMDA receptors require both NMDA and glycine for activation, the conformationally restricted analogs may compete with these agonists. Alternatively, these compounds may uncompetitively block NMDA receptors. In order to elucidate the mode of action of these compounds, we investigated the effect of PPDC on the concentration–response curves for NMDA and glycine.

The concentration–response curve for NMDA in the presence of a fixed concentration of glycine (10 μM) approached a saturation level at ~300 μM (Fig. 4A). The concentration–response curve for glycine with a fixed concentration of NMDA (30 μM) reached a saturation level at ~1 μM (Fig. 4B). PPDC decreased these saturation levels without shifting the respective concentration–response curves. These results indicate that PPDC blocks NMDA receptors without competing with NMDA or glycine.

In order to test this conclusion, we compared the concentration–response curves shown in Figure 4 with an equation that assumes the uncompetitive block of

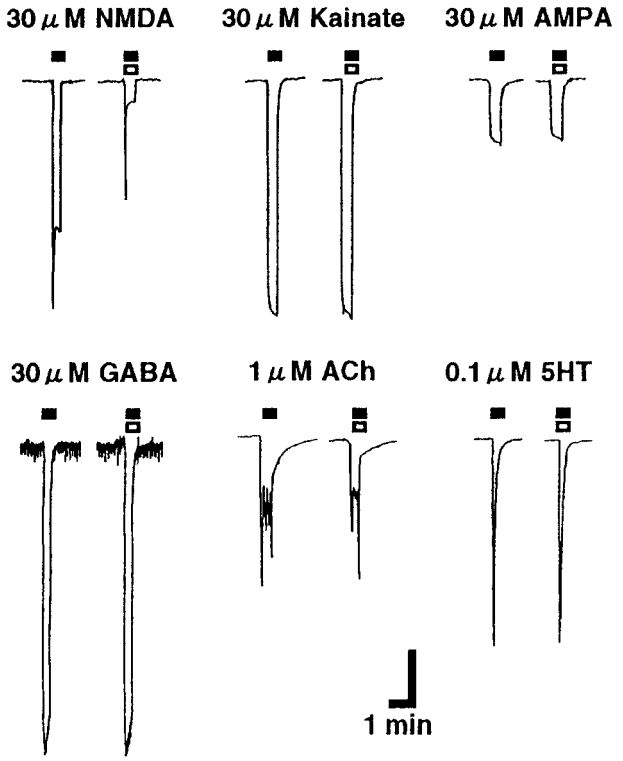

Fig. 3. Responses of various neurotransmitter receptors to their respective agonists in the absence and presence of 10 μM PPDC. The meanings of horizontal bars are the same as in Figure 2. Scale: 100 nA for Ach and 5HT and 10 nA for the others.

TABLE II. Effect of PPDC on neurotransmitter receptors

NMDA	14.7 ± 8.07*
Kainate	99.7 ± 1.84
AMPA	100.0 ± 1.20
$GABA_A$	97.9 ± 9.18
ACh_{M1}	76.2 ± 15.8
$5HT_{2C}$	94.9 ± 14.1

Numerals are the mean ± SD of residual responses to neurotransmitters obtained from six oocytes where the respective control responses are taken as 100.
*The blocking effect is significant (two-tailed *t*-test, $P < 0.05$).

NMDA receptors:

$$R = \frac{\left(\frac{K_d}{[A_{control}]}\right)^n + 1}{\left(\frac{K_d}{[A]}\right)^n + \frac{[PPDC]}{K_{PPDC}} + 1} \qquad (2)$$

where R is the ratio of the response to the test solution to the response to the control-stimulating solution, $[A_{control}]$ and [A] are the concentration of the agonist to be investigated in the control and test stimuli (NMDA in Fig. 4A and glycine in Fig. 4B), K_d and K_{PPDC} are the dissociation constants for agonists (NMDA in Fig. 4A and glycine in Fig. 4B) and PPDC, respectively, [PPDC] is the fixed concentration of PPDC, 3 μM, and n is the empirical Hill coefficient. The Hill coefficient for PPDC is taken as unity based on the analysis of the suppression curve shown in Figure 2.

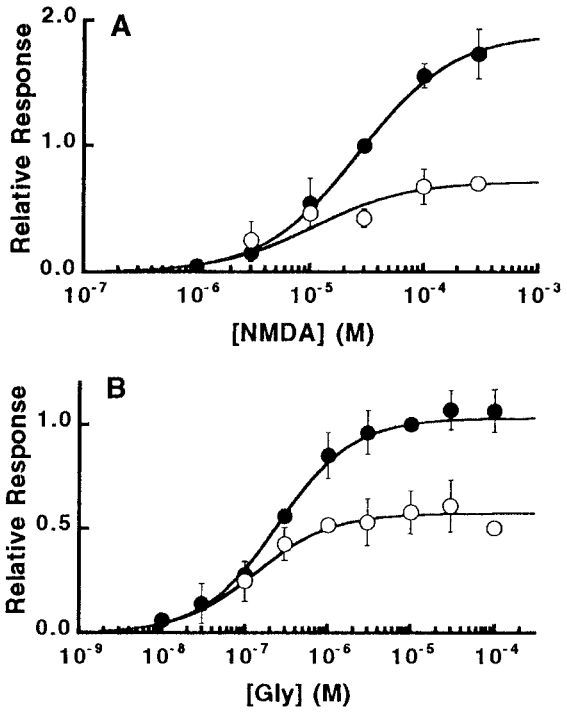

Fig. 4. Concentration–response curves for NMDA in the presence of 10 μM glycine (**A**) and those for glycine in the presence of 30 μM NMDA (**B**) in the absence (●) and presence of 10 μM PPDC (○). Holding potential, −50 mV. Plotted points of data are calculated relative to whole cell currents evoked by 30 μM NMDA supplemented with 10 μM glycine in the absence of PPDC. Lines are drawn by a nonlinear least-square method by Eq. 2.

As Figure 4 shows, the concentration curves for NMDA and glycine are well described by Eq. 2 using the nonlinear least squares method. This good fit supports the above conclusion that the mode of action is uncompetitive. The estimated dissociation constants were 1.81 μM in the presence of 10 μM glycine and 3.72 μM in the presence of 30 μM NMDA, and comparable to each other. The estimated dissociation constants for NMDA and glycine were 27.0 μM and 0.25 μM, respectively. The empirical Hill coefficients were 1.04 for NMDA and 0.96 for glycine.

Voltage-dependent block

The slope of a current–voltage curve is a measure of the voltage dependence of channel openings. In the absence of Mg^{2+}, the current–voltage curve of NMDA receptors produced by *Xenopus* oocytes was almost linear in the range of examined membrane potentials. However, it became nonlinear in the presence of 3 and 10 μM PPDC; the blocking effect of PPDC was enhanced on hyperpolarization and a negative slope appeared on

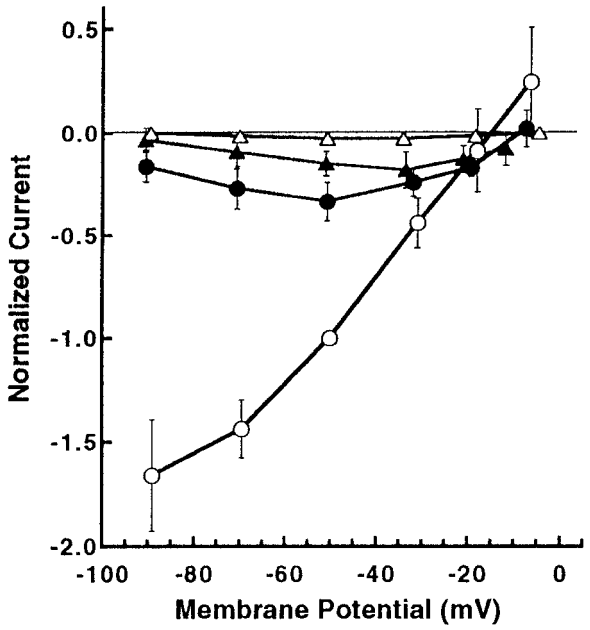

Fig. 5. Current–voltage relations in the absence and presence of PPDC and MK-801. Oocytes were voltage-clamped at different potentials and then stimulated with 30 μM NMDA supplemented with 10 μM glycine in the absence (○) and presence of 3 μM PPDC (●), 10 μM PPDC (▲), and 1 μM MK-801 (△). Plotted are steady-state voltage-clamp currents calculated relative to the currents evoked by the control at −50 mV. Plotted points represent the mean ± SD obtained from five oocytes for the control and PPDC and two for MK-801.

each current–voltage curve (Fig. 5). These results suggest that the dissociation constant for PPDC is decreased on hyperpolarization. MK-801, a typical open channel blocker (Foster and Wong, 1987; Huettner and Bean, 1988; Wong et al., 1986) yielded a similar current–voltage curve, though its blocking effect was more remarkable (Fig. 5).

Block of elementary currents

PPDC was also effective at blocking elementary currents evoked by NMDA in outside-out patches voltage-clamped at −100 mV (Figs. 6, 7). Since the analysis of the channel mechanism becomes difficult when a patch contains multiple channels, we first tried to record from patches that had only one NMDA receptor. However, we found that the open probability of NMDA receptors was lower than 0.007 (Fig. 6). Since the lifetime of a patch is limited, we recorded from patches containing several NMDA receptors (Fig. 7) in addition to the recordings from patches having a few NMDA receptors (Fig. 6), and analyzed only the parts of records that had no overlapping of elementary currents.

Figure 6 shows a typical record obtained from a patch containing a few NMDA receptors; although it appears to contain only one NMDA receptor, ironically, the only one overlapping an opening occurred in a 68-sec recording in the presence of 3 μM PPDC, but not in an 89-sec recording before the application of 3 μM PPDC. PPDC decreased the number of events in concentration-dependent manner, which was recovered, though not completely, by the exposure to 100 μM NMDA in the absence of PPDC (Fig. 6A). As amplitude histograms (Figs. 6B,C) show, 3 μM PPDC decreased the number of events without decreasing the elementary currents of ~4 pA, indicating PPDC has no effect on the elementary conductance of NMDA receptors. PPDC (3 μM) decreased the mean open times obtained from patches with a few NMDA receptors from 3.0 ms to 2.5 ms (Fig. 6) and from 2.8 ms to 2.3 ms. Similar results were obtained from a patch containing several NMDA receptors; 3 μM PPDC decreased the mean open time from 3.1 ms to 2.4 ms without decreasing elementary currents of ~4 pA.

The difference between these mean open times in the absence and presence of PPDC was significant (one-tailed paired *t*-test, $P < 0.01$). Thus, PPDC was found to decrease the mean open time of NMDA receptors by 0.6 ± 0.1 ms (mean ± SD, n = 3).

Protective effects

Since the conformationally restricted analogs blocked NMDA receptors, as described above, we investigated their protective effect on cerebral neuronal cells in culture against the neurotoxicity induced by 300 μM NMDA supplemented with 10 μM glycine. As the following results (Fig. 9A) show, 300 μM NMDA with 10 μM glycine elicited maximum neurotoxicity. Among examined compounds at 10 μM, PPDC, PEDC, and PPEDC significantly protected cultures against the neurotoxicity (one-way ANOVA and Scheffe's multiple comparison, $P < 0.01$). All these compounds had a (1*S*, 2*R*, 1′*S*)-configuration (Fig. 8A). Examined compounds (10 μM) alone had no neurotoxic effects (Fig. 8B). On the other hand, PPDC had no protective effect against kainate-induced neurotoxicity (two-tailed *t*-test, $P > 0.05$): The mean (±SD) of relative LDH efflux induced by 1 mM kainate was 2.05 ± 0.65 without PPDC and 2.20 ± 0.68 with 10 μM PPDC, where the background LDH efflux was taken as 1.0. These results indicate that the protective effect results from the blocking of NMDA receptors, since PPDC did not block kainate receptor subtypes, as Figure 3 and Table II show.

The neurotoxicity of NMDA was increased with increasing concentration in the presence of a fixed concentration of glycine (10 μM) and reached a maximum level at 300 μM NMDA (Fig. 9A). PPDC (10 μM) significantly decreased the maximum LDH releases (two-way ANOVA, Scheffe's multiple comparison, $P < 0.01$). The protective effect increased with increasing PPDC concentration, with an IC_{50} value of 4.6 μM, and became significant at 5 μM and higher (one-way ANOVA, Scheffe's multiple comparison, $P < 0.01$) (Fig. 9B). In the presence of 100 μM PPDC, the maximum LDH efflux decreased to the background level.

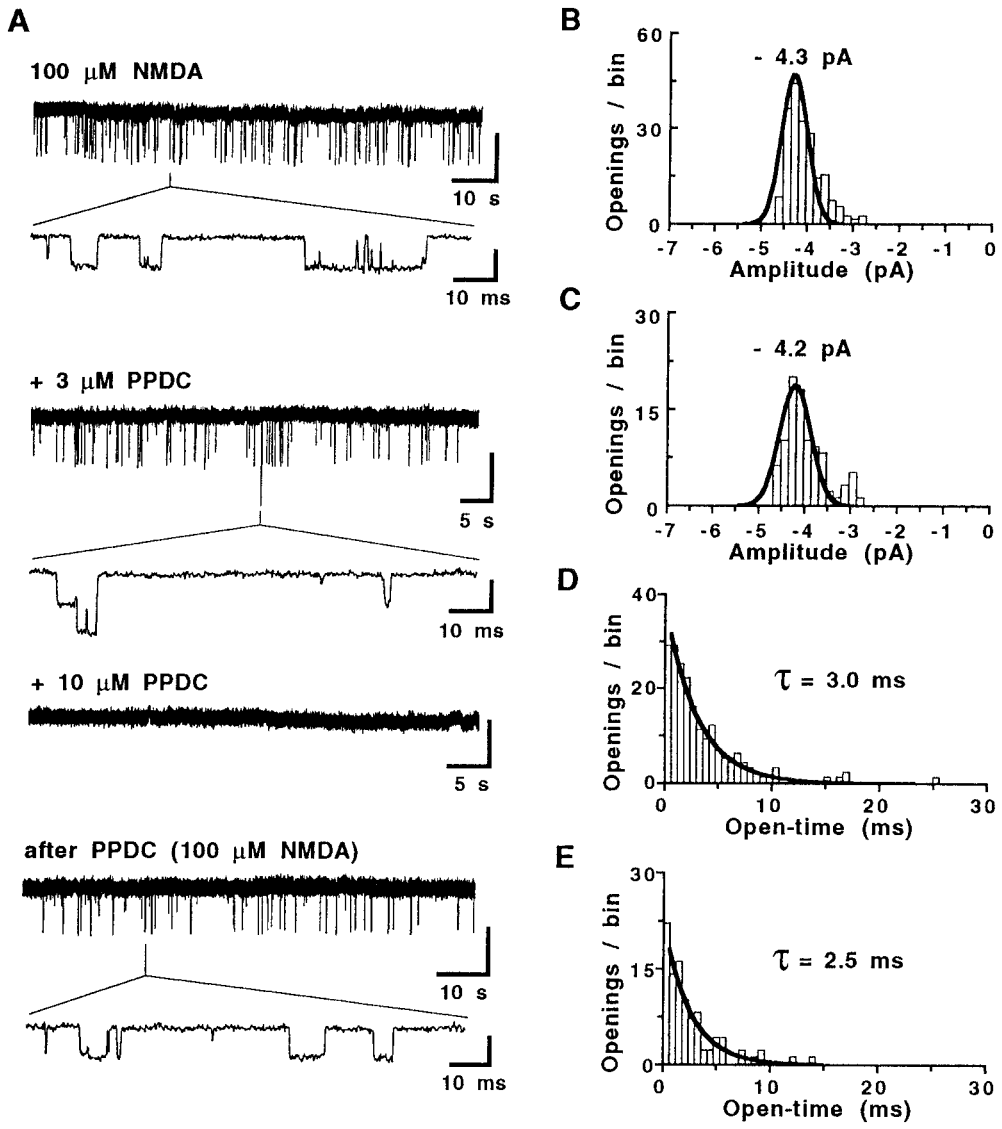

Fig. 6. PPDC block of elementary currents in an outside-out patch containing a few NMDA receptors of cultured hippocampal neurons. (**A**) Elementary currents evoked by 100 μM NMDA with no added glycine. After an initial burst lasting for ~15 sec, we recorded a control for 89 sec, elementary currents with 3 μM PPDC for 68 sec, 10 μM PPDC for 47 sec, and then a control for 78 sec again. (**B, C**) Amplitude histograms in the absence and presence of 3 μM PPDC. (**D, E**) Open time histograms in the absence and presence of 3 μM PPDC. Histograms were made from idealized records constructed by using a half-amplitude threshold criterion and the channel amplitude obtained from the amplitude histogram; overlapping events (it occurs only one time in the presence of 3 μM PPDC in **A**) are omitted in analysis. Vertical bars in A correspond to 4pA

DISCUSSION

In this study, we investigated the basic characteristics of the action of the conformationally restricted analogs of milnacipran, typically PPDC, because of its negligible effect on 5HT uptake (Shuto et al., 1996a,b), on NMDA receptors. The results allow us to reach three firm conclusions.

First, the compounds with a (1*S*, 2*R*, 1′*S*)-configuration were potent blockers against NMDA receptors, which clearly shows that this configuration increases the affinity of the compounds to NMDA receptors. Among the compounds with this configuration, PPDC was found to be a selective, uncompetitive NMDA receptor blocker; it selectively blocked NMDA receptors among examined neurotransmitter receptors, including ionic and metabotropic types (Fig. 3 and Table II), and decreased the maximum responses without shifting the concentration–response curves of NMDA receptors for both NMDA and glycine (Fig. 4). It remains to be investigated if the mode of action and the selectivity are derived from this (1*S*, 2*R*, 1′*S*)-configuration or the structure of milnacipran itself.

Second, PPDC blocks open channels of NMDA receptors. PPDC blocked only the activated NMDA receptors

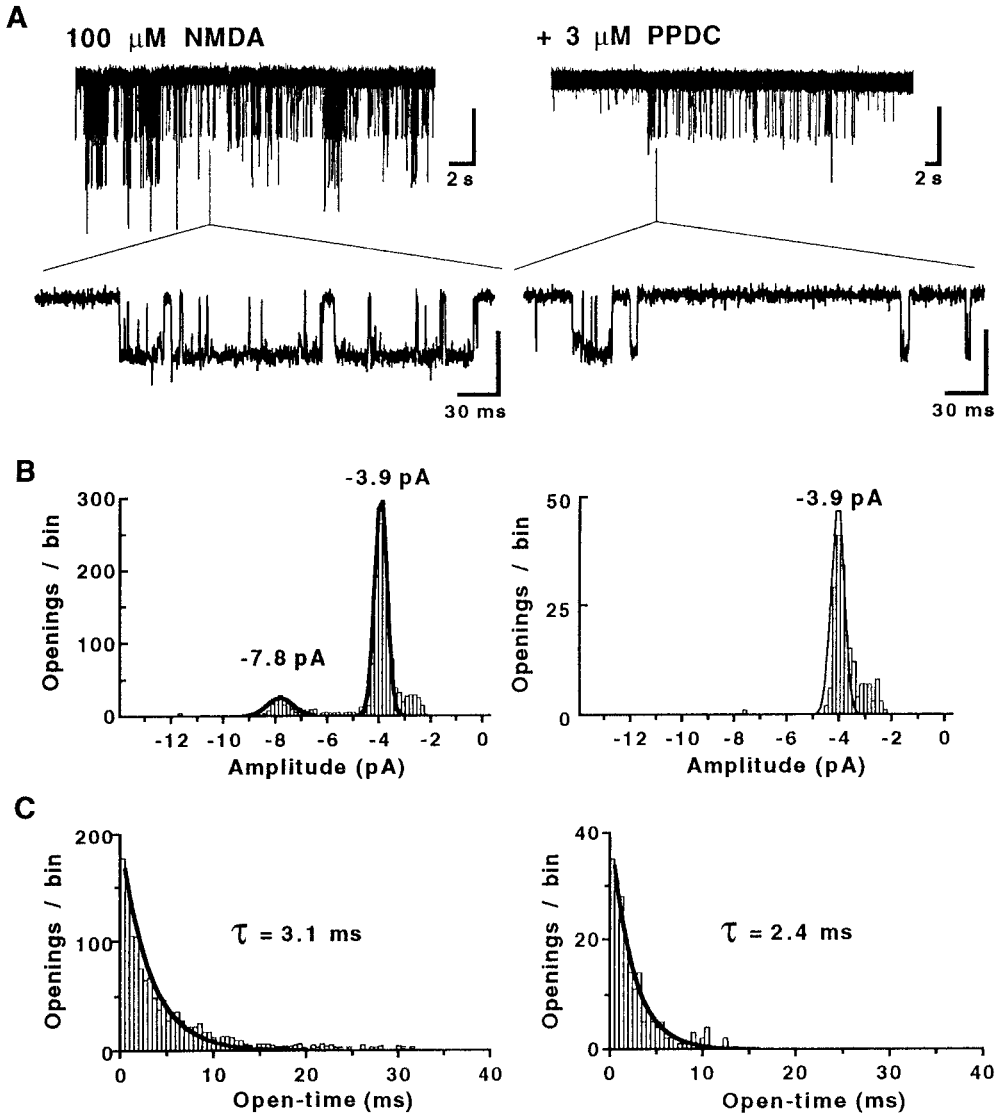

Fig. 7. PPDC block of elementary currents in an outside-out patch containing several NMDA receptors of cultured hippocampal neurons. (**A**) Elementary currents evoked by 100 μM NMDA with no added glycine in the absence (left) and presence of 3 μM PPDC (right). After an initial burst of ~15 sec, we recorded a control for 26 sec (1,222) and then tested the effect of 3 μM PPDC for 43 sec (197), where the numbers in each parenthesis show nonoverlapping events. (**B, C**) Amplitude and open time histograms for patch in **A**. Histograms were made similarly to those shown in Figure 6. Vertical bars in A correspond to 4pA.

(inset of Fig. 2) in an uncompetitive manner (Fig. 4). In addition, the blocking effect of PPDC was increased on hyperpolarization (Fig. 5), which supports the above conclusion since the transmembrane field only occurs across the pathway of open ion channels and, hence, PPDC molecules must enter the pathways to sense the field.

There is a simple channel-block mechanism (Adams, 1976; Armstrong, 1971) which assumes that agonist binding is much faster than the open-close reaction.

$$\text{Close} \underset{\beta}{\overset{\alpha}{\rightleftarrows}} \text{Open} \underset{k_{block}}{\overset{k_{unblock}}{\rightleftarrows}} \text{Blocked}$$

In this mechanism, the transition rate (blocking rate constant) from an open to a blocked state is $k_{block} \cdot [PPDC]$, the mean open time in the absence of PPDC (τ_{close}) is $1/\alpha$, and the mean blocked time in the presence of PPDC (τ_{block}) is the reciprocal of the sum of α and $k_{block} \cdot [PPDC]$. These transformations lead to:

$$k_{block} = (1/\tau_{block} - 1/\tau_{close})/[PPDC] \quad (3)$$

$$\alpha = 1/\tau_{close} \quad (4)$$

The k_{block} thus obtained was $2.8 \times 10^7 \pm 5.7 \times 10^6$ $M^{-1}s^{-1}$ ($n = 3$) at -100 mV, which is close to the theoretical maximum from diffusion limitation. This

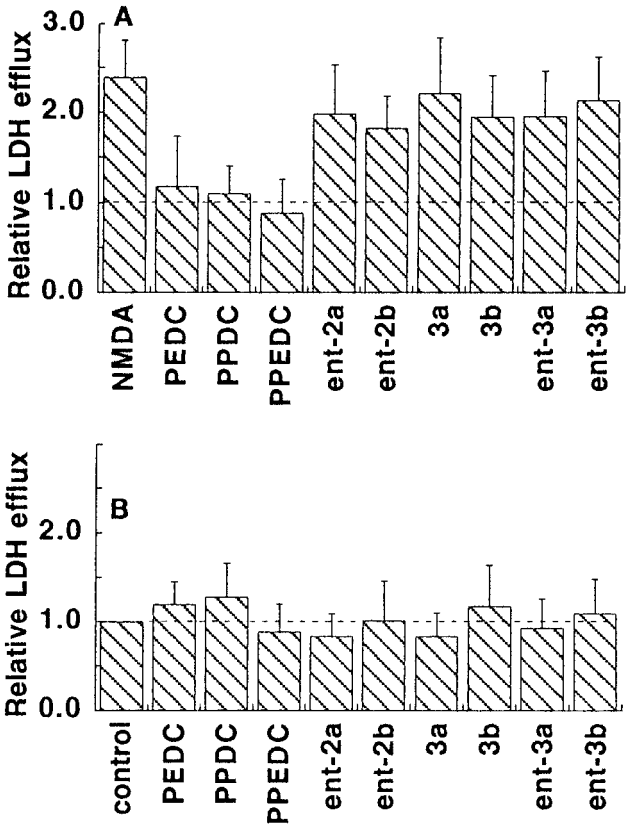

Fig. 8. Effects of conformationally restricted analogs against neurotoxicity induced by 300 μM NMDA supplemented with 10 μM glycine (**A**) and their effects on the background LDH efflux (**B**). A: PEDC, PPDC, and PPEDC significantly protected the cultures (Scheffe's multiple comparison, $P < 0.01$). (**B**) The changes induced by compounds alone were not significant (one-way ANOVA, $P > 0.05$). Each bin and vertical bar is the mean and SD of relative LDH efflux where the background LDH efflux was taken as 1.0.

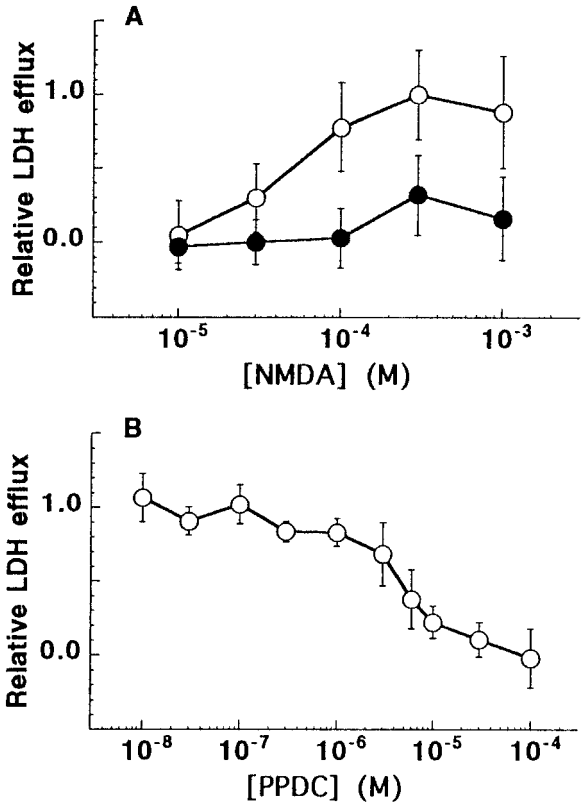

Fig. 9. Protective effect of PPDC against NMDA-induced neurotoxicity. (**A**) Concentration–response curves for NMDA supplemented with 10 μM glycine in the absence (○) and presence of PPDC (●). PPDC significantly decreased the maximum LDH efflux (two-way ANOVA; $P < 0.01$, Schette's multiple comparison, $P < 0.01$). (**B**) A suppression curve against LDH efflux induced by 300 μM NMDA with 10 μM glycine (control) as a function of coapplied PPDC concentration. PPDC significantly decreased the LDH efflux on the concentration of 5 μM and higher (one-way ANOVA, Scheffe's multiple comparison, $P < 0.01$). Each point and vertical bar is the mean and SD of relative LDH efflux calculated from the equation; relative LDH efflux = (LE-LB)/(LM-LB), where LE is LDH efflux determined on each well, LB is the background LDH efflux, and LM is the maximum LDH efflux (mean LDH efflux induced by control).

rate constant for blocking is similar to that of another uncompetitive NMDA blocker, MK-801, 2.37×10^7 $M^{-1}s^{-1}$ at −60 mV (Jahr, 1992).

The rate constant for unblocking was not determined because of the limited lifetimes of outside-out patches and the low open probability of NMDA receptors in outside-out patch, ~0.007 (upper limits), as the present results and previous ones (Huettner and Bean, 1988) show. As Figure 10 qualitatively shows, however, that the unblocking process of macroscopic currents for PPDC is much faster than that for MK-801. The macroscopic unblocking rate constant depends both on the unblocking time constant and the fraction of open channels. Since the traces shown in Figure 10 were obtained from the same oocyte, the fraction appears similar. Therefore, the difference between the unblocking processes show that the unblocking rate constant for PPDC was much faster than MK-801, indicating the different role of PPDC in experimental and clinical usage.

Third, the conformationally restricted analogs with the (1*S*, 2*R*, 1′*S*)-configuration protected cultured neu-

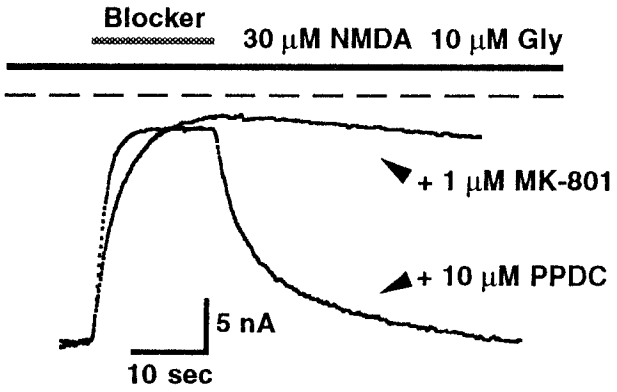

Fig. 10. Blocking and unblocking processes by 10 μM PPDC and 1 μM MK-801 in the continuous presence of 30 μM NMDA supplemented with 10 μM glycine. Compared traces were obtained from the same oocyte to eliminate the differences in the open probability of NMDA receptors. Broken line: zero current level. Holding potential −50 mV.

ronal cells against NMDA-induced neurotoxicity, even though the compounds with the other configurations failed to protect the culture. On the other hand, the effective compounds failed to protect against kainate-induced neurotoxicity. These results indicate that protective effects result from the potent blocking action on NMDA receptors.

Neuroprotective drugs are required to obtain an appropriate hydrophobicity in order to permeate the blood–brain barrier (BBB). Milnacipran was shown to permeate the BBB as an antidepressant drug (Barone et al., 1994; Moret et al., 1985). Since PPDC was synthesized by replacing a proton of milnacipran with an ethyl group (Shuto et al., 1996b), PPDC has increased hydrophobicity and satisfies this demand. PPDC had no blocking effect on other neurotransmitter receptors. Also, PPDC had a negligible effect on 5HT uptake (Shuto et al., 1996a,b). These characteristics are suitable when PPDC is used as a drug against neuronal cell death evoked by brain disorders.

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