Effects of the Novel Tricyclic Quinoxalinedione Derivatives, SM-18400 and Its Analogs, on *N*-Methyl-D-aspartate (NMDA) Receptor-Mediated Synaptic Transmission in the Isolated Neonatal Rat Spinal Cord In Vitro

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Received August 27, 1997 Accepted December 17, 1997

ABSTRACT—We examined the effects of novel tricyclic quinoxalinedione derivatives, SM-18400 ((*S*)-9-chloro-5-[*p*-aminomethyl-*o*-(carboxymethoxy)phenylcarbamoylmethyl]-6,7-dihydro-1*H*,5*H*-pyrido[1,2,3-*de*]quinoxaline-2,3-dione hydrochloride trihydrate) and its analogs (i.e., ID-17263 and ID-17332), on the *N*-methyl-D-aspartate (NMDA) receptor-mediated polysynaptic reflex (PSR) in the isolated spinal cord of neonatal rats in vitro. Application of SM-18400 selectively suppressed the PSR activity in a concentration-dependent manner without affecting the monosynaptic reflex (MSR). Differential suppression of the PSR was also obtained with ID-17263, ID-17332 and other known NMDA receptor glycine-binding site antagonists, 5,7-dichlorokynurenate (5,7-diClkyn) and L-689,560 (4-*trans*-2-carboxy-5,7-dichloro-4-phenylamino-carbonylamino-1,2,3,4-tetrahydroquinoline). Relative potencies of the test drugs for inhibition of the PSR were as follows: SM-18400 $\gg$ L-689,560 > ID-17332 > ID-17263 > 5,7-diClkyn. In addition, the inhibitory effects of SM-18400 on PSR were markedly antagonized by simultaneous application of D-serine, an agonist for NMDA receptor glycine-binding sites. These findings suggest that SM-18400 is a potent NMDA receptor glycine-binding site antagonist and blocks the NMDA receptor-mediated synaptic neurotransmission in the spinal cord in vitro.

Keywords: SM-18400, NMDA receptor, Glycine-binding site, D-Serine, Spinal reflex (in vitro)

The *N*-methyl-D-aspartate (NMDA) receptors are ligand-gated ion channels that are regulated through multiple, allosterically coupled modulatory sites including glycine-binding sites, polyamine modulatory sites and phencyclidine (PCP)/dizocilpine-binding sites, in addition to NMDA (glutamate)-binding sites (1, 2). Previous studies have suggested that NMDA receptors play an important role in the pathogenesis of epilepsy, pain processing and hypoxic-ischemic brain injury (e.g., stroke) (3–5). Among the various NMDA antagonists, channel site and NMDA-binding site antagonists have been studied as neuroprotectants in clinical trials. However, the utility of these antagonists was found to be limited because of their psychotomimetic side effects (5). Alternatively, antagonists specific for the NMDA receptor glycine-binding sites are expected to be the effective remedy for the treatment of brain ischemia and other

neurodegenerative disorders with reduced central nervous system adverse actions (6–9).

We recently synthesized a series of tricyclic quinoxalinedione derivatives and found that several of these compounds selectively interact with glycine-binding sites at the NMDA receptors. Among the compounds tested, SM-18400 ((*S*)-9-chloro-5-[*p*-aminomethyl-*o*-(carboxymethoxy)phenylcarbamoylmethyl]-6,7-dihydro-1*H*,5*H*-pyrido[1,2,3-*de*]quinoxaline-2,3-dione hydrochloride trihydrate) and its analogs, ID-17263 (9-bromo-5-(carboxymethyl)-6,7-dihydro-1*H*,5*H*-pyrido[1,2,3-*de*]quinoxaline-2,3-dione) and ID-17332 (*trans*-6-methyl-ID-17263), showed very high affinity for the glycine-binding sites, as determined by radioligand binding assay with [³H]5,7-dichlorokynurenate (5,7-diClkyn) (10, 11). Thus, these drugs may be useful for treating brain ischemia in humans (12, 13).

In previous studies, we demonstrated that the in vitro neonatal spinal cord served as a useful model for eval-

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uating the actions of NMDA receptor antagonists (14–16). Various NMDA antagonists including channel site (e.g., dizocilpine and PCP analogs) and NMDA-binding site antagonists (e.g., DL-2-amino-5-phosphonovalerate (APV) and 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonate (CPP)) all preferentially inhibited the Mg^{2+} -sensitive polysynaptic reflex (PSR), while negligibly affecting the monosynaptic reflex (MSR) in the isolated spinal cord *in vitro*. In addition, some of the antagonists at NMDA receptor glycine-binding sites (e.g., 7-chlorokynureate (7-Clkyn) and HA-966) also inhibited the PSR in a manner reversible by simultaneous application of D-serine, an agonist at NMDA receptor glycine-binding sites (16). In the present study, we investigated the effects of SM-18400 and its analogs, ID-17332 and ID-17263, on PSR activity in *in vitro* spinal cord preparations and compared their actions with those of other glycine-binding site antagonists, 5,7-diClkyn and L-689,560 (4-*trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline) (17, 18).

MATERIALS AND METHODS

Preparation of the spinal cord and recording of the reflex activity

Isolation of the spinal cord and recording of the ventral root potentials were performed as described previously (15, 16). Briefly, the spinal cord was dissected out from male Sprague-Dawley rats (6- to 9-days-old) under ether

anesthesia, hemisected longitudinally and superfused with Mg^{2+} -free physiological solution in a recording chamber (flow rate: 1.5–2.5 ml/min). The superfusion medium was bubbled continuously with 95% O_2 – 5% CO_2 , and the temperature was kept at $25.0 \pm 0.5^\circ C$.

Test stimuli (0.15-msec duration and 5- to 30-V intensity) were applied to the L_{4–6} dorsal root through a glass suction electrode every 10 sec. The discharge of the corresponding ventral root was recorded with a glass suction electrode, amplified, monitored on an oscilloscope (VC-11; Nihon Kohden, Tokyo) and stored on FM tape. Acquired responses were signal-averaged with on-line computer assistance (ATAC-3300, Nihon Kohden) and analyzed for amplitude, area and latency as described previously (19). The mean value of the reflex activity area was obtained from 10 successive responses in each experiment before and after drug application (at the plateau phase of the drug effect).

Solutions and drugs

The Mg^{2+} -free physiological solution contained: 116.4 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 0.92 mM NaH_2PO_4 , 26.2 mM $NaHCO_3$ and 11.0 mM glucose. When bubbled with 95% O_2 – 5% CO_2 , the solution had a pH of 7.3. SM-18400, ID-17332, ID-17263, 5,7-diClkyn and L-689,560 were synthesized in our laboratories. D-Serine was purchased from Aldrich Chem. (Tokyo). All drug solutions were freshly prepared with the Mg^{2+} -free physiological solution in each experiment.

Fig. 1. Effects of SM-18400, 5,7-diClkyn and L-689,560 on MSR and PSR in the isolated spinal cord *in vitro*. Spinal cords were superfused with Mg^{2+} -free physiological solution throughout the experiment. After the control recordings were obtained, the spinal cord was exposed to SM-18400 (9 nM), 5,7-diClkyn (3 μM) or L-689,560 (100 nM) until the response reached the plateau. The traces are signal-averaged records of ten successive responses before and after drug application. The traces in each column were obtained in the same preparation. The calibrations are 2 mV and 5 msec.

Fig. 2. Concentration-response curves for the depression of MSR (open circles) and PSR (closed circles) by SM-18400, ID-17332 and ID-17263 in the isolated spinal cord *in vitro*. After the control recordings were obtained, the spinal cord was exposed to SM-18400, ID-17332 or ID-17263 until the response reached the plateau. Each point indicates the mean $\pm$ S.E.M. of 3 separate experiments.

Statistical procedure

Reflex activities were expressed as a percentage of the pre-drug control. The IC_{50} value that produced 50% inhibition of the reflex activity for each drug was determined by the Litchfield and Wilcoxon method (20). Statistical significance of the effects of D-serine on the SM-18400-induced PSR inhibition was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Effects of tricyclic quinoxalinedione derivatives, 5,7-diClkyn and L-689,560 on the spinal reflex

Stimulation of the L_{4-6} dorsal root under Mg^{2+} -free conditions evoked two temporally distinct reflex potentials, MSR and PSR, in the corresponding ventral root (Fig. 1). The mean amplitude, area and latency of the MSR were 3.27 ± 0.13 mV, 4.47 ± 0.23 mV·msec and 6.47 ± 0.16 msec, respectively; and those for the PSR were 1.36 ± 0.06 mV, 3.55 ± 0.24 mV·msec and 11.7 ± 0.2 msec ($N=33$), respectively.

Bath application of SM-18400 (0.3–9 nM) markedly inhibited the PSR in a concentration-dependent manner, while the drug reduced MSR activity by about 10% (Figs. 1 and 2). Application of ID-17332 and ID-17263, analogs of SM-18400, also produced preferential and concentration-dependent inhibition of the PSR (Fig. 2). The activity of PSR was completely suppressed by 9 nM SM-18400, 300 nM ID-17332 and 1000 nM ID-17263, while

these concentrations of each drug reduced MSR by about 10–30%. The IC_{50} values of SM-18400, ID-17332 and ID-17263 for the PSR were 2.2, 60 and 250 nM, respectively (Table 1).

Preferential inhibition of the PSR was also obtained with 5,7-diClkyn and L-689,560 (Figs. 1 and 3). The IC_{50} values of 5,7-diClkyn and L-689,560 for the PSR inhibition were 770 and 24 nM, respectively, and the

Table 1. Potencies of various NMDA receptor antagonists for inhibition of the PSR in the isolated spinal cord *in vitro*

NMDA antagonists	PSR inhibition	
	IC_{50} value (nM)	Relative potency ^{a)}
Glycine-binding site		
SM-18400	2.2	1.0
L-689,560	24	0.092
ID-17332	60	0.037
ID-17263	250	0.0088
5,7-diClkyn	770	0.0029

7-Clkyn	1700 ^{b)}	0.0013
HA-966	40000 ^{b)}	0.000055
Channel site		
Dizocilpine	12 ^{b)}	0.18
NMDA-binding site		
CPP	150 ^{b)}	0.015
APV	1200 ^{b)}	0.0018

^{a)}Relative potency to SM-18400. ^{b)}Values are quoted from Ref. 16 (Gen Pharmacol, 29, 645–649, 1997).

Fig. 3. Concentration-response curves for the depression of MSR (open circles) and PSR (closed circles) by 5,7-diClkyn and L-689,560 in the isolated spinal cord in vitro. After the control recordings were obtained, the spinal cord was exposed to 5,7-diClkyn or L-689,560 until the response reached the plateau. Each point indicates the mean $\pm$ S.E.M. of 3–4 separate experiments.

PSR activity was almost completely suppressed by 3 μ M of 5,7-diClkyn and 300 nM of L-689,560 (Table 1). In contrast, the activity of MSR was slightly reduced (about 10–20%) by 5,7-diClkyn and L-689,560 (Fig. 3).

We have previously shown that various NMDA antagonists, including those for glycine-binding sites, channel sites and NMDA-binding sites, inhibit PSR activity under the same conditions used here (16). As shown in Table 1, the relative potencies of the NMDA antagonists tested in the present and previous studies were as follows: SM-18400 > dizocilpine > L-689,560 > ID-17332 > CPP > ID-17263 > 5,7-diClkyn > APV = 7-Clkyn > HA-966. The potency of SM-18400 in inhibiting the PSR was the greatest among the NMDA glycine-binding site antagonists examined and was about fivefold more potent than the channel site antagonist dizocilpine (21).

Effects of D-serine on SM-18400-induced inhibition of PSR

We next examined the effects of D-serine, an agonist of glycine-binding sites of NMDA receptors, on the SM-18400-induced inhibition of PSR. The spinal cords were first exposed to 30, 100 or 300 μ M of D-serine for 15 min and then to SM-18400 (9 nM) in the presence of D-serine. Under these conditions, the inhibitory effects of SM-18400 were markedly antagonized by the simultaneous application of 100 or 300 μ M D-serine (Fig. 4). The magnitude of the PSR was reduced to $8 \pm 4\%$ of the control by 9 nM SM-18400, but the value significantly increased to $84 \pm 11\%$ or $81 \pm 7\%$ in the presence of 100 or 300 μ M

D-serine, respectively. Pretreatment of the spinal cord with D-serine (30, 100 or 300 μ M) alone only slightly increased the magnitude of the MSR and PSR by 1–3% and 5–10%, respectively (data not shown).

Fig. 4. Effects of D-serine on the SM-18400-induced depression of the PSR in the isolated spinal cord in vitro. The spinal cord was first exposed to D-serine at 30, 100 or 300 μ M for 15 min and subsequently to SM-18400 (9 nM) in the presence of D-serine. Columns with vertical bars indicate the mean $\pm$ S.E.M. of 3–4 separate experiments. * $P < 0.01$, Significantly different from the value without D-serine (one-way ANOVA followed by Dunnett's multiple comparison test).

DISCUSSION

In the present study, we demonstrated that a novel tricyclic quinoxalinedione derivative SM-18400 and its analogs, ID-17332 and ID-17263, produced selective suppression of the spinal PSR in *in vitro* spinal cord preparations maintained under Mg^{2+} -free conditions. Selective suppression of the PSR was also observed with other known NMDA receptor glycine-binding site antagonists, 5,7-diClkyn and L-689,560. The potency of SM-18400 in inhibiting the PSR was greatest among various NMDA receptor glycine-binding site antagonists examined in the present and previous studies (16), and the relative potencies of the drugs (SM-18400 $\gg$ L-689,560 $>$ ID-17332 $>$ ID-17263 $>$ 5,7-diClkyn $>$ 7-Clkyn $\gg$ HA-966) seemed to be consistent with their binding affinities for the glycine-binding site (10, 11, 22). These results support the idea (15, 23–26) that the spinal PSR is mediated by NMDA receptors and suggest that glycine-binding sites play a crucial role in activation of NMDA receptors in the spinal cord *in vitro*. In addition, the fact that the potency of SM-18400 was about 5- and 70-fold more potent than those of dizocilpine and CPP, well-known channel site and NMDA-binding site antagonists, respectively, suggests that SM-18400 may be one of the most potent antagonists of NMDA receptors.

We have previously shown that D-serine, an agonist of NMDA receptor glycine-binding sites, selectively reversed the suppression of the PSR induced by 7-Clkyn, but not the channel site antagonist dizocilpine or the NMDA-binding site antagonist CPP (16). In this study, the suppression of PSR by SM-18400 was also antagonized by D-serine. These results strongly suggest that SM-18400 inhibited PSR activity by antagonizing glycine-binding sites, but not the channel sites or the NMDA-binding sites at the NMDA receptors. This is consistent with the biochemical observation that SM-18400 has very low binding affinity for the latter two sites (12).

Previous studies have shown that NMDA antagonists can ameliorate hypoxic-ischemic brain injury (3–5). However, the clinical utility of channel site and NMDA-binding site antagonists is very limited because of their psychotomimetic side effects in humans (5). Interestingly, several studies have shown that antagonists of NMDA receptor glycine-binding sites do not produce excitement (e.g., hyperlocomotion) in animals (6, 7) and fail to substitute for either dizocilpine or PCP in the drug discrimination studies (8, 9), suggesting that they would have minimal psychotomimetic side effects. Thus, antagonists of NMDA receptor glycine-binding sites may be useful as alternative agents for the treatment of ischemic brain injury. In our preliminary studies, peripheral administration of SM-18400, ID-17332 and ID-17263 inhibited the

convulsions elicited by intracerebroventricular administration of NMDA in mice (10–12). In addition, SM-18400 effectively prevented edema formation in the global ischemia model in spontaneously hypertensive rats and reduced infarction volume in the focal brain ischemia model in normotensive rats (12, 13). These findings, taken together with the present results, suggest that SM-18400 is a potent NMDA receptor glycine-binding site antagonist that is active with peripheral administration and may be useful as a neuroprotectant for the treatment of hypoxic-ischemic brain injury. Further studies are required to determine the pharmacological characteristics of SM-18400.

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