ABSTRACT

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is thought to play an important role in the pathogenesis of several neurological disorders as well as normal brain function. The search for AMPA receptor antagonists as potential therapeutics is ongoing. Here, we describe the functional characterization of a novel noncompetitive AMPA receptor antagonist, 2-[N-(4-chlorophenyl)-N-methylamino]-4H-pyrido[3,2-e]-1,3-thiazin-4-one (YM928). This compound inhibited AMPA receptor-mediated toxicity in primary rat hippocampal cultures with an IC50 of 2 μM. Its manner of inhibition was noncompetitive as the agonist concentration was increased. YM928 blocked AMPA-induced intracellular calcium influx with an IC50 of 3 μM and antagonized AMPA-induced inward currents with an IC50 of 1 μM in cultured cells. YM928 displaced neither [3H]AMPA binding nor other existing glutamate receptor-related ligand binding in rat brain membranes. In terms of in vivo activity, YM928 had an anticonvulsant effect in sound-induced seizures in DBA/2 mice 45 min after oral administration at 3 mg/kg. Thus, YM928 has potential as an oral therapeutic drug for various types of neurological disorders.

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor belongs to the ionotropic glutamate receptor family that is regulated by the neurotransmitter glutamate (for review, see Seeburg, 1993). Other members of this family are the N-methyl-D-aspartate (NMDA) and kainate (KA) receptors. The AMPA receptor provides the majority of fast excitatory transmission in the brain. It is composed of four subunits (GluR1–4) that can assemble to form functional ion channels through which Na+/K+ or Ca2+ is permeable, depending on the subtype composition. Excessive activation of ionotropic glutamate receptors is thought to be implicated in the pathogenesis of a diverse group of neurological disorders (for review, see Gill et al., 1999; Lees, 2000). These disorders include epilepsy, focal and global ischemia, central nervous system trauma, and various forms of neurodegeneration such as Parkinson's disease and Huntington's disease. Indeed, glutamate can induce neuronal death in vitro, and several glutamate receptor antagonists have been shown to have neuroprotective effects in animal models of brain ischemia and neurodegenerative disorders. Both the AMPA and the NMDA receptor seem to play an important role in such pathological conditions. The cerebroprotective effects of NMDA receptor antagonists have been well documented in focal ischemia models (Park et al., 1988; Gill et al., 1991). However, NMDA receptor antagonists may have limited utility as therapeutic agents, since these also produce psychotomimetic effects (Koek et al., 1988), impairment of learning and memory (Morris et al., 1986), and ultrastructural changes in cortical neurons (Olney et al., 1989). Therefore, the development of AMPA receptor antagonists has been encouraged to create therapeutics for neurological disorders.
There are two prototype AMPA receptor antagonists, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[|]quinazoline-7-sulfonamide (NBQX) and, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-[5H-2,3]-benzodiazepine (GYKI52466) (Fig. 1) for review, see Nikam and Kornberg, 2001). NBQX belongs to the quinoxalinedione class and is a competitive AMPA receptor antagonist. On the other hand, GYKI52466 belongs to the 2,3-benzodiazepine class and is a noncompetitive AMPA receptor antagonist. They have been excellent tools for investigating the function of the AMPA receptor. They were shown to be neuroprotective in global (Sheardown et al., 1990, 1993; Buchan et al., 1991; Judge et al., 1991; Le Peillet et al., 1992; Li and Buchan, 1993; Lodge et al., 1996) and focal (Gill et al., 1992; Smith and Meldrum, 1992; Xue et al., 1994; Graham et al., 1996) models of ischemia. Their anticonvulsant activities were also described in several animal models (Chapman et al., 1991; Smith et al., 1991; Yamaguchi et al., 1993; Durmuller et al., 1994). NBQX, however, is poorly soluble and precipitates in the kidney at projected therapeutic plasma levels. Although some efforts have provided an improvement in the water solubility of this class of compounds, for example YM872, the problem of brain penetrability still remains (Kohara et al., 1998). Recently decahydroisoquinolines typified by LY293558 (Bullock et al., 1994) and quinazolinones typified by CP-465,022 (Lazzaro et al., 2002; Menniti et al., 2000) have become known as new classes of competitive and noncompetitive AMPA receptor antagonists, respectively.

To create new orally active AMPA receptor antagonists, a hundred thousand compounds have been screened against KA-induced toxicity in rat primary cortical cultures, which is mediated by the AMPA receptor (Ohno et al., 1997). Several active compounds with potentially useful chemical structures were found, and from these, a pyridothiazine derivative was selected as a lead compound, 2-[N-(4-Chlorophenyl)-N-methylamino]-4H-pyrido[3,2-e]-1,3-thiazin-4-one (YM928) arose from medicinal chemistry based on this compound (Fig. 1). In the present study, we describe the functional characterization of YM928.

**Fig. 1.** Chemical structures of YM928, NBQX, and GYKI52466.
(Axopatch 1D patch-clamp amplifier, Digidata 1200 digitizer, pCLAMP6 acquisition and analysis computer program; Axon Instruments Inc., Union City, CA) and a thermal pen recorder (rectihoriz.

8K20; NEC, Tokyo, Japan). The pipette solution contained 140 mM CaF and 5 mM CsCl in 10 mM HEPES, adjusted to pH 7.2 with CsOH. The perfusion solution contained 140 mM NaCl, 5 mM KCl, 2.4 mM CaCl₂, and 10 mM glucose in 10 mM HEPES, adjusted to pH 7.4 with NaOH. Cells were perfused at 5 to 8 ml/min of perfusion solution at room temperature. Inward currents were induced by application of 20 μM AMPA for 10 s. YM928 was perfused from 1 min before AMPA application at the indicated concentrations. IC₅₀ values were determined as previously. Values are expressed with their 95% confidence intervals.

Radioligand Binding Competition Assays. The studies were performed at NovaScreen (Hanover, MD), using published protocols. Values are expressed as percentage of inhibition of specific binding and represent the average of two tubes at each concentration tested.

Sound-Induced Seizure in DBA/2 Mice. Male DBA/2 mice, weighing 9.5 to 12.5 g (Charles River Japan, Inc., Yokohama, Japan) were exposed to auditory stimulation (12 kHz, 120 dB for 60 s or until tonic extension occurred) in a soundproof box at 45 min after oral administration of vehicle or YM928. The drug was suspended in 0.5% aqueous methylcellulose as vehicle. The dosing volume was 0.3 ml/10 g, which was calculated on the basis of the body weight on the day of the experiment. Anticonvulsant effects were evaluated according to the following scores: 0, no response; 1, wild running; 2, clonic seizure; 3, tonic seizure; 4, death (De Sarro et al., 1988). Maximum response was measured for each mouse.

Results

KA-Induced Toxicity. The effect of YM928 was examined on KA-induced toxicity in primary rat hippocampal cultures, which is mediated by the AMPA receptor (Ohno et al., 1997). Neurons were exposed to KA with or without a test drug, and 24 h later, cell death was assessed by the amount of LDH activity in the culture media. YM928 inhibited KA-induced toxicity completely and concentration dependently (Fig. 2A). YM928 reduced the maximum response of KA-dose response curves (Fig. 2B). Higher KA concentrations than those shown here were not used because of its poor solubility and nonspecific effects. The IC₅₀ value for YM928 was 2.0 (1.5–2.6) μM. Other AMPA antagonists were also evaluated (Table 1). As has already been shown, NBQX showed quite strong activity. However, two noncompetitive antagonists, GYKI52466 and LY300164/talampanel, had weaker activity than that of YM928.

Intracellular Calcium Influx. The effect of YM928 on AMPA-induced [Ca²⁺]i, in primary rat hippocampal cultures was investigated. YM928 inhibited AMPA-induced [Ca²⁺]i completely and concentration dependently (Fig. 3). The IC₅₀ value of YM928 was 3.0 (2.3–3.7) μM.

To examine the effect of YM928 on NMDA receptors, voltage-dependent Na⁺ channels, and voltage-dependent Ca²⁺ channels in hippocampal neurons, the compound was tested against NMDA- and veratridine-induced [Ca²⁺]i (Fig. 4). Against NMDA-induced [Ca²⁺]i, YM928 showed no effect at 30 μM. At 100 μM, YM928 slightly inhibited NMDA-induced [Ca²⁺]i (Fig. 4A). On the other hand, the competitive NMDA receptor antagonist CGS19755 markedly inhibited NMDA-induced [Ca²⁺]i at 10 μM. Against veratridine-induced [Ca²⁺]i, YM928 had a slight effect at 30 and 100 μM (Fig. 4B). NBQX, a relatively selective inhibitor of the AMPA receptor, inhibited it by approximately 15% at 10 μM as well.

TTX, at 1 μM, completely inhibited veratridine-induced [Ca²⁺]i.

AMPA-Induced Inward Currents. To confirm the effect of YM928 on the AMPA receptor electrophysiologically, AMPA-induced inward currents were examined by whole-cell patch-clamp analysis in rat hippocampal cultures (Fig. 5). YM928 inhibited 20 μM AMPA-induced inward currents completely and concentration dependently. The IC₅₀ value was 1.03 (0.91–1.12) μM.
Radioligand Binding Competition Assays. The interaction of YM928 with known glutamate-related ligand binding sites was investigated. YM928 showed pIC\textsubscript{50} values 4 at rat brain sites labeled by \textsuperscript{3}H]AMPA, \textsuperscript{3}H]KA, \textsuperscript{3}H]CGP39653, \textsuperscript{3}H]glycine, \textsuperscript{3}H]MK-801, or \textsuperscript{3}H]glutamate, indicating no significant affinity for ionotropic glutamate channels, or the glycine or MK-801 site of the NMDA receptor complex, chloride channels, or glutamate uptake sites (Table 2). In an additional competition assay screen using 36 ligands for the main types of autonomic and ion-channel receptors, YM928 exerted less than 50% inhibition at 100 \textmu M (data not shown), indicating no relevant affinity for any of the investigated receptor types.

**Table 1**

<table>
<thead>
<tr>
<th>AMPA Antagonist</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM928</td>
<td>2.0</td>
<td>1.5 - 2.6</td>
</tr>
<tr>
<td>NBQX</td>
<td>0.46</td>
<td>0.39 - 0.52</td>
</tr>
<tr>
<td>GYKI53466</td>
<td>17.0</td>
<td>13.7 - 20.3</td>
</tr>
<tr>
<td>LY300164</td>
<td>6.0</td>
<td>4.9 - 7.1</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effect of YM928 on AMPA-induced intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in rat hippocampal cultures. A, fluorometric measurements of [Ca\textsuperscript{2+}]\textsubscript{i} induced by AMPA and inhibition by 8 \textmu M YM928. Data are the mean from 15 neurons. B, concentration-dependent inhibition of AMPA-induced [Ca\textsuperscript{2+}]\textsubscript{i}, by YM928. Values in parentheses are the number of cells tested.

**Fig. 4.** Effect of YM928 on NMDA- and veratridine-induced [Ca\textsuperscript{2+}]\textsubscript{i} in rat hippocampal cultures. A, effects of YM928 and CGS19755 on NMDA-induced [Ca\textsuperscript{2+}]\textsubscript{i}. NMDA (100 \textmu M)-induced [Ca\textsuperscript{2+}]\textsubscript{i} is scaled to the basal level of [Ca\textsuperscript{2+}]\textsubscript{i}. Data represent mean ± S.E.M. and values in parentheses are the number of cells tested. B, effects of YM928, TTX (1 \textmu M) and NBQX (10 \textmu M) on veratridine-induced [Ca\textsuperscript{2+}]\textsubscript{i}. Veratridine-induced [Ca\textsuperscript{2+}]\textsubscript{i} is scaled to the basal level of [Ca\textsuperscript{2+}]\textsubscript{i}. Data represent mean ± S.E. and values in parentheses are the number of cells tested.

**Discussion**

The present studies show that YM928 is an orally active and noncompetitive AMPA receptor antagonist. Thus, the...
compound blocked KA-induced toxicity, AMPA-induced 
[Ca^{2+}], and AMPA-induced inward currents in rat primary
hippocampal cultures in a concentration-dependent manner.
In the KA-induced toxicity assay, potency was observed sim-
ilar to that of existing AMPA receptor antagonists. YM928
inhibited the maximum response of KA and it did not dis-
place [3H]AMPA binding to rat forebrain membranes at con-
centrations up to 100μM, suggesting that the manner of its
inhibition is noncompetitive. In terms of in vivo activity,
YM928 significantly reduced sound-induced seizures in
DBA/2 mice after oral administration at 3 mg/kg.
YM928 was able to inhibit AMPA-induced current com-
pletely in whole-cell patch-clamp experiments, suggesting
that YM928 directly acts on the AMPA receptor. YM928 also
blocked the maximum response of the KA dose-response
curve in KA-induced toxicity experiments. However,
[3H]AMPA binding experiments indicated that YM928 does
not act on the glutamate binding site on the AMPA receptor.
Therefore, YM928 seems to be a noncompetitive AMPA recep-
tor antagonist and to act at a distinct site on the AMPA
receptor. Recently, another class of noncompetitive AMPA
receptor antagonists typified by CP-465,022 and CP-526,427
was identified (Menniti et al., 2000; Lazzaro et al., 2002).
Interestingly, Menniti and colleagues did identify [3H]CP-
526,427 binding in rat forebrain membranes; however, the
[3H]CP-526,427 binding site did not interact directly with the
glutamate binding site. The binding affinity of a series of
compounds for the [3H]CP-526,427 binding site was well
related to potency for inhibition of a functional AMPA recep-
tor-mediated response. Among noncompetitive AMPA recep-
tor antagonists, 2,3-benzodiazepines can displace [3H]CP-
526,427 binding, but Evans blue cannot. Therefore, on the
AMPA receptor, there seem to be at least two allosteric
modulatory sites that noncompetitive AMPA receptor antag-
onists can interact with. YM928 might bind to these sites.
Further investigation is needed on this matter.
In terms of the selectivity of YM928 for other receptors,
YM928 did not have any affinity for glutamate-related ligand
binding sites. Although YM928 had a slight inhibitory effect
on veratridine- and NMDA-induced [Ca^{2+}], it was much less
effective than known antagonists at these sites. Since vera-
tridine activates sodium influx, causing depolarization and
increasing [Ca^{2+}], in cells, these experiments suggest that
YM928 does not interact with voltage-dependent sodium
channels or calcium channels on rat hippocampal neurons,
and that the inhibitory effect of YM928 on [Ca^{2+}], is specific
for the AMPA-induced response. Moreover, in dozens of typ-
cal neurotransmitter-ligand binding assays, YM928 at 10
μM showed no inhibitory activity. Taken together, these re-
results suggest that YM928 is specific for the AMPA receptor.
YM928 significantly inhibited sound-induced seizures in
DBA/2 mice 45 min after oral administration at 3 mg/kg,
suggesting that its brain penetrability might be excellent.
Quinoxalinediones typified by NBQX have poor brain pene-
trability, and this restricts their potential use in the treat-
ment of chronic diseases. The oral activity of YM928 may
extend its application to several kinds of disease conditions.
Moreover, its noncompetitive action may be preferable to
competitiveness for protection against neurological disorders
with high synaptic glutamate levels, such as stroke and epi-
lepsy, because high synaptic concentrations of glutamate

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Radioligand</th>
<th>Membrane Source</th>
<th>Percent Inhibition (Average; n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPA site</td>
<td>[3H]AMPA</td>
<td>Rat forebrain</td>
<td>0.98 -5.36 11.70</td>
</tr>
<tr>
<td>Kainate site</td>
<td>[3H]Kainate</td>
<td>Rat forebrain</td>
<td>9.87 -6.30 11.18</td>
</tr>
<tr>
<td>NMDA agonist site</td>
<td>[3H]CGP 39653</td>
<td>Rat forebrain</td>
<td>-14.94 2.34 18.90</td>
</tr>
<tr>
<td>NMDA glycine site</td>
<td>[3H]Glycine</td>
<td>Rat cortex</td>
<td>3.34 3.97 5.39</td>
</tr>
<tr>
<td>NMDA MK801 site</td>
<td>[3H]MK801</td>
<td>Rat forebrain</td>
<td>-10.29 0.03 -3.64</td>
</tr>
<tr>
<td>Chloride-dependent site</td>
<td>[3H]Glutamate</td>
<td>Rat cerebellum</td>
<td>-1.34 5.22 16.54</td>
</tr>
<tr>
<td>Glutamate uptake site</td>
<td>[3H]Glutamate</td>
<td>Rat cortex</td>
<td>3.98 6.09 9.87</td>
</tr>
</tbody>
</table>
YM928 has potential as an oral therapeutic drug for various types of neurological disorders.

Acknowledgments

We thank Drs. Toichi Takenaka, Shinji Usuda, Gensei Kon, Toshiyasu Mase, Kazuo Honda, and Michael Minchin of Yamanouchi Pharmaceutical Co. Ltd. for invaluable advice. We also thank Yukiko Funatsu, Mika Sudoh, and Hanae Hattori for assistance.

References


Le Perugia E, Arvin B, Menegatti G, and Meldrum BS (1992) The non-NMDA receptor antagonists, NBQX and GYK325466, protect against cortical and striatal cell loss following transient global ischaemia in the rat. Brain Res 571:115–120.


Fig. 6. Anticonvulsant effect of YM928 against sound-induced seizure (SIS) in DBA/2 mice. Anticonvulsant effects were evaluated according to the following score: 0, no response; 1, wild running; 2, clonic seizure; 3, tonic seizure; 4, death. Maximum response was measured for each mouse. YM928 was orally administered 45 min before SIS. Horizontal bar represents median score. *, p < 0.05; **, p < 0.01 significant difference relative to control (Steel test).


Address correspondence to: Dr. Kazushige Ohno, Neuroscience Research, Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba 305-8585, Japan.

E-mail: ohno@yamanouchi.co.jp