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# Interaction of the neuroprotective drug riluzole with $GABA_A$ and glycine receptor channels

Bahram Mohammadi, Klaus Krampfl, Hafis Moschref, Reinhard Dengler, Johannes Bufler *

*Neurological Department, Medical School Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany*

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**Abstract**

Riluzole is used as therapeutic agent in amyotrophic lateral slerosis. We investigated the interaction of riluzole with recombinant GABA ($\gamma$-aminobutyric acid)$_A$ receptor channels ($\alpha_1\beta_2\gamma_2$-subunits) and glycine receptor channels ($\alpha_1\beta$-subunits) transiently expressed in HEK293 cells. For electrophysiological experiments, the patch–clamp technique in combination with tools for ultrafast solution exchange was used. Saturating concentrations of GABA or glycine were applied with different concentrations of riluzole to outside-out patches containing $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels or $\alpha_1\beta$-glycine receptor channels on their surface, respectively. The current declined after application of GABA or glycine with three time constants of desensitization to a steady-state current amplitude. Application of riluzole resulted in a shift to fast desensitized states at both receptors. The proportion of the time constants of fast desensitization increased and the time constants of slow desensitization and the steady-state current decreased whereas the maximal current amplitudes were not affected by riluzole. The data of the study demonstrate for the first time interaction of GABAergic and glycinergic currents with riluzole under physiological conditions. 

*Keywords:* $GABA_A$ receptor channel, recombinant $\alpha_1\beta_2\gamma_2$; Glycine receptor channel, recombinant $\alpha_1\beta$; Patch–clamp technique; Rapid application; Riluzole

## 1. Introduction

Riluzole has a mild effect on survival of patients with amyotrophic lateral sclerosis (Bensimon et al., 1994; Lacomblez et al., 1996). It has been shown to bind to various membrane proteins and to interact with voltage-dependent neuronal sodium channels (Benoit and Escante, 1991), NMDA receptor channels (Debono et al., 1994; Hubert et al., 1994), glutamate release (Hebert et al., 1994) and G-protein mediated signalling pathways (Doble et al., 1992). It is now widely used as a neuroprotective agent in the treatment of amyotrophic lateral sclerosis.

Amyotrophic lateral sclerosis is characterized by a progressive degeneration of cortical and spinal motor neurons. Excitotoxicity is an important factor in the pathogenesis of the disease. A dysbalance of excitatory (glutamatergic) and inhibitory (GABAergic) input may result in overactivation of cortical motorneurons. Motor cortical hyperexcitability was demonstrated in studies with transcranial magnetic stimulation (TMS) (Eisen et al., 1993). It was postulated that decreased inhibition is crucial for cortical hyperexcitability of amyotrophic lateral sclerosis patients. Positron emission tomography (PET) studies supported the hypothesis showing increased activity of the motor cortical surrounds during hand and finger movements (Kew et al., 1993). Direct evidence of decreased cortical inhibition came from recent histochemical studies on the motor cortex of amyotrophic lateral sclerosis patients, showing marked depletion of nonpyramidal local inhibitory interneurons prior to loss of cortical motor neurons (Nihei et al., 1992).

The therapeutic effect of riluzole in amyotrophic lateral sclerosis is generally attributed to its antiglutamatergic action. Side effects of riluzole such as sedation and adynamia, and anticonvulsant properties (Doble, 1996) suggest that inhibitory receptors are also involved in the action of riluzole. The most widespread inhibitory receptors in the central nervous system are the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels whereas the closely related $\alpha_1\beta$ glycine receptor channels are prevailing in the brain stem and

* Corresponding author. Tel.: +49-511-532-2439; fax: +49-511-532-3115.

*E-mail address:* Bufler.Johannes@MH-Hannover.de (J. Bufler).

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spinal cord of mammals (Betz, 1992). In the present study, a potential interaction of riluzole with $GABA_A$ receptor coupled channels and glycine receptor channels was studied on outside-out patches from human embryonic kidney (HEK)293 cells expressing $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels or $\alpha_1\beta$ glycine receptor channels. The patch–clamp technique (Hamill et al., 1981) was used in combination with tools for ultrafast solution exchange (Franke et al., 1987).

## 2. Methods

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% $CO_2$/95% air incubator. Cells were suspended in a buffer containing: 50 mM $K_2HPO_4$; 20 mM K-acetate; pH 7.35. cDNA of rat $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subunits (subcloned in pCDM8 expression vectors, Invitrogen, Groningen, Netherlands) or $\alpha_1\beta$ glycine receptor subunits (subcloned in pCIS2 expression vectors; Gormann et al., 1990) were added to the suspension of HEK293 cells and transfected by a device for electroporation (EquiBio, Kent, UK). To visualize transfected cells, they were cotransfected with cDNA of green fluorescent protein (GFP). Transfected cells were plated on glass coverslips and incubated for 15–24 h prior to the patch–clamp experiments.

Electrophysiological experiments were performed at room temperature (20°C), and cells were continuously superfused with extracellular solution containing: 162 mM NaCl, 5.3 mM KCl, 0.6 mM $Na_2HPO_4$, 0.22 mM $KH_2PO_4$, 15 mM HEPES, and 5.6 mM glucose. pH was adjusted to 7.4 with NaOH. Patch pipettes were pulled from thin-walled borosilicate glass tubing with filament (Clark, Pangbourne, UK) using a DMZ-Universal puller (Zeitz, Augsburg, Germany). Pipette tips were filled with high $K^+$ solution (140 mM KCl, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM glucose; pH 7.4) and had a resistance between 8 and 12 MΩ. Outside-out patches were obtained using standard methods (Hamill et al., 1981). Currents were recorded with an EPC9 patch–clamp amplifier (List Instrumnts, HEKA, Germany). Data were stored on hard disk with a sampling rate of 10 kHz and filtered for analysis with 2 kHz. GABA and glycine were obtained from Sigma (St. Louis, USA). Riluzole was kindly provided by Aventis (Paris, France). Solutions were freshly prepared prior to each experiment. Riluzole was dissolved in dimethylsulfoxiside (DMSO, Fluca Chemika, Buchs, Switzerland) and further diluted to final concentrations.

Fast application of agonist was done using a piezo-driven device for concentration–clamp measurements (Franke et al., 1987). A smooth liquid filament was achieved with a single outflow (glass tubing 0.15 mm inner diameter). 2 s or 2 ms pulses of 1 mM GABA or glycine with different concentrations of riluzole were applied to outside-out patches expressing the respective receptor channels. Between pulses, patches were bathed in a continuously flowing background solution. Addition of riluzole to the background solution did not alter the experimental results. To minimize run-down of currents, the interval between pulses were at least 30 s. Three to five single current traces were averaged for analysis. Desensitization was fitted with the Simplex method. The number of exponential components was incremented until addition of another current component did not significantly improve the fit (Haas and Macdonald, 1999). Experimental data were given as mean ± S.D. Statistical analysis was performed using the independent *t*-test. The data after application of 1 mM glycine were compared to the data obtained in presence of different riluzole concentrations for statistical analysis. Time for solution exchange was $< 100$ μs measured with an open pipette and high electrolyte gradient (Bufler et al., 1996).

## 3. Results

Experiments were performed by application of steps of saturating concentrations of GABA and glycine (1 mM) + different riluzole concentrations to outside-out patches of HEK293 cells with transiently expressed recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ or $\alpha_1\beta$ glycine receptor channels.

Application of 1 mM GABA to outside-out patches of HEK293 cells containing $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels resulted in a current transient with a maximum amplitude of −212 pA in the experiment of Fig. 1A (upper trace). The fast increase of the current was followed by a triphasic decay due to desensitization with time constants of 25 ms ($\tau_{fast}$; mean 28.6 ± 5.1 ms S.D., $n = 8$), 106 ms ($\tau_{intermediate}$; mean 112.2 ± 8.3 ms S.D., $n = 8$) and 1450 ms ($\tau_{slow}$; mean 1385.4 ± 154.3 ms S.D., $n = 8$). The relative proportion of $\tau_{fast}$, $\tau_{intermediate}$ and $\tau_{slow}$ on the whole current amplitude was 5.9% (mean 6.9 ± 3.3% S.D., $n = 8$), 14.1% (mean 16.1 ± 5.5% S.D., $n = 8$) and 50% (mean 47.8 ± 6.2% S.D., $n = 8$), respectively. A steady-state current with an amplitude of 30% (mean 31.4 ± 8.3% S.D., $n = 8$) of the whole current amplitude persisted as previously described (Krampfl et al., 2000). Addition of riluzole to the 1 mM GABA containing test solution had different concentration-dependent effects (Fig. 1A, lower traces). The current decay at 0.03 and 0.1 mM riluzole was best fitted with three time constants and at 0.3 and 1 mM riluzole with two time constants. The peak current amplitude was not altered in presence of riluzole concentrations up to 1 mM (Fig. 2A, $p > 0.5$). However, the proportion of $\tau_{slow}$ and the persistent current on the whole current amplitude decreased with increasing concentrations of riluzole and was completely abolished at concentrations ≥ 0.3 mM riluzole (Figs. 1A and 4A). Additionally, the proportion of $\tau_{intermediate}$ on the whole current amplitude in-

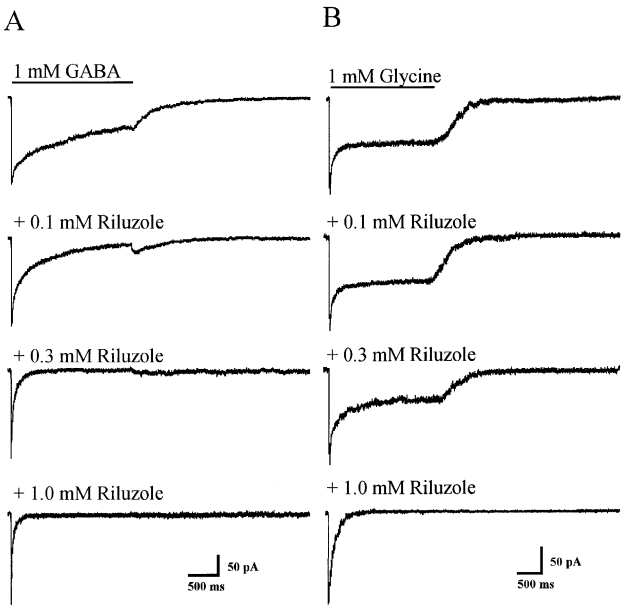


Fig. 1. (A) Current responses of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels recombinantly expressed on HEK293 cells to pulses of 1 mM GABA (upper trace) or 1 mM GABA + different concentrations of riluzole (lower traces). (B) Current responses of $\alpha_1\beta$ glycine receptor channels recombinantly expressed on HEK293 cells to pulses of 1 mM glycine (upper trace) or 1 mM glycine + different concentrations of riluzole (lower traces). Each trace is the average current of three to five single 2-s pulses. The bar above current traces indicates the time of application of the test solution. The outside-out patches from HEK293 cells were clamped to a holding potential of −60 mV during the experiments.

creased to 43% (mean 37.1 ± 3.8% S.D., $n = 7$, $p < 0.001$) at 0.3 mM riluzole and decreased at 1 mM riluzole to 8% (mean 8.2 ± 2.2% S.D., $n = 7$). The proportion of $\tau_{fast}$ on the whole current amplitude increased to 92% (mean 92.7 ± 2.1% S.D., $n = 7$, $p < 0.001$) when 1 mM riluzole was added to the test solution. At this concentration, the current decayed nearly monoexponentially with a time constant of current decay of 27 ms (mean 30.4 ± 5.5 ms S.D., $n = 6$), corresponding to $\tau_{fast}$ (Fig. 1, lower trace; Fig. 4A). As shown in Fig. 3A, the values of $\tau_{fast}$, $\tau_{intermediate}$ and $\tau_{slow}$ did not change at all riluzole concentrations tested ($p > 0.5$).

After the end of a 2-s pulse of 1 mM GABA + 0.1 mM riluzole, a small recovery current occurred. This pointed to a transition from a non-conductive to an open state of the receptor after the end of GABA + riluzole pulses. At higher riluzole concentrations, the recovery current decreased and was no more measured when 1 mM riluzole was added to the test solution (Fig. 1A).

Similar experiments were performed on outside-out patches from HEK293 cells transfected with $\alpha_1\beta$ glycine receptor channels. Application of a saturating concentration of 1 mM glycine (Legendre, 1998) resulted in a current with peak amplitude of −177 pA in the experiment of Fig. 1B (upper trace). The current decreased in presence of glycine best fitted with three time constants ($\tau_{fast} = 3.5$ ms, mean 4.3 ± 1.3 ms S.D., $n = 4$; $\tau_{intermediate} = 27$ ms, mean 24.3 ± 5.3 ms S.D., $n = 4$; $\tau_{slow} = 125$ ms, mean 146.4 ± 37.5 ms S.D., $n = 4$) approaching a persistent current of 59% (mean 55.4 ± 3.9% S.D., $n = 4$). In the experiment of Fig. 1B (upper trace), the proportion of $\tau_{fast}$, $\tau_{intermediate}$ and $\tau_{slow}$ on the whole current amplitude was 6% (mean 5.2 ± 0.2% S.D., $n = 4$), 21% (mean 21.9 ± 0.6% S.D., $n = 4$) and 14% (mean 16.5 ± 7.5% S.D., $n = 4$), respectively. Riluzole application in combination with 1 mM glycine had similar effects on $\alpha_1\beta$ glycine receptor channel currents as on $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channel currents. The peak current amplitude was not affected at all riluzole concentrations tested (0.03–1 mM riluzole, Fig. 2B, $p > 0.5$). However, corresponding to the results observed at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channel currents (Fig. 4B), the proportion of current decay in presence of riluzole was shifted to faster time constants of current decay, whereas the absolute values of the time constants of current decay were not significantly affected (Fig. 3B, $p > 0.5$). When 1 mM riluzole was added to the glycine containing test solution, $\tau_{fast}$ and $\tau_{intermediate}$ had mean values of 4.2 ± 1.8 ms S.D. ($n = 4$) and 26.4 ± 5.0 ms

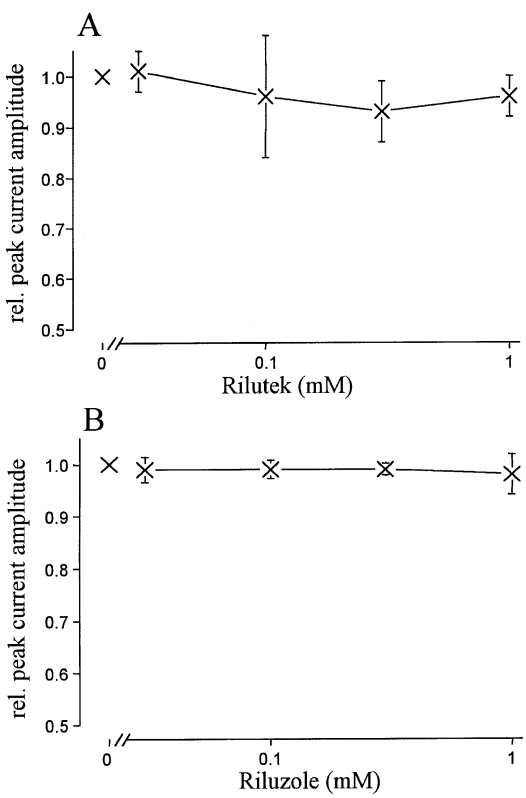


Fig. 2. Dose–response curve for the relative maximal current amplitude of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channel currents or $\alpha_1\beta$ glycine receptor channel currents upon activation by GABA (A) or glycine (B) + riluzole as indicated, respectively. Each point is the average current ± S.D. of seven independent experiments in case of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channel currents and four independent experiments in case of $\alpha_1\beta$ glycine receptor channel currents.

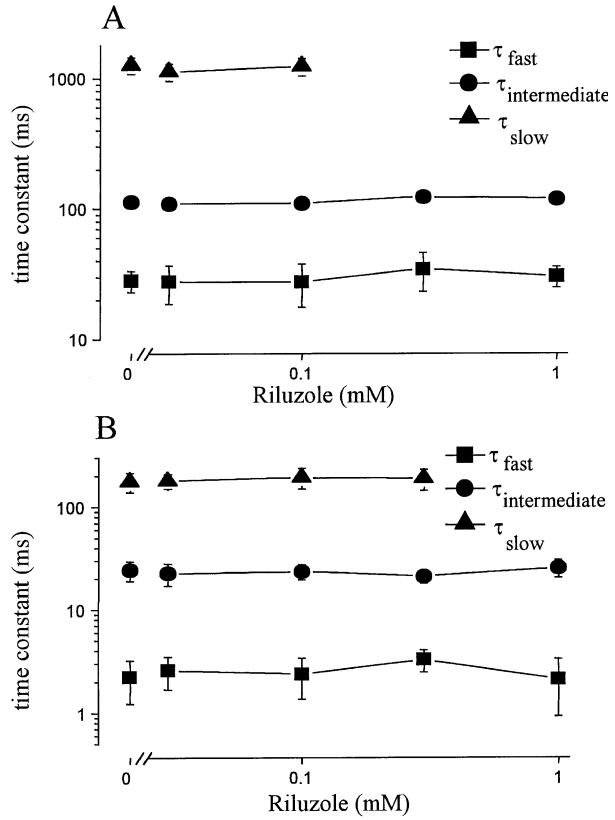


Fig. 3. Dependence of the time constants of current decay $\tau_{fast}$, $\tau_{intermediate}$ and $\tau_{slow}$ on the riluzole concentration. Each point is the average current ± S.D. of seven independent experiments in case of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channel currents (A) or four independent experiments in case of $\alpha_1\beta$ glycine receptor channel currents (B).

S.D. ($n = 4$), respectively. The proportion of $\tau_{fast}$ and $\tau_{intermediate}$ on the whole current amplitude increased to 27.1 ± 3.2% S.D. ($n = 4$, $p < 0.001$) and 72.5 ± 3.9% S.D. ($n = 4$, $p < 0.001$), respectively, whereas $\tau_{slow}$ + the persistent current tended to zero (Fig. 4B).

As can be perceived in Figs. 1–4, the effect of riluzole on $\alpha_1\beta$ glycine receptors was similar but occurred at higher riluzole concentrations than at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channel currents. At 0.1 mM riluzole, $GABA_A$ receptor channel currents activated by 1 mM GABA showed a distinct decrease of the time course of current decay and of the persistent current amplitude whereas glycine receptor channels were nearly not affected by 0.1 mM riluzole (Fig. 4). The sum of the proportion of $\tau_{slow}$ + the persistent current on the whole current amplitude tended to zero when 1 mM riluzole was added to the 1 mM containing GABA or glycine containing test solution (Fig. 4). At high concentrations of riluzole applied to $GABA_A$ receptor channels, the current decreased nearly exclusively with $\tau_{fast}$. In contrast, when 1 mM riluzole was applied to glycine receptor channels, the greatest part of the current decayed with $\tau_{intermediate}$ (Fig. 4B).

A recovery current was not observed after the end of 2-s pulses of 1 mM glycine + riluzole (Fig. 1) as was the case at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels.

Under physiological conditions of synaptic transmission, GABA or glycine is present at the postsynaptic membrane in high concentrations of at least 1 mM for some milliseconds (Jones and Westbrook, 1995; Legendre, 1998). The kinetics of short pulses of 1 mM GABA or 1 mM glycine parallel that of inhibitory postsynaptic currents (IPSC). Therefore, to simulate synaptic transmission, short 2-ms pulses of 1 mM GABA + 1 mM riluzole or 1 mM glycine + 1 mM riluzole were applied to $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels or $\alpha_1\beta_1$ glycine receptor channels.

The current activated by 2-ms pulses of 1 mM GABA reached a maximum amplitude within 0.6 ms and decreased due to unbinding of GABA from the receptor (Fig. 5A). The time course of current decay after application of 1 mM GABA alone was fitted with one time constant of deactivation of 10.1 ms (mean 9.8 ± 1.8 ms, $n = 3$). When 1 mM riluzole was added to the test solution, the time constant of current increased to 20.7 ms (mean 21.4 ± 2.7 ms, $n = 3$, $p < 0.001$). As a result, the hyperpolarizing $Cl^-$ current flow over the membrane with time (calculated as the area under the current traces) increased by 33%

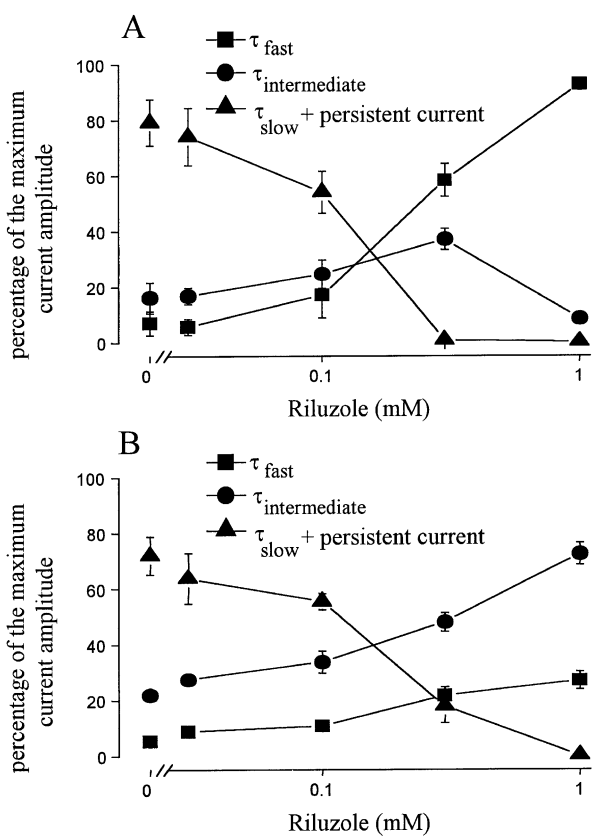


Fig. 4. Dose–response curve of the proportion of $\tau_{fast}$, $\tau_{intermediate}$ and $\tau_{slow}$ + persistent current amplitude on the riluzole concentration added to the 1 mM GABA (A) or 1 mM glycine (B) containing test solution. Each point is the average ± S.D. of seven independent experiments in case of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channel currents or four independent experiments in case of $\alpha_1\beta$ glycine receptor channel currents.

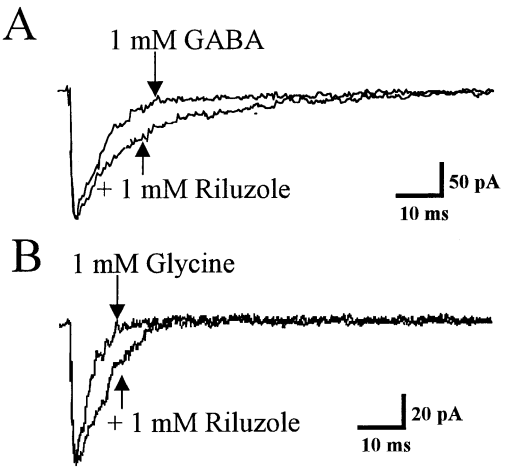


Fig. 5. Outside-out patch current responses of recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels (A) or $\alpha_1\beta$ glycine receptor channels (B) to 2-ms pulses of 1 mM GABA and 1 mM GABA + 1 mM riluzole or 1 mM glycine and 1 mM glycine + 1 mM riluzole, respectively. Outside-out patches were clamped to a holding potential of −60 mV during the experiments.

from 20.2 to 26.9 pA ms (mean 37.4 ± 5.2%, $n = 3$) when 1 mM riluzole was added to the test solution.

Similar results were obtained in the experiments with $\alpha_1\beta$ glycine receptor channels (Fig. 5B). Application of 2-ms pulses of 1 mM glycine resulted in current transient with a time constant of current decay fitted with 4.9 ms (mean 4.5 ± 0.8 ms, $n = 3$). The current decay was also slower in presence of 1 mM riluzole to the test solution (the time constant of current decay was 9.1 ms [mean 9.0 ± 0.5 ms, $n = 3$, $p < 0.001$]).

## 4. Discussion

Riluzole is the first neuroprotective agent where a positive effect on the live span of patients suffering from the neurodegenerative disease amyotrophic lateral sclerosis could be shown in double blind placebo-controlled trials. It has been shown to bind to various membrane proteins and to interact directly with voltage-dependent neuronal sodium channels (Benoit and Escante, 1991), NMDA receptor channels (Debono et al., 1994; Hubert et al., 1994), glutamate release (Hebert et al., 1994) and G-protein mediated signalling pathways (Doble et al., 1992). In the present study, we analyzed direct functional modulation of inhibitory receptor-coupled GABAergic and glycinergic channel complexes by riluzole. Outside-out patches containing recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels or $\alpha_1\beta$ glycine receptor channels showed current transients after application of 1 mM GABA or 1 mM glycine with three time constants of desensitization (Fig. 1) (Jahn et al., 1997; Haas and MacDonald, 1999; Krampfl et al., 2000; Legendre, 1998). Addition of riluzole to the test solution resulted in a concentration-dependent increase of fast desensitization, and decrease of the amplitude of the slower time constants of desensitization and the persistent current (Fig. 4). Neither the maximum amplitude nor the values of the time constants were affected by riluzole also at high concentrations (Figs. 2 and 3). This points to a mechanism different from open channel block (Bufler et al., 1996). At $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels, after reaching the peak current amplitude, the current decayed mainly with $\tau_{fast}$ in presence of 1 mM riluzole whereas the major part of the current of $\alpha_1\beta$ glycine receptor channels decayed with $\tau_{intermediate}$ after application of 1 mM riluzole. $\alpha_1\beta$ glycine receptor channels were less sensitive to riluzole than $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels.

Under physiological conditions, GABA or glycine is present at the postsynaptic receptor at best for some milliseconds. Most $Cl^-$ current during IPSCs flows after removal of GABA or glycine from the synaptic cleft (Jones and Westbrook, 1995; Legendre, 1998). The physiological situation of the postsynaptic site of the synapse was mimicked by experiments with application of short (2 ms) agonist pulses to $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels or $\alpha_1\beta$ glycine receptor channels with and without riluzole added to the test solution of the fast application system (Fig. 5). The increase of the deactivation time constant with increase of current flow after cessation of agonist application in presence of riluzole at both receptors (however more effective in $GABA_A$ receptor channels) points to increased open probability of the receptors after removal of the agonist in presence of riluzole. Recently, a similar effect with increasing deactivation time constants was shown at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels in presence of diazepam (Krampfl et al., 1998).

Recenty published TMS-studies (Prout and Eisen, 1994; Ziemann et al., 1997) showed that cortical hyperexcitability is a characteristic feature of amyotrophic lateral sclerosis. A dysbalance of excitatory (glutamatergic) and inhibitory (GABAergic) input to cortical pyramidal motor neurons may be pathogenetically relevant. It was shown in pharmacological TMS studies that cortical hyperexcitability was reversible after application of Diazepam and gabapentin (Caramia et al., 2000). Similarly, reduced intracortical inhibition could be normalized after treatment of amyotrophic lateral sclerosis patients with riluzole (as shown in double pulse TMS experiments). The effect had a maximum three months after onset of therapy and was still demonstrable after 12 months (Desiato et al., 1999). If these neurophysiological findings can be correlated to the progression of the disease or to a therapeutic benefit is not yet clear so far.

The concentrations of riluzole used in the present study were clearly over the clinical relevant concentration range. However, the results seem to be relevant under two aspects: first, the data show for the first time an interaction of riluzole with inhibitory ligand-gated receptor channels; and second, a molecular mechanism different from open channel or competitive block seems to underlay the ob-

served interaction between $GABA_A$ or glycine receptor channels and riluzole.

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