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Direct Effects of Riluzole on 5-Hydroxytryptamine (5-HT)₃ Receptor–Activated Ion Currents in NCB-20 Neuroblastoma CellsKi Jung Kim¹, Hyeong Seok Cho¹, Se Joon Choi¹, Seung Hyun Jeun¹, Seong Yun Kim¹, and Ki-Wug Sung^{1,*}¹Department of Pharmacology, Medical Research Center, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea

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Abstract. The pharmacological action of riluzole, a drug that has been approved as a neuroprotective agent for the treatment of amyotrophic lateral sclerosis, has not yet been established. We examined the effects of riluzole on 5-hydroxytryptamine (5-HT)₃ receptors in NCB-20 neuroblastoma cells using the whole-cell voltage clamp technique combined with a fast drug application method. Co-application of riluzole (1–300 μ M, 5 s) produced a dose-dependent reduction in peak amplitudes and in the rise slope of the currents induced by 2 μ M 5-HT. In addition, 5-HT₃–mediated currents evoked by dopamine, a partial 5-HT₃–receptor agonist, were inhibited by riluzole co-application. These inhibitory effects were clearly shown at low concentrations of 5-HT. The decay time constants of the receptor desensitization and deactivation were also significantly attenuated by riluzole. G-protein inhibitors (pertussis toxin and guanosine 5'-[β -thio] diphosphate) did not completely block these inhibitory actions of riluzole. These results indicate that riluzole inhibits 5-HT₃–induced ion currents directly by slowing channel activation in NCB-20 neuroblastoma cells.

Keywords: riluzole, 5-HT₃ receptor, ion channel, patch clamp, NCB-20 cell

Introduction

Riluzole (2-amino-6-trifluoromethoxy-benzothiazole) has been used as a neuroprotective agent to treat amyotrophic lateral sclerosis (ALS), possessing several pharmacological properties such as analgesic, anesthetic, anticonvulsant, anti-ischemic, and sedative (1–3). Although the cellular and molecular mechanisms of riluzole are still controversial, it has been proposed that the beneficial neuroprotective effects can be ascribed to the block of presynaptic release of glutamate in the brain and corticostriatal neurons in vivo (2). Recently, it has been reported that riluzole directly modulates the function of various ligand-gated ion channels, including γ -aminobutyric acid (GABA), kainate, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and *N*-methyl-D-aspartate (NMDA) receptors (4–7).

Among the subfamilies of 5-hydroxytryptamine (5-HT) receptors, the 5-HT₃ receptor is the only ligand-

gated ion channel that mediates a transient inward current (8, 9). 5-HT₃ receptors are widely distributed in the central nervous system and are localized to pre-synaptic as well as postsynaptic sites (10). Several studies have reported that the 5-HT₃ receptor mediates fast synaptic transmission at the amygdala and visual cortex (11, 12). It also has been suggested that pre-synaptic 5-HT₃ receptors facilitate neurotransmitter release from the striatum, cortex, hippocampus, hypothalamus, and raphe nuclei (13–17). Studies analyzing the mechanisms underlying 5-HT₃ receptor–mediated synaptic facilitation suggest that receptor stimulation enhances calcium entry via both voltage-gated Ca²⁺ channels and through the 5-HT₃–receptor channel itself (18). All of these results suggest that 5-HT₃ receptors may mediate the neuronal excitability, probably resulting from calcium entry, which is the common mechanism in excitotoxic neuronal damage due to calcium overload.

Although riluzole modulates the function of various ligand-gated ion channels, which may participate in the regulation of neuronal excitability, there have been no reports that riluzole affects 5-HT₃ receptors. Thus, we

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tested the effects of riluzole on 5-HT₃-receptor function using patch-clamp recording combined with a fast drug application technique. Through this experiment, we aimed to provide an additional pharmacological mechanism for the neuroprotective effect of riluzole.

Materials and Methods

Cell culture

NCB-20 neuroblastoma cells (kindly provided by Dr. Lovinger, National Institute on Alcohol Abuse and Alcoholism, USA) were maintained under conditions as previously described (19). Frozen cell stocks were maintained in liquid nitrogen, and thawed as needed. Cells were routinely grown in medium containing 90% Dulbecco's modified Eagle's medium, which contained 3.7 g/l NaHCO₃ (pH adjusted to 7.4 with NaOH), 10% fetal bovine serum, and 1% hypoxanthine aminopterin thymidine supplement (HAT, which gave final concentration of 13.6 mg/l hypoxanthine, 0.178 mg/l aminopterin, and 3.88 mg/l thymidine) and were cultured in an incubator containing 5% CO₂ at 37°C. Cells were seeded onto 35-mm culture dishes at least 2 days prior to electrophysiological examination.

Electrophysiological recordings

Whole-cell patch-clamp recordings were performed in 35-mm culture dishes at room temperature (21°C–24°C) on the stage of an inverted microscope (TS 100; Nikon, Tokyo), as described previously (19). Cells were continuously superfused with an extracellular solution containing 150 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl₂, 10 mM HEPES, and 10 mM D-glucose (pH adjusted to 7.4 with NaOH and osmolality adjusted to 340 mOsm/kg with sucrose) at a rate of 2–3 ml/min. Patch pipettes were pulled from thin-walled borosilicate glass tubing with a filament using a puller (PP-830; Narishige, Tokyo). Pipette tips had resistances of 2.0–3.0 MΩ when filled with an internal solution containing 140 mM CsCl, 2 mM MgCl₂, 5 mM EGTA, and 10 mM HEPES (pH adjusted to 7.4 with CsOH, osmolality adjusted to 310 mOsm/kg with sucrose). Whole-cell currents were amplified using an EPC-7 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) and saved onto a PC using DigiData1322 and pClamp 8.2 software (Axon Instruments, Foster City, CA, USA). All experiments were carried out at a holding potential of –50 mV unless otherwise stated. Currents were filtered at 10 kHz using a three-pole Bessel filter and digitized at 10 kHz. Capacitive currents were compensated with analog compensation, but leak subtraction was not used in this study.

Drug preparation and application

After establishing a whole-cell configuration, cells were placed in front of one side of a θ -tubing pipette. The extracellular solution, which contained the agonist, flowed through a different side of the θ -tubing, which was pulled to an inner diameter of ~300 μ m. Solutions were rapidly switched around the cell using a piezoelectric translator (P-287.70; Physik Instrumente, Walldbronn, Germany), which displaced the θ -tubing laterally so that the cell could be exposed for a defined period of time to the drug-containing solution, and then rapidly returned to the drug-free solution. The piezoelectric translator was controlled by a high-voltage amplifier (E-507, Physik Instrumente) and triggered by pClamp 8.2 software (Axon Instruments). The rate of solution exchange was usually 24.4 ± 5.3 ms ($n = 11$) in the open pipette tip configuration (19). Solution flow was driven by gravity from wells placed above the preparation, and the application of the solutions was controlled by a valve placed upstream of the drug-containing tubing. The agonist and other drugs were diluted in the extracellular solution from stock solutions in distilled water. Riluzole was dissolved in dimethyl sulfoxide (DMSO) and further diluted into the extracellular solution containing the agonist, and thereby delivered simultaneously with the agonist. GDP- β -S was dissolved in the pipette solution from stock solutions (10 mM) and applied directly to the inside of the cells. Pertussis toxin (PTX) was dissolved in distilled water to make stock solutions (100 μ g/ml) and pretreated for 24 h via cell culture medium. Riluzole was purchased from Tocris (Ellisville, MO, USA) and PTX was purchased from Calbiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA). Cell culture reagents were obtained from Gibco BRL (Rockville, MD, USA). Solutions were freshly prepared prior to each experiment. For several minutes after achieving whole-cell configuration, a variable amount of current run-down was typically observed. However, the morphology of current traces stabilized after 5–10 min. To minimize the run-down condition, we waited at least 10 min, and drug applications were carried out by 2-min intervals. However, at the end of the experiments, 5-HT-induced currents were not 100% of the initial current, so the 5-HT-induced currents that decreased more than 20% in comparison with the first current were excluded.

Data analysis

Peak current amplitudes were measured using Clampfit 9.2 software (Axon Instruments) by determining the difference in current at time points set by cursors positioned to measure the current prior to the onset of

agonist application and at the peak current during agonist application. We also analyzed the 20%–80% rise slope (the slope to increase from 20% to 80% of the peak) using the built-in statistical tools in Clampfit 9.2 software, which are commonly used to determine current activation. Since the peak current amplitudes and rise slope induced by agonist application varied between different cells, these values were normalized to the maximal values in each cell. Receptor desensitization was measured as the decay in the agonist-induced response in the continuous presence of agonist. Receptor deactivation was measured as the decay in the response after brief application of the agonist for 50 ms. The decay time constant for desensitized or deactivated currents were calculated by monoexponential fits using Clampfit 9.2 software.

Concentration–response data were fitted to a sigmoid curve, as calculated by the four-parameter logistic equation using Prism 4.0 software (GraphPad Software, San Diego, CA, USA):

$$Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) \text{Hillslope})})$$
, where bottom is the baseline response, top is the maximal response, X is the logarithm of concentration, and IC₅₀ is the concentration of riluzole resulting in 50% inhibition.

All averaged measurements are expressed as the mean ± S.E.M. Statistical significance was determined by Student's *t*-test. The criterion for statistical significance was *P* < 0.05.

Results

Effects of riluzole on 5-HT₃-receptor activation

Firstly, the concentration–response relationship for 5-HT on 5-HT₃ receptor-mediated currents was determined. The application of 0.1–30 μM 5-HT for 5 s at 2-min intervals to NCB-20 cells caused a concentration-dependent inward current at –50 mV, and 10 μM 5-HT induced a near-maximal response. The EC₅₀ value was 2.19 ± 0.11 μM, with a Hill coefficient of 2.67 ± 0.24 (*n* = 6).

We tested the concentration-dependent effects of riluzole on the current activated by 2 μM 5-HT (near EC₅₀). At 2 μM, 5-HT elicited an inward current, which desensitized slowly during the application of the agonist for 5 s (Fig. 1A). Application of 100 μM riluzole alone (without 5-HT) did not induce any current (Fig. 1A). The peak current amplitudes induced by 2 μM 5-HT were decreased by riluzole co-application in a concentration-dependent manner (Fig. 1: A and B; data were normalized to the value of the currents induced by 2 μM 5-HT alone, which was taken as 100%). In addition to reducing the peak current amplitudes, riluzole also

decreased the rise slope of the currents induced by 2 μM 5-HT (Fig. 1: A and B). There was an apparent dose-dependent reduction in the rise slope when compared with the peak amplitude data. The IC₅₀ value of riluzole, based on the rise slope of the currents, was 3.55 ± 0.59 μM, with a Hill coefficient of 1.35 ± 0.23 (*n* = 10). The rapid withdrawal of 5-HT and riluzole induced a transient increase in the tail currents above 100 μM riluzole (Fig. 1A, arrow heads). The amplitudes of the tail currents increased in a dose-dependent manner (Fig. 1C; 12.22 ± 2.77 pA with 100 μM riluzole and 79.68 ± 10.60 pA with 300 μM riluzole, *n* = 10, *P* < 0.001, paired *t*-test).

To investigate the effect of riluzole on the activation process, we used dopamine (DA), a partial agonist at 5-HT₃ receptors. Current activated by 1 mM DA exhibited a slow rise time, smaller peak amplitude when compared with that of the full agonist, and either no decay or a small, slow decay during agonist application for 5 s. These patterns have been well described in previous reports (20), and these reflect that DA might activate the 5-HT₃ receptor without desensitization. Therefore, it was possible to clarify further the effect of riluzole on the activation or opening process of 5-HT₃ receptors. Figure 1D shows that riluzole decreased the 5-HT₃-receptor currents induced by DA in a concentration-dependent manner. Co-application of riluzole reduced the peak current amplitude and the rise slope. The plot of normalized currents as a function of the riluzole concentration yielded an IC₅₀ value for peak amplitude of 12.13 ± 1.57 μM, with a Hill coefficient of 1.08 ± 0.09 (Fig. 1E, *n* = 9; data were normalized to the value of the currents induced by 1 mM DA alone, which was taken as 100%). IC₅₀ value of riluzole based on the rise slope was 4.82 ± 0.92 μM, with a Hill coefficient of 0.78 ± 0.05 (*n* = 9). These results support the idea that the inhibitory action of riluzole on the 5-HT₃ receptor is primarily through the blocking of the channel activation process.

The mode of antagonistic action was further investigated by constructing 5-HT dose–response curves in the absence or presence of 100 μM riluzole. Figure 2A shows that riluzole profoundly inhibited the 5-HT₃-receptor current at low agonist concentration. At concentrations above 3 μM 5-HT, riluzole had little or no effect on the peak amplitude: the EC₅₀ for 5-HT was 2.19 ± 0.11 μM and 2.26 ± 0.09 μM (*n* = 5, *P* = 0.8630, paired *t*-test) and the Hill coefficient was 2.67 ± 0.24 and 3.58 ± 0.17 (*n* = 5, *P* < 0.001, paired *t*-test) for experiments conducted in the absence or presence of 100 μM riluzole, respectively (Fig. 2B; data were normalized to the value of the currents induced by 10 μM 5-HT alone, which was taken as 100%).

Fig. 1. Effect of riluzole on 5-HT₃-receptor activation. A: Sample traces showing the inhibition of 5-HT-evoked (2 μ M, open bar) currents by riluzole (30, 100, and 300 μ M, closed bar). When we treated the cells with riluzole only (100 μ M, top trace), currents were not induced. Note that tail currents (arrow heads) were obtained upon termination of 5-HT and high concentration of riluzole (100, 300 μ M) application. B: Dose-response effects of riluzole on 2 μ M 5-HT-evoked currents. Non-linear regression fit of rise slope data yielded an IC₅₀ value of 3.55 μ M and a Hill slope value of 1.35 ($n = 10$; data were normalized to the value of the currents induced by 2 μ M 5-HT alone, which was taken as 100%). C: Bar graph showing that averaged tail currents were induced upon termination of drug application and were increased by a higher dose of riluzole (300 μ M). * $P < 0.001$, when compared with 100 μ M riluzole ($n = 10$). D: Sample traces showing the inhibition of dopamine-evoked (1 mM, open bar, DA) currents by riluzole (3, 30, and 300 μ M, closed bar). Note that tail currents were still obtained upon termination of 1 mM DA and 300 μ M riluzole co-application. E: Dose-response relationship of riluzole on 1 mM DA-evoked currents. Non-linear regression fit of both peak amplitude and rise slope yielded an IC₅₀ value of 12.13 μ M and 4.82 μ M, respectively, and a Hill slope value of 1.35 and 0.78, respectively ($n = 9$; data were normalized to the value of the currents induced by 1 mM DA alone, which was taken as 100%). Data are expressed as means $\pm$ S.E.M.

However, the rise slope was inhibited by 100 μ M riluzole at all of the concentrations of agonist tested: the EC₅₀ for 5-HT was 7.67 ± 0.06 μ M and 11.47 ± 1.09 μ M ($n = 6$, $P < 0.01$, paired t -test) and the Hill coefficient was 2.29 ± 0.39 and 2.44 ± 0.30 ($n = 6$, $P = 0.2448$, paired t -test) for experiments conducted in the absence or presence of 100 μ M riluzole, respectively (Fig. 2C; data were normalized to the value of the currents induced by 10 μ M 5-HT, which was taken as 100%). These results suggest that riluzole acts in a non-competitive manner.

5-HT₃-receptor currents in cells that had been pre-treated with 100 μ M riluzole for 1 min prior to simultaneous application with agonist were indistinguishable from co-application experiments (Fig. 3: A and B). The rate of current activation (Fig. 3B; $37.29 \pm 6.36\%$ or $47.72 \pm 6.42\%$ of the rise slope of currents induced by 2 μ M 5-HT for co-application with or without 1 min pretreatment with riluzole, respectively, $n = 5$, $P =$

0.2821 , paired t -test) and the normalized peak amplitude (Fig. 3B; $72.58 \pm 9.66\%$ or $68.44 \pm 8.74\%$ of peak amplitude induced by 2 μ M 5-HT for co-application with or without 1 min pretreatment of riluzole, respectively, $n = 8$, $P = 0.7200$, paired t -test; data were normalized to the value of the currents induced by 2 μ M 5-HT alone, which was taken as 100%) were not significantly different with or without 1-min pretreatment of riluzole. These results suggest that riluzole is unlikely to bind to the closed state of the receptor but binds readily to the open or active state.

We also tested the dissociation of riluzole from 5-HT₃ receptors. When 100 μ M riluzole was co-applied with 10 μ M 5-HT for 5 s, a characteristic tail current occurred upon removal in all of the cells tested; however, removal (and wash out) of agonist prior to removal of riluzole did not result in the appearance of such a tail current (Fig. 3C). Figure 3D shows that the normalized rise slope ($75.32 \pm 3.72\%$ or $73.71 \pm 4.38\%$ of the peak

Fig. 2. Non-competitive action of riluzole on 5-HT₃ receptors. A: Sample traces showing that 5-HT-evoked currents (1, 3, 10, and 30 μ M, open bar) were inhibited by riluzole (100 μ M, closed bar). B and C: Dose-response relationship of peak current amplitude and rise slope for 5-HT in the absence (open circles) or presence (closed circles) of 100 μ M riluzole. Non-linear regression fit of the peak amplitude yielded an EC₅₀ value of 2.19 μ M and 2.26 μ M and a Hill slope value of 2.67 and 3.58, respectively (n = 6). Non-linear regression fit of the rise slope yielded an EC₅₀ value of 7.67 μ M and 11.47 μ M and a Hill slope value of 2.29 and 2.44, respectively (n = 6; data were normalized to the value of the currents induced by 10 μ M 5-HT alone, which was taken as 100%). Data are expressed as means $\pm$ S.E.M.

amplitude induced by 10 μ M 5-HT for co-application followed by washing with external containing 100 μ M riluzole, respectively, n = 5, P = 0.8469, paired t -test; data were normalized to the value of the currents induced by 10 μ M 5-HT alone, which was taken as 100%) and the decay time constant (1521.61 ± 294.93 or 1224.65 ± 206.54 ms for co-application followed by washing with external containing 100 μ M riluzole, respectively, n = 5, P = 0.0949, paired t -test) were not significantly different in external solution with or without containing 100 μ M riluzole. These results suggest that riluzole is likely to dissociate from the open state of the receptor, and the dissociation rate of riluzole from the receptor is faster than that of 5-HT.

Effects of riluzole on 5-HT₃-receptor deactivation and desensitization

To examine whether riluzole could alter 5-HT₃-receptor deactivation, a short agonist application was immediately followed by exposure to normal external solution with or without 100 μ M riluzole. We used a high concentration of 5-HT (10 μ M) to induce more

profound channel deactivation following activation, and because the rise time of the current induced by 10 μ M 5-HT for 5 s was 51.30 ± 4.54 ms, we set the agonist application time at 50 ms for this experiment. As shown in Fig. 4A, the presence of riluzole in the wash phase slowed the current decay. On average, the decay time constant was 1095.24 ± 58.11 ms when washed with normal external solution and 2369.89 ± 212.66 ms when washed with external solution containing riluzole (n = 11, P < 0.001, paired t -test).

We also examined the effect of riluzole on receptor desensitization by analyzing the decay time constant of the 5-HT₃-receptor current induced by 10 μ M 5-HT for 5 s with or without 100 μ M riluzole. Figure 4B shows that 100 μ M riluzole significantly increased the decay time constant: 967.66 ± 215.38 ms for 5-HT alone and 2210.81 ± 460.96 ms for riluzole co-application (n = 4, P < 0.05, paired t -test). The tail current was clearly observed in all four of the cells we examined.

Direct action of riluzole on the 5-HT₃ receptor

Other studies have shown that riluzole exerts its

Fig. 3. Effects of riluzole on 5-HT₃ receptors in the open state. **A:** Sample traces showing the currents evoked by 5-HT (2 μ M, open bar) with riluzole (100 μ M, closed bar) co-application with or without riluzole pretreatment for 1 min. **B:** Averaged rise slope and peak amplitude normalized to 2 μ M 5-HT-induced currents. The inhibition by riluzole of the rise slope and peak amplitude was not significantly changed by 1-min pretreatment with riluzole. $P = 0.2821$ for rise slope and $P = 0.7200$ for peak amplitude, when compared with 5-HT with riluzole co-application without pretreatment, respectively ($n = 8$). Data were normalized to the value of the currents induced by 5-HT (2 μ M) alone, which was taken as 100%. **C:** Sample traces showing the 5-HT₃ receptor-activated currents evoked by 5-HT (10 μ M, open bar) with riluzole (100 μ M, closed bar) co-application with or without washing with the external solution containing 100 μ M riluzole. Note that tail currents were abolished by washing with the external solution containing 100 μ M riluzole. **D:** Bar graphs showing the averaged rise slope and decay time constant. The rise slope was normalized to 10 μ M 5-HT-induced currents. $P = 0.8469$ for rise slope and $P = 0.0949$ for decay time constant, when compared with co-application without riluzole washing, respectively ($n = 5$). Data are expressed as means $\pm$ S.E.M. Data were normalized to the value of the currents induced by 5-HT (10 μ M) alone, which was taken as 100%.

effect by intracellular signaling mechanisms through the activation of the G_i/G_o protein (21, 22). To elucidate whether the inhibitory effects of riluzole were mediated via the activation of intracellular G proteins, we used G protein inhibitors, GDP- β -S in the pipette solution and PTX in the culture medium and extracellular solution. Intracellular application of GDP- β -S (2 mM), a non-hydrolyzable GDP analog that inhibits G protein-mediated responses, failed to prevent the inhibitory effects of riluzole. Even after treatment with GDP- β -S, riluzole (100 μ M) still decreased the peak amplitude induced by 2 μ M 5-HT to 85.77 ± 3.46 ($P < 0.001$, paired t -test, $n = 7$) (Fig. 5: A, and B; data were normalized to the value of the currents induced by 2 μ M 5-HT alone, which was taken as 100%). Similar effects have been shown after pre-incubation with 2 mM PTX, a G_i inhibitor, for 24 h in culture medium. When 100 μ M

riluzole was applied with 2 μ M 5-HT, PTX did not completely block the peak amplitude ($84.71 \pm 1.44\%$ of the peak current induced by 2 μ M 5-HT, $P < 0.001$, paired t -test $n = 7$, Fig. 5: A and B; data were normalized to value of currents induced by 2 μ M 5-HT alone, which was set as a standard for normalization 100%). Neither GDP- β -S nor PTX affected the peak current induced by 5-HT alone. There was also significant difference between riluzole effects with and without G-protein inhibitors. However, riluzole still significantly decreased 5-HT₃ receptor-mediated currents even in the presence of GDP- β -S or PTX (Fig. 5B; $P < 0.0001$, unpaired t -test, respectively). These results suggest that riluzole inhibits 5-HT₃-receptor function both through the direct receptor targeting mechanism and G-protein mediated signaling mechanism. Further studies might be needed to clarify which mechanism is relevant significantly to the action

Fig. 4. Effect of riluzole on 5-HT₃-receptor deactivation and desensitization. A: Sample traces showing currents evoked by 5-HT (10 μ M, open bar) for 50 ms (indicated by arrow) in the absence or presence of riluzole (100 μ M, closed bar). The bar graph shows the average effect of riluzole on 5-HT₃-receptor deactivation. The decay time constant for the deactivation was significantly increased by washing with the external solution containing 100 μ M riluzole. ** $P < 0.001$, when compared with 10 μ M 5-HT ($n = 11$). B: Sample traces showing currents evoked by 5-HT (10 μ M, open bar) for 5 s in the absence or presence of riluzole (100 μ M, closed bar). The bar graph shows the average effect of riluzole on 5-HT₃-receptor desensitization. The decay time constant for desensitization was significantly increased by riluzole co-application. * $P < 0.05$, when compared with 10 μ M 5-HT ($n = 4$). Data are expressed as means $\pm$ S.E.M.

Fig. 5. Direct effect of riluzole on 5-HT₃ receptors, by a mechanism other than intracellular signaling. A: Representative currents evoked by 5-HT (2 μ M, open bar, black trace) with riluzole (100 μ M, closed bar, gray trace) in the presence of guanosine 5'-[β -thio]diphosphate (GDP- β -S) or pertussis toxin (PTX). GDP- β -S (2 mM, $n = 7$) was included in the intracellular pipette solution. Cells were incubated with 200 ng/ml PTX ($n = 7$) for 24 h prior to recording. B: Bar graphs showing that the inhibition of 5-HT₃ receptor-activated currents by riluzole was not prevented by the intracellular application of GDP- β -S or by incubation with PTX. Averaged peak amplitude was normalized to 2 μ M 5-HT-induced currents. * $P < 0.001$ when compared with peak currents activated by 2 μ M 5-HT alone; ** $P < 0.0001$, when compared with peak currents activated by 2 μ M 5-HT with 100 μ M riluzole. Data were normalized to the value of the currents induced by 5-HT (2 μ M) alone, which was taken as 100%. Data are expressed as means $\pm$ S.E.M.

of riluzole on 5-HT₃ receptors.

Discussion

Our results suggest that riluzole inhibits 5-HT₃-receptor function by blocking activation, desensitization, and deactivation of the receptor channels because riluzole reduced the peak current amplitude and the rise slope of the current activation phase, and it also increased the decay time constant during agonist application (5 s) and after short agonist application (50 ms). This inhibitory

effect was more intense at relatively low concentrations (below the EC₅₀) of 5-HT, in which the channels were more likely to be in the active state, but lesser extent in the desensitized state (20). Therefore, the effects of riluzole in blocking activation were clearly shown at low agonist concentrations (Fig. 2). Our finding that riluzole also inhibits the effect of DA, a partial agonist at 5-HT₃ receptors (20), with a more reflective dose-dependent pattern (Fig. 1), supports the above explanation because the DA-induced 5-HT₃-receptor currents did not show decay during partial agonist application, implying that

the receptors are mainly in the open state, without profound desensitization (20). These results suggest that riluzole inhibits the channel opening associated with activation of 5-HT₃ receptors. Pretreatment of riluzole for 1 min did not show any inhibitory effects (Fig. 3), and these findings also support the idea that riluzole exerts its effects mainly on the channel-opening step. However, the parameters for the desensitization and deactivation of 5-HT₃-receptor channels were also changed by riluzole (Fig. 4). In many ligand-gated ion channels, the channel desensitization and deactivation processes are correlated with the opening of the ion channels – for example, if more channels are activated, they will be more strongly desensitized and deactivated. In other words, if the rise slope, a measure of channel activation, is increased, the rates of both desensitization and deactivation are more accelerated in an agonist dose-dependent manner (23, 24). Therefore, the inhibition of 5-HT₃-receptor desensitization and deactivation by riluzole in our experiment could be explained by the secondary changes following the inhibition of channel opening. Of course, we could not rule out a direct effect of riluzole on the desensitization and deactivation steps of the 5-HT₃ receptor. Therefore, we need to carry out a further study to elucidate the effect of riluzole on receptor desensitization and deactivation.

The inhibitory effects of riluzole might be mediated by a direct action on 5-HT₃ receptors. As GDP- β -S or PTX are generally used to block G-protein-mediated intracellular signaling mechanisms (25, 26), if riluzole inhibits 5-HT₃-receptor function through the G-protein-related intracellular mechanism, both of these drugs should block the effect of riluzole. However, neither GDP- β -S nor PTX treatment within the pipette solution or culture medium blocked the inhibitory effect of riluzole (Fig. 5: A and B). All of these findings suggest that riluzole exerts its inhibitory effect through 5-HT₃ receptors directly.

One point that needs more explanation is the appearance of the tail currents after cessation of combined riluzole and agonist application (Figs. 1 and 2). The tail currents were not observed when 5-HT was applied alone. Zhou et al. (23) suggested that two distinct mechanisms underlie these tail currents. The first is likely due to the fact that a drug shifts the balance between the open and desensitized states of the receptor and favors the open state. The second possible mechanism is the removal of the strong receptor-blocking effect induced by high concentrations of a drug. Recently, the open channel blocking activity of an agonist has been shown to be the underlying mechanism for the appearance of tail currents in homomeric 5-HT_{3A} receptors (27). All of these reports suggest that the fast

removal of the blocking action is the most likely explanation for the appearance of tail currents by a drug that exerts an inhibitory effect on ion channels. In some of the cells in our experiments (three of eight cells), small tail currents were also evoked by co-application of riluzole and DA, which acts as a weak partial agonist, and therefore only desensitizes 5-HT₃ receptors to a small extent (Fig. 1); in addition, the tail current was not observed when the cells were washed with riluzole (Fig. 3C). These results support the idea that riluzole might block 5-HT₃ receptors and evoked tail currents following abrupt cessation of drug application. These proposals might not offer sufficient explanation because they are all only valid when the dissociation rate constant of 5-HT is slower than that of riluzole (23). More experiments will be necessary to clarify the exact mechanism of the appearance of the tail current after cessation of riluzole and 5-HT co-application.

Finally, presynaptic 5-HT₃ receptors modulate the release of various neurotransmitters in the central nervous system (13 – 17, 28, 29). Despite the fact that riluzole is used as a neuroprotective drug and its pharmacological mechanism is primarily derived from the antagonism of neuronal glutamatergic transmission in the central nervous system, these reports suggest the possibility that riluzole could exert its effect through an action on presynaptic 5-HT₃ receptors. It has been reported that riluzole increased the time constant of fast desensitization and decreased the time constant of slow desensitization and steady-state current of GABA_A and glycine receptors (5). Also, riluzole decreased the activity of AMPA receptors and the kainate-induced currents (4, 7). These results indicate that riluzole inhibits neuronal excitability and protects against excitotoxicity. Another report suggests that 5-HT₃-receptor activation might mediate the neuronal excitability through the enhancement of calcium entry via the 5-HT₃ receptor itself (18). In the present study, we showed that riluzole inhibited 5-HT₃-receptor activation and 5-HT₃ receptor-induced currents. Taken together, these results suggest the possibility that riluzole might contribute to the therapeutic efficacy by potentially acting on the 5-HT₃ receptors. Our data provide experimental evidence to support this possibility. Although our data could not provide exact evidence whether riluzole affects the presynaptic or postsynaptic 5-HT₃ receptors, they suggest that riluzole inhibits 5-HT₃-receptor activation and thereby prevents neuronal hyperexcitability, no matter what the pre- or postsynaptic mechanism is. To our knowledge, this is the first study examining the effect of riluzole on 5-HT₃-receptor function.

In summary, riluzole decreased the amplitudes and the rise slopes of currents induced by low concentration

of 5-HT. Therefore, riluzole directly blocks 5-HT₃ receptor-mediated currents, probably by slowing channel activation.

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