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Inhibition by riluzole of glycinergic postsynaptic currents in rat hypoglossal motoneurones

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1 Riluzole has been shown to have beneficial effects in motoneurone disease, yet its effect on motoneurones is not known. To address this question, we investigated synaptic modulation by riluzole in hypoglossal motoneurones by recording glycinergic inhibitory postsynaptic currents evoked by stimulation of nearby single interneurones.

2 Glycinergic inhibitory postsynaptic currents were evoked by electrical stimulation of single interneurones and were recorded from visually identified hypoglossal motoneurones. Riluzole (10 μM) inhibited mean amplitude of evoked glycinergic inhibitory postsynaptic currents by 87%.

3 We found that riluzole suppressed sodium currents in brainstem interneurones by 23.8%. Riluzole did not modulate barium currents through voltage-activated calcium channels (98% of control). Therefore, the effect of riluzole on synaptic transmission may be mediated, in part, by stabilizing presynaptic neurones through inhibition of voltage-activated sodium currents.

4 In the presence of tetrodotoxin (0.5 μM), riluzole reduced the frequency (1.2 Hz in control to 0.6 Hz in riluzole) of spontaneous transmitter release recorded in motoneurones.

5 Riluzole was found to have no effect on mean miniature inhibitory postsynaptic current amplitude, therefore the reduction in spontaneous transmitter release cannot be due to an action on postsynaptic glycine receptors.

6 We conclude that riluzole inhibits synaptic transmission presynaptically, independent of a reduction in the excitation of presynaptic neurones.

Keywords: Voltage-activated sodium channel; voltage-activated calcium channel; miniature i.p.s.cs; brainstem; glycine; synaptic modulation; amyotrophic lateral sclerosis.

Introduction

Riluzole (2-amino-6-(trifluoromethoxy)benzothiazole), an anticonvulsant and neuroprotective compound, has been shown to have beneficial effects in degenerative motoneurone diseases such as amyotrophic lateral sclerosis (Bensimon *et al.*, 1994; Rowland, 1994). It has been reported that riluzole suppresses voltage-activated sodium channels expressed in *Xenopus* oocytes and in various neuronal systems (Benoit & Escande, 1991; Herbert *et al.*, 1994; Böhme *et al.*, 1994). As sodium currents are essential for neuronal excitation, it is suggested that riluzole stabilizes neurones and as a result, suppresses evoked glutamate release in the central nervous system (CNS). Excessive activation of the NMDA(N-methyl-D-aspartate) type of glutamate receptor results in neuronal degeneration (Abele *et al.*, 1990). Although riluzole inhibited NMDA receptors expressed in *Xenopus* oocytes (Debono *et al.*, 1993), it is believed that the neuroprotective effect of riluzole depends on its suppression of neuronal excitability in the CNS.

Riluzole has been shown to suppress glutamate release by various mechanisms in addition to its action on sodium currents (Stutzmann *et al.*, 1993; Ch'eramy *et al.*, 1992; Martin *et al.*, 1993). In the presence of tetrodotoxin (TTX), riluzole suppresses the high potassium solution-induced release of glutamate and asparate from hippocampal slices (Martin *et al.*, 1993). Riluzole and the muscarinic agonist, carbachol, have been shown to inhibit glutamate-stimulated D-[^{3}H]-asparate release in cultured cerebellar granule cells (Doble *et al.*, 1992). As pertussis toxin-sensitive G-protein was shown to be involved in this inhibition, it is possible that riluzole inhibits neurotransmitter release by mechanisms that are distinct from those requiring sodium current inhibition.

At present, the effect of riluzole on synaptic transmission to motoneurones has not been studied. Recently, we reported on a preparation for the study of both unitary evoked and spontaneous synaptic transmission to rat hypoglossal motoneurones (Umemiya & Berger, 1994b). We have used this preparation to investigate the effect of riluzole on glycinergic synaptic transmission to neonatal rat hypoglossal motoneurones. A study of such cranial motoneurones may be particularly relevant as riluzole is believed to be more beneficial on patients with bulbar onset than on those with spinal onset amyotrophic lateral sclerosis (Bensimon *et al.*, 1994; Rowland, 1994). We found that riluzole suppresses glycinergic synaptic transmission probably by a presynaptic action.

Methods

Preparation

Procedures for whole-cell recording were essentially identical to those described previously (Edwards *et al.*, 1989; Umemiya & Berger, 1994a,b). Briefly, neonatal Sprague-Dawley rat pups (2 to 6 days old) were rapidly decapitated and their brainstems removed and cut into transverse sections (150-200 μm) with a slicer. Slices were incubated at 37°C for at least 1 h.

Recording

Brainstem slices were viewed with Nomarski optics (400×). Patch electrodes were pulled from borosilicate glass capillaries (Clark, England) to a d.c. resistance of 5 MΩ. Whole-cell recordings were made with an EPC-7 amplifier (List, Germany) at room temperature (23-25°C) using the pCLAMP system

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(Axon Instruments). Access resistance was usually <20 MΩ and was compensated by 60–70%. Synaptic currents recorded from motoneurones were evoked by extracellular electrical stimulation of nearby single neurones with a glass pipette containing external solution (Takahashi, 1992; Umemiya & Berger, 1994b). The pipette was placed on the surface of the soma of the stimulated interneurone. Both evoked inhibitory postsynaptic currents (i.p.s.cs) and miniature i.p.s.cs (m.i.p.s.cs) were filtered at 1 kHz with a four-pole Bessel filter (Cornerstone, Dagan), sampled at 5 kHz by the pCLAMP system and analyzed using the pCLAMP system or the programme developed by Dr W.R. Satterthwaite in our laboratory. Voltage-activated sodium currents were filtered at 2 kHz and sampled at 10 kHz. Barium currents passing through voltage-activated calcium channels were filtered at 1 kHz and sampled at 5 kHz. Data from m.i.p.s.cs were analyzed statistically with Student's paired t tests to evaluate hypothesized differences between group means. In all cases significance was accepted if $P<0.05$.

Solutions

Slices were prepared in solutions containing (mM):NaCl 130, $NaHCO_3$ 26, $NaHPO_4$ 1.25, KCl 3, glucose 10, $CaCl_2$ 1, $MgCl_2$ 5. The solution used to maintain slices was similar in composition, but $CaCl_2$ was raised to 2 mM, $MgCl_2$ was reduced to 2 mM and lactic acid (4 mM) was added. External solution contained (mM): NaCl 140, KCl 3, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10 (pH = 7.4 by NaOH). Glycinergic i.p.s.cs were recorded in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 2 μM), bicuculline (10 μM) and D-2-amino-5-phosphonopentanoate (AP5, 10 μM) to isolate glycinergic currents. To record miniature i.p.s.cs, TTX (0.5 μM) was added to the external solution. To measure barium currents through voltage-activated calcium channels, 2 mM $BaCl_2$ was substituted for $CaCl_2$ to minimize run-down of calcium channels and to block potassium conductances, and 1 μM TTX was added to block voltage-activated sodium currents. For voltage-activated sodium current recording, calcium currents were blocked by 0.2 mM $CdCl_2$. The internal solution for synaptic current recording contained (mM): CsCl 120, NaCl 4, $MgCl_2$ 4, $CaCl_2$ 0.5, HEPES 10, EGTA 10 ($E_{Cl} = -3.3$ mV; pH = 7.2 by CsOH). The internal solution for barium and sodium current recording contained (mM): $CsMeSO_4$ 100, TEACl 30, $MgCl_2$ 1, $CaCl_2$ 0.5, NaCl 5, HEPES 10, EGTA 10, ATP-Mg 3, GTP-Tris 0.3 (pH = 7.2 by TEAOH).

Drugs

The following drugs were used: Riluzole (gift from Rhône Poulenc Rorer, Vitry sur Seine, France); tetrodotoxin (Calbiochem, San Diego, CA, U.S.A); AP5, CNQX (Research Biochemicals Inc., Natick, MA, U.S.A); bicuculline (Sigma, St. Louis, MO, U.S.A).

Results

Using whole-cell current recording techniques, we recorded glycinergic inhibitory postsynaptic currents (i.p.s.cs) in visualized neonatal rat hypoglossal motoneurones (HMs). HMs were identified from their location, size and shape (Umemiya & Berger, 1994a). Unitary evoked glycinergic i.p.s.cs were activated by extracellular stimulation of single nearby interneurones and were isolated following blockade of GABA and glutamate receptors (Umemiya & Berger, 1994b). We used symmetrical Cl^- in internal and external solutions ($E_{Cl} = -3$ mV) and held the motoneurone membrane potential at -65 mV; in this condition, Cl^- currents were inward. Bath-applied riluzole (10 μM) reversibly reduced the mean evoked glycinergic i.p.s.c. amplitude by $87.3 \pm 11.3\%$ (mean ± s.e.) ($n = 5$) (Figure 1).

It has been suggested that synaptic inhibition by riluzole is mediated by conduction failure caused by inhibition of voltage-activated sodium channels (Böhme *et al.*, 1994). To test the possible inhibition by riluzole of sodium currents, we measured sodium currents from interneurones (Figure 2a). These neurones were in the same location and had similar morphology to those that were electrically stimulated to produce i.p.s.cs in HMs. To improve space- and voltage-clamp of sodium currents, we recorded mostly from small interneurones (<10 μm). In addition we held the membrane potential at -50 mV to obtain partial inactivation of the sodium currents (Roy & Narahashi, 1992). From this potential, sodium currents were activated by depolarization steps to 10 mV. The mean peak current amplitude was 1250 ± 700 pA ($n = 5$). Riluzole inhibited sodium currents by $23.8 \pm 5.0\%$ (Figure 2a) and the inhibition was partially reversible. Therefore, it is possible that riluzole inhibits conduction in axons and/or excitation of the presynaptic terminal of glycinergic interneurones.

Another possible mechanism for presynaptic inhibition is a reduction in calcium influx when the action potential invades the presynaptic terminal. Since voltage-activated calcium channels are responsible for calcium influx that triggers

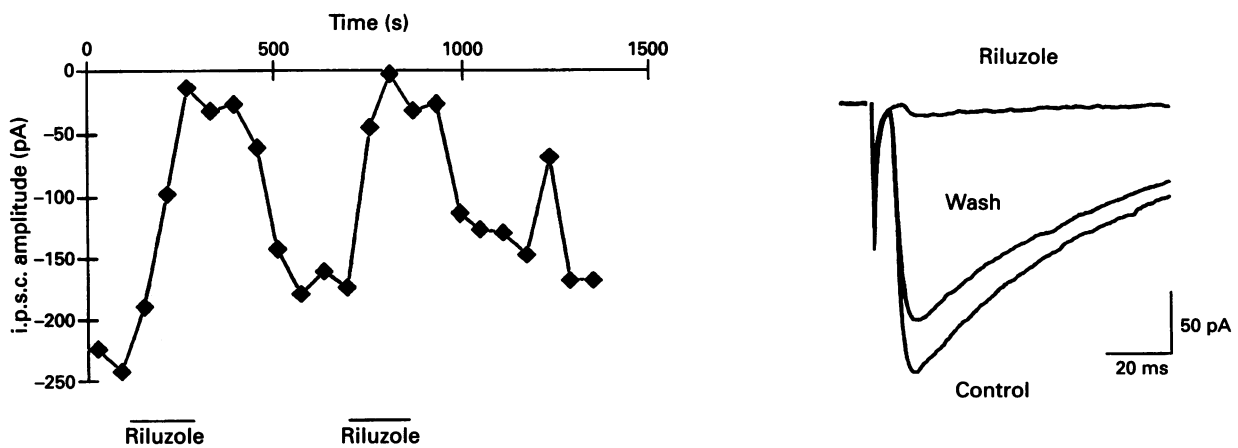

Figure 1 Riluzole inhibits glycinergic i.p.s.cs in rat hypoglossal motoneurones. (a) Time course of the effect of two sequential applications of riluzole (10 μM) on glycinergic i.p.s.cs. Glycinergic i.p.s.cs were activated by stimulation of a nearby interneurone every 3 s and recorded from a HM. Each point indicates average peak amplitude of 20 i.p.s.cs. HM membrane holding potential: -65 mV; E_{Cl} of the recording solution: -3 mV. (b) Sample traces averaged from 20 trials before, during and after application of riluzole.

transmitter release, we tested the effect of riluzole on voltage-activated calcium channels recorded from the somata of interneurones (Figure 2b). Barium currents flowing through voltage-activated calcium channels were activated at 0 mV from a holding potential of −70 mV. The mean amplitude was 762±227 pA ($n=7$) and riluzole did not alter the current amplitude (97.9±1.3% of control) ($n=7$). It is possible, though unlikely, that riluzole inhibits calcium channels in the presynaptic terminal by mechanisms distinct from those in the soma.

Additional evidence supporting the hypothesis that the site of inhibition of synaptic transmission by riluzole is the presynaptic terminal was obtained by recording spontaneous inhibitory postsynaptic currents (s.i.p.s.cs) in HMs (Figure 3). Initially these s.i.p.s.c. were obtained without blockade of sodium currents with TTX. In control conditions, mean s.i.p.s.cs amplitude was 25.2 ± 4.4 pA and mean frequency was 4.3 ± 1.5 Hz ($n=6$). Riluzole reduced the mean frequency of s.i.p.s.cs to 2.0±0.7 Hz ($P<0.05$) without changing mean s.i.p.s.cs amplitude (23.7±5.0 pA in riluzole, $P<0.53$) (Figure 3). Since riluzole did not change the mean amplitude of spontaneous i.p.s.cs, it is unlikely that riluzole postsnaptically inhibits glycine receptors. On the other hand, sodium currents were not blocked by TTX; therefore, it remains possible that the reduction of the s.i.p.s.c. frequency by riluzole may be due to inhibition of sodium currents in glycinergic interneurones. To test this we investigated the effect of riluzole on spontaneous miniature i.p.s.cs (m.i.p.s.cs) recorded in the presence of TTX (0.5 μM). TTX reduced spontaneous i.p.s.c. frequency from 4.7±1.7 Hz to 1.2 ±0.4 Hz ($n=5$) without changing the mean amplitude of the spontaneous i.p.s.cs. Riluzole further reduced m.i.p.s.c. frequency to 0.62±0.31 Hz($P<0.05$) without changing the mean amplitude of m.i.p.s.cs; the mean amplitude was 27.8±4.3 pA and 25.3±4.8 pA in the presence and absence of riluzole, respectively ($P>0.14$). That riluzole significantly reduced the frequency of m.i.p.s.cs supports the hypothesis that the site of inhibition by riluzole is presynaptic. In addition, the result shows that riluzole inhibits transmitter release independent of a presynaptic sodium current-dependent mechanism.

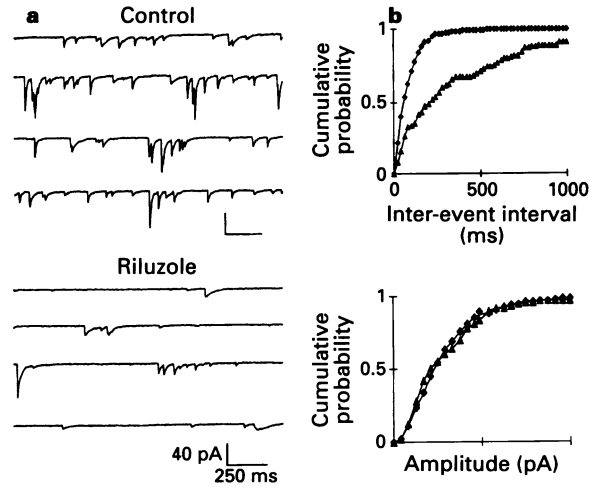

Figure 3 Riluzole reduced the frequency of spontaneous i.p.s.cs without changing their mean amplitude. (a) Current traces of s.i.p.s.cs recorded in a HM in the absence and presence of riluzole (10 μM). In this HM, riluzole reduced s.i.p.s.c. frequency from 10.2 Hz to 5.0 Hz without changing mean s.i.p.s.c. amplitude (34.3 pA and 36.0 pA, in the absence and presence of riluzole, respectively). Motoneurone membrane holding potential: −65 mV. (b) Cumulative inter-event interval distribution (upper panel) and amplitude distribution histogram (lower panel) in Control (◆) and in riluzole (▲).

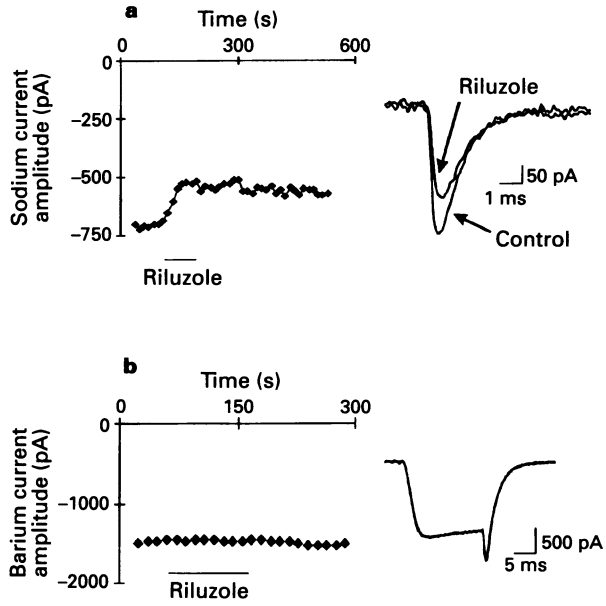

Figure 2 Riluzole inhibits voltage-activated sodium channels but does not modulate voltage-activated calcium channels in interneurones. (a) Voltage-activated sodium current inhibition by riluzole (10 μM). Interneurone membrane holding potential was −50 mV and currents were activated by 5-ms step depolarizations to 10 mV every 12 s. In this neurone, riluzole inhibited peak current amplitude by 24.4% and the inhibition was partially reversible. Right-hand panel: current traces in the absence and presence of riluzole are superimposed. (b) Effect of riluzole (10 μM) on barium current passing through voltage-activated calcium channels. Interneurone membrane holding potential was −70 mV and currents were activated by 20-ms step depolarizations to 0 mV every 12 s. Right-hand panel: current traces in the absence and presence of riluzole are superimposed.

Discussion

Our results show that riluzole has an inhibitory effect on glycinergic synaptic transmission to HMs. It is probable that riluzole has no effect on postsynaptic glycine receptors because riluzole did not change the mean s.i.p.s.c amplitude. Thus, it is likely that riluzole inhibits glycine release to presynaptic mechanisms.

In this study we found that riluzole inhibited voltage-activated sodium currents, but did not modulate voltage-activated calcium currents recorded from the somata of interneurones. Therefore, riluzole may cause an overall reduction in the excitability of the CNS through inhibition of voltage-activated sodium channels (Hebert *et al.*, 1994; Böhme *et al.*, 1994). It is possible that riluzole inhibits conduction along the axon from the stimulated interneurone soma to its presynaptic terminal (Benoit & Escande., 1992). Previously, it has been shown that riluzole inhibits voltage-activated sodium channels in axons (Benoit & Escande., 1991; Hebert *et al.*, 1994). However, our finding that riluzole also inhibits spontaneous transmitter release in the presence of TTX indicates that riluzole also inhibits transmitter release by mechanisms that are independent of inhibition of voltage-activated sodium channels.

Calcium influx through NMDA receptor channels plays a major role in glutamate-induced excitotoxicity (Thomson, 1990; Abele *et al.*, 1990; Kemp & Leeson, 1993). Even though we did not test the effects of riluzole on glutamatergic synaptic transmission, it is possible that riluzole modulates similar presynaptic transmitter release mechanisms that contribute to both glycinergic and glutamatergic synaptic transmission. Recent developments in molecular biology show that proteins at the presynaptic terminal may be common in many types of synapses (Bajjalieh & Scheller, 1995). If riluzole modulates one or more of these proteins, its effect may be generalized to different types of synapses.

A speculative mechanism by which riluzole may reduce excitotoxicity by glutamate is through inhibition of glycine

release, which may in turn reduce NMDA receptor channel activity, as glycine is co-activator of NMDA receptor channels (Thomson, 1990; Johnson & Ascher, 1992; Kemp & Leeson, 1993; McBain & Mayer, 1994). Synaptic boutons on motoneurones abundantly cover the surface of motoneurones (Hagger & Barr, 1950), and, as a consequence, if neurotransmitters from single quanta saturate postsynaptic receptors (Tang *et al.*, 1994), then transmitter could diffuse to neighbouring synapses (Barbour *et al.*, 1994). Thus it is possible that glycine diffuses from its synapse to nearby glutamatergic synapses. Additionally, the effect of synaptically released glycine could be to relieve NMDA receptor channel desensitization, since glycine has been found to suppress desensitization of NMDA receptor channels (Vyklick'y *et al.*, 1990). Therefore, we believe it is possible that a reduction in glycine release may result in reduction in NMDA receptor channel activity, and, as a result, riluzole could retard motoneurone glutamate excitotoxicity.

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