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Effects of riluzole on rat cortical neurones: an *in vitro* electrophysiological study

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1 The electrophysiological effects of riluzole on rat prefrontal and frontal cortical neurones were investigated by using both extracellular (field) and intracellular recording techniques in brain slices.

2 Bath applied riluzole (3–200 μM) depressed the cortico-cortical stimulus-evoked field potential in a concentration-related manner (EC_{50} = 29.5 μM).

3 Riluzole (3–100 μM) reduced the tonic firing of the neocortical neurones which was caused by intracellular current injection, while it did not have any effect on the resting membrane potential and apparent input resistance of these cells.

4 In the presence of tetrodotoxin (1 μM) and tetraethylammonium (30 mM), the injection of a depolarizing current step generated a calcium spike in the neocortical neurones. Riluzole (30 μM) abolished this calcium-dependent action potential. However, when the amount of the depolarizing current was increased the calcium-dependent regenerative potential was evoked again.

5 The depolarization of the membrane (10–20 mV) caused by brief (8–15 s) bath applications of glutamate (300 μM–1 mM) were not changed in the presence of riluzole (30 μM).

6 It is concluded that riluzole has direct actions on rat neocortical neurones: (a) it blocks the repetitive discharge of sodium action potentials and (b) it increases the threshold for the generation of the calcium spike. These two cellular mechanisms might at least in part account for the depression of the cortico-cortical field potential caused by this drug.

Keywords: Neuroprotection; epilepsy; field potentials; sodium and calcium spikes; brain slices; repetitive firing

Introduction

Riluzole is a new neuroprotective drug, which has been suggested to be effective in amyotrophic lateral sclerosis (Bensimon *et al*., 1994; Rowland, 1994), in HIV-induced neocortical lesions (Sindou *et al*., 1994) and in animal models of ischaemic (Malgouris *et al*., 1989; Pratt *et al*., 1992) and traumatic (Stutzmann *et al*., 1996) neuronal damage. In addition, riluzole possesses antiepileptic properties (Mizoule *et al*., 1985; Stutzmann *et al*., 1991). Although, this drug does not interfere with the binding of excitatory amino acid (EAA)-agonists to glutamate receptors (Debono *et al*., 1993), it is generally thought that riluzole reduces the function of the EAA-mediated transmission in the brain (Mizoule *et al*., 1985). In fact, it has been found that riluzole inhibits the release of glutamate and aspartate *in vivo* (Cheramy *et al*., 1992), in slices (Benavides *et al*., 1985; Martin *et al*., 1993) and in neuronal cultures (Hubert & Doble, 1989; Hubert *et al*., 1994). A common pathogenic feature of neurodegeneration and epilepsy is an overactivity of the brain excitatory transmission (Meldrum & Garthwaite, 1990; McNamara, 1994). Thus, a reduction of the efficacy of the glutamatergic transmission has been claimed as an important mechanism of action of anticonvulsant/neuroprotective drugs (Walker & Sander, 1994; Rothstein & Kuncl, 1995). Accordingly, it has been demonstrated that anticonvulsant and neuroprotective agents such as lamotrigine and oxacarbazepine reduce the intracortical field potential which is mediated by the activation of EAA receptors (Calabresi *et al*., 1996).

Although current research suggests that riluzole is certainly a promising drug having neuroprotective and anticonvulsant properties, the mechanisms of action of this compound within the neocortex have not yet been investigated. Therefore, the aim of this work was to examine the effects of riluzole on the cortico-cortical excitatory synaptic transmission and on the intrinsic excitability of the rat frontal and prefrontal neurones maintained *in vitro*, by use of extracellular and intracellular recordings.

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Methods

Preparation and maintenance of cortical slices

Adult male Wistar rats were used and the experimental procedures used have been described previously (Calabresi *et al*., 1996). The brain was removed and coronal slices (200–300 μm) from rat (Wistar, Morini, Reggio Emilia) prefrontal and frontal probes were prepared with the use of a vibratome. A single slice was transferred to a recording chamber continuously perfused with artificial cerebrospinal fluid (ACSF). The ionic composition of the ACSF was in mM: NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 10 and $NaHCO_3$ 18; the solution was gassed with 95% O_2 and 5% CO_2. When $CdCl_2$ was added to the solution NaH_2PO_4 was omitted. The temperature of the perfusing ACSF was maintained in the bath chamber between 34 and 35°C and the perfusion rate varied between 2 and 3 ml min^{-1}.

Electrophysiological recordings

Extracellular recording electrodes were filled with 2 M NaCl (1–10 MΩ resistance); intracellular recording electrodes were filled with 2 M KCl (30–70 MΩ). For synaptic stimulation, bipolar electrodes were used. The stimulating electrodes were positioned 0.5–3 mm distant from the recording electrode. The frequency of the stimulation was 0.05 Hz. The field potentials were obtained following the orthodromic electrical stimulation (10–40 V, 0.03–0.05 ms) of superficial (II-III) as well as deep (IV-V) cortical layers. The field potential amplitude was defined as the average of the amplitude from the peak of the early positivity to the peak of the negativity and the

amplitude from the negativity to the peak of the late positivity. To elicit repetitive firing of action potentials, 500 ms depolarizing current pulses of varying intensity (0.3–0.8 nA) were injected into the cells under current clamp conditions. Tetrodotoxin (TTX, 1 μM) and tetraethylammonium (30 mM) were used to block Na^+ and K^+ currents, respectively. Under these conditions a depolarizing pulse (50–150 ms, 0.7–1.5 nA) evoked a Ca^{2+} spike. An Axoclamp 2B amplifier was used in Bridge mode for recordings. Traces were displayed on an oscilloscope and stored in a digital system (pClamp 5.5). Data are presented as a mean ± s.e.mean.

Drugs

The following drugs were used: riluzole (2-amino-6-trifluoromethoxy-benzothiazole) (a gift from Dr Doble–Rhone Pou-

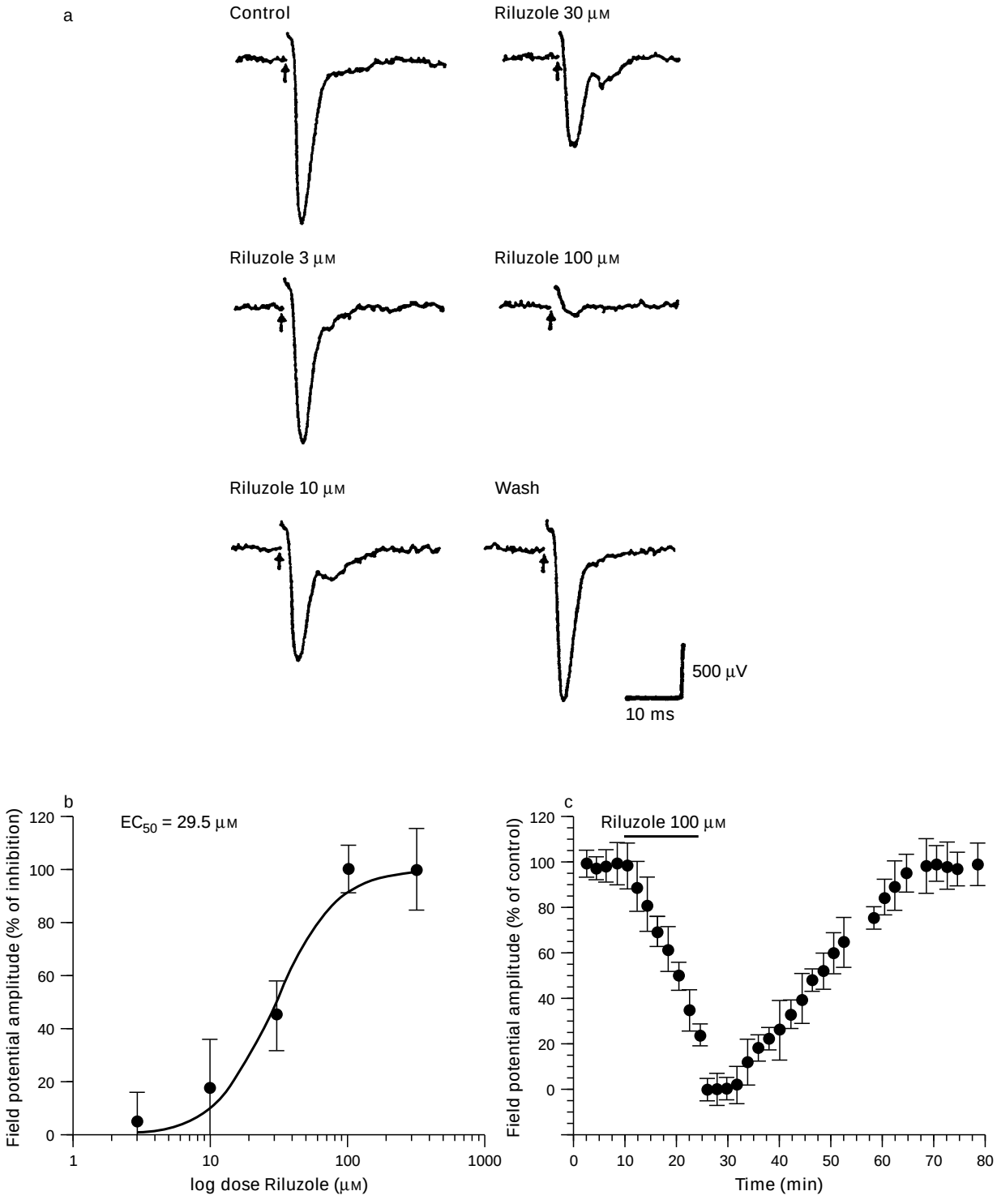


Figure 1 Effects of riluzole on the cortical field potential. (a) The traces (average of four sweeps) obtained from a single experiment illustrate the concentration-dependent and reversible depression of the cortical field by bath application of riluzole. Arrows indicate synaptic stimulation. Note that the stimulus artefact has been erased. (b) Dose-response relationship for the depressant effect of riluzole. Each point (6–9 experiments) shows mean effects and vertical lines indicate s.e.mean. (c) The depression of the field amplitude and its recovery (each point represents the mean of 3–5 experiments). The control amplitude of the field was 1.5 ± 0.2 mV, $n = 5$.

lenc Rorer), tetrodotoxin (TTX, Calbiochem), glutamic acid, cadmium chloride and tetraethylammonium chloride (TEA) (Sigma). Drugs were bath-applied by switching the superfusing solution to one containing known concentrations of substances.

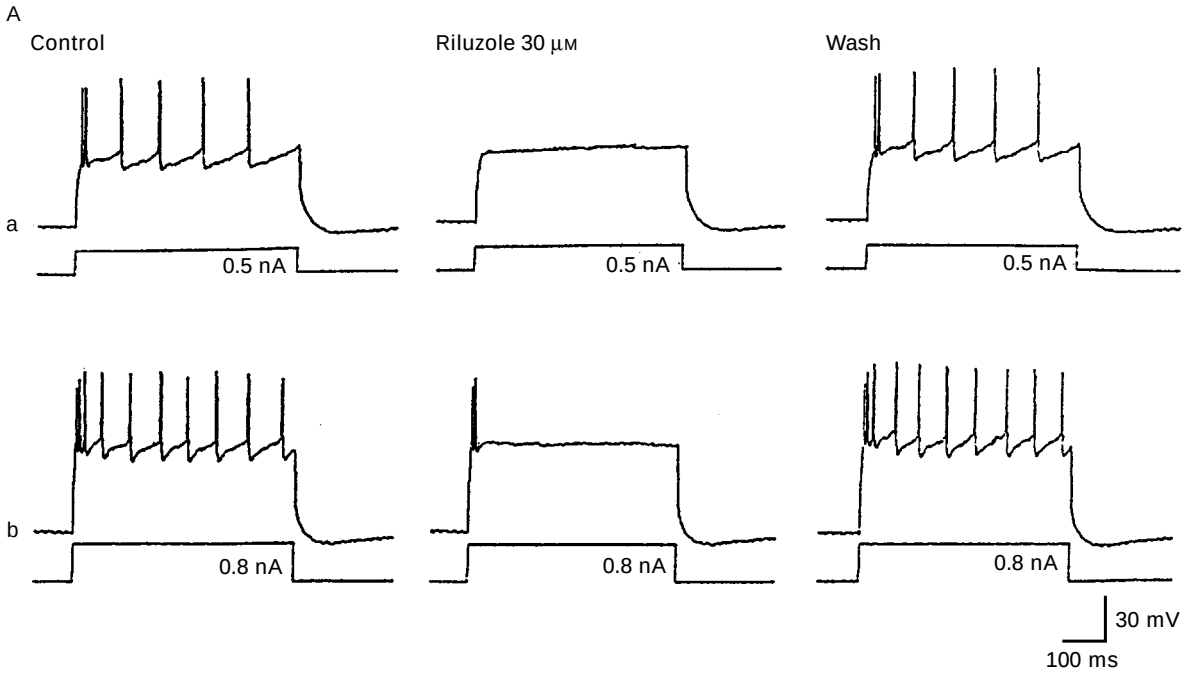
Results

Effects of riluzole on the stimulus-evoked cortico-cortical field potential

In control conditions, cortico-cortical field potentials were recorded from prefrontal and frontal cortical slices ($n=42$). The amplitude of the field potential ranged from 0.5 to 2 mV. Bath application of riluzole (3–200 μM) decreased the amplitude of field potential in a dose-dependent fashion ($EC_{50}=29.5$ μM). As shown in Figure 1 the threshold concentration was 3 μM while the maximal inhibition was obtained with 100 μM riluzole. The latter concentration caused a complete block of the field potential. The depression of the field potential caused by 100 μM riluzole occurred slowly (8–15 min) and recovered after 35–40 min of washout (Figure 1c).

Effect of riluzole on the neuronal firing induced by depolarizing current steps

Stable intracellular recordings were obtained from neurones of the prefrontal and frontal cortical area. These cells had a mean resting potential of -73.1 ± 3.2 mV ($n=22$), spike amplitude from resting membrane potential >75 mV and input resistance of 48 ± 7 MΩ ($n=7$). The electrophysiological and pharmacological properties of these cor-



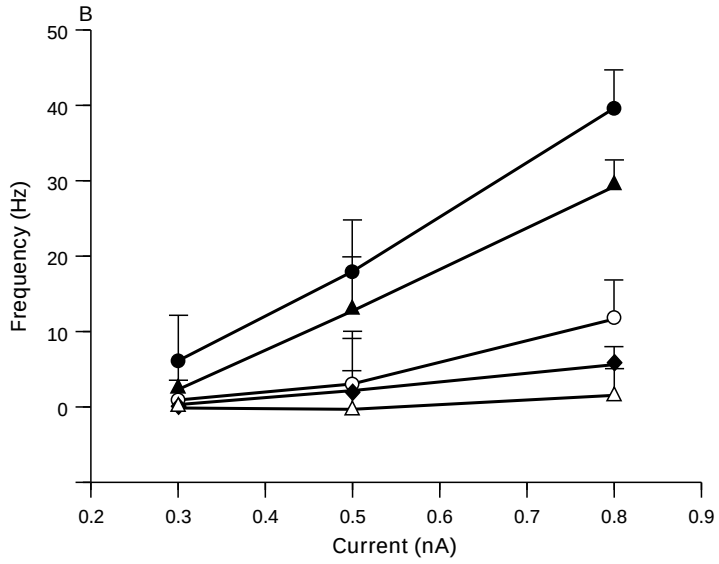


Figure 2 Effects of riluzole on the current evoked firing activity. (A) Riluzole (30 μM) reduced in a reversible manner the number of action potentials elicited by the depolarizing pulses of different intensity. The lower traces in (a) and (b) are current traces; resting potential, −72 mV. (B) Relationship between the number of the action potentials and the intensity of the depolarizing current. The effects of different concentrations of riluzole are shown: (▲) 3 μM, (○) 10 μM, (◆) 30 μM and (△) 100 μM riluzole; (●) control responses. Each point represents mean of 4–5 observations.

tical neurones have been described previously (Connors *et al*., 1982; Connors & Gutnick, 1990). The cells responded to a 500 ms depolarizing pulse (0.3–0.8 nA) with a sustained repetitive firing of action potentials (Figure 2) which were blocked by TTX (1 μM) (not illustrated). As shown in Figure 2 the increase in current intensity increased the frequency of the firing. All the neurones responded to the injection of current with a tonic discharge. Bath application of riluzole (3–100 μM) decreased the number of sodium-dependent action potentials elicited by the current step in 8–15 min. Lower concentrations of 3 μM had no significant effects (Figure 2B). The inhibitory effect of riluzole on firing activity was more evident for the higher rates of firing and could be reversed by washing in approximately 40 min (Figure 2). At concentrations of 10–200 μM riluzole did not change the resting membrane potential and the apparent input resistance of the cortical cells (measured by small hyperpolarizing current pulses, 8–10 mV) (not shown).

Effect of riluzole on the voltage activated Ca^{2+} conductance

A regenerative calcium-dependent action potential was evoked in cortical cells by the injection of a depolarizing current pulse (0.7–1.5 nA, 50–150 ms) during the superfusion of TTX (1 μM) and TEA (30 mM) (Stafstrom *et al*., 1985; Franz *et al*., 1986) and maintaining the holding potential at about −70 mV. Under these conditions, bath application of riluzole (30 μM) suppressed the calcium spike in 8–12 min. The inhibitory effect of riluzole on this potential reversed in 30–40 min of washing ($n=6$) (Figure 3). The inorganic calcium channels antagonist Cd^{2+} (0.3 mM) blocked the calcium spikes ($n=3$) (Franz *et al*., 1986) (Figure 3). It appears that riluzole induces an elevation of the threshold for eliciting the calcium-dependent action potential because an increase of the intensity of the depolarizing current pulse caused the reappearance of this potential (Figure 4).

Effect of riluzole on the glutamate-induced depolarization of the membrane

In order to assess whether riluzole could interact with glutamate ionotropic receptors located at postsynaptic sites, glutamate (300 μM–1 mM for 8–15 s) was superfused on cortical cells before, during and after the application of riluzole 30 μM for 15 min ($n=3$). The repeated application of glutamate caused reproducible subthreshold depolarizations of the membrane (10–20 mV amplitude). It was observed that riluzole had no effect on the glutamate-induced responses (not shown).

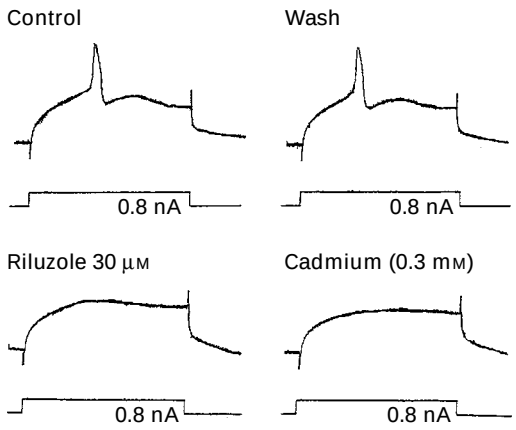


Figure 3 Calcium-dependent regenerative potentials and the effect of riluzole. Bath application of riluzole 30 μM abolished the calcium spike. This effect was reversed after 35 min of washing. Addition of cadmium blocked the tetrodotoxin (TTX)-insensitive action potential. Lower traces in each panel are current traces. The membrane potential was held at −70 mV during the experiment. All the traces were obtained in the presence of TTX and tetraethylammonium (TEA).

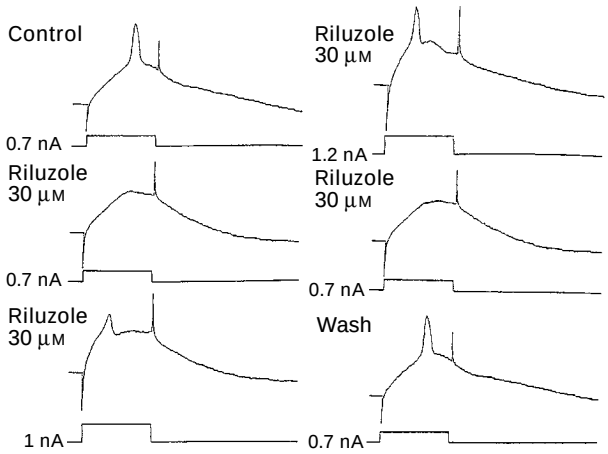


Figure 4 Riluzole increases the threshold for the activation of the calcium spike. After the suppression of the calcium potential by riluzole 30 μM, this could be generated again by increasing the amount of the injected current from 0.7 to 1 and 1.2 nA. The membrane potential was held at −72 mV. Note that the current pulse of 1.2 nA was able to restore the full amplitude of the regenerative potential. All the traces were obtained in the presence of tetrodotoxin and tetraethylammonium.

Discussion

The main finding of the present study was that riluzole inhibits in a concentration-dependent and reversible manner the cortical field potential evoked by intracortical electrical stimulation. We have recently shown that the cortico-cortical field potential of prefrontal and frontal lobe is mainly dependent on the activation of non-N-methyl-D-aspartate (NMDA) glutamate receptors in physiological concentrations of extracellular magnesium (1.2 mM) (Calabresi *et al*., 1996). In accordance with these results previous electrophysiological studies on frontal (Hablitz & Sutor, 1990) and sensorimotor cortex (Hwa & Avoli, 1992) have demonstrated that the intracortical excitatory transmission is mainly mediated by non-NMDA-dependent mechanisms. The depressant effect of riluzole on the non-NMDA-mediated EAA transmission is somehow similar to that produced by lamotrigine and GP 47779, the active metabolite of oxacarbazepine, in the cerebral cortex (Calabresi *et al*., 1996).

Depression of the repetitive firing

We have also found that riluzole inhibits the discharge of the sodium-dependent action potentials (TTX-sensitive) (Stafstrom *et al*., 1985; Connors & Gutnik, 1990) and elevates the threshold for the generation of the calcium spike in neocortical neurones. The suppression of a voltage-dependent sodium current, manifested by the inhibition of the firing activity, is a common mechanism of action of many neuroprotective and anticonvulsant drugs (Matsuki *et al*., 1984; Yaari *et al*., 1986; McLean & MacDonald, 1986; Cheung *et al*., 1992; Van den Berg *et al*., 1993; Walker & Sander, 1994; Capek & Esplin, 1994; Wamil & McLean, 1994; Pisani *et al*., 1995) and might play an important role in the reduction of the field potential amplitude both at pre- and postsynaptic sites. In previous electrophysiological studies it has been found that riluzole blocks sodium channels in the inactivated state (Benoit & Escade, 1991) and inhibits the release of some putative neuro-

transmitters (Benavides *et al*., 1985; Drejer *et al*., 1986; Doble *et al*., 1992; Martin *et al*., 1993; Umemiya & Berger, 1995). In accordance with these findings, we have found that riluzole inhibits the repetitive firing of sodium-dependent action potentials preferentially in the late phase of the depolarizing step. It is worth mentioning that the concentrations of riluzole shown to depress the direct-evoked firing also depress the cortical field with the same time-course. A similar range of doses has been found to reduce the release of glutamate and aspartate in hippocampal slices (Martin *et al*., 1993).

Depression of the calcium-dependent spike

Cortical neurones possess all three major types (L, N, P) of high voltage-activated (HVA) Ca^{2+} channels (Sayer *et al*., 1990; Brown *et al*., 1993). An interference of riluzole with these voltage-activated calcium channels could also explain the reduction of the field potential. In fact, during current clamp recordings in the presence of TTX and TEA which block the voltage-activated sodium and some potassium currents, respectively, the current-evoked Ca^{2+} spikes were depressed by riluzole. The fact that the suppression of Ca^{2+}-dependent regenerative potential was overcome by injecting larger depolarizing pulses, suggests that the suppression of the calcium spike might be due to an increased threshold for its generation. Although riluzole reduces the intracellular increase of calcium evoked by EAA agonists (Hubert *et al*., 1994), to our knowledge this is the first account of a direct depression of a regenerative calcium-dependent event caused by this drug. Thus the reduction of the voltage-dependent influx of calcium ions into the cortical cells might partially account for a diminished EAA transmitter release by limiting the influx of calcium into the presynaptic terminals. It is well known that inhibition of HVA Ca^{2+} currents reduces transmitter release from presynaptic terminals (Takahasi & Momiyama, 1993). A similar depressant mechanism on calcium currents has been recently suggested for the depression of excitatory transmission by oxycarbazepine within the corticostriatal pathway (Stefani *et al*., 1995).

Lack of effect of riluzole on the glutamate induced depolarization

Although Debono *et al*. (1993) demonstrated a direct but non-competitive action of riluzole on glutamate ionotropic receptors, it is unlikely that an interaction of this compound with glutamate receptor-operated channels could account for the depression of the field excitatory postsynaptic potentials (e.p.s.ps). In fact, the postsynaptic sensitivity of cortical neurones to glutamate was not altered by 30 μM riluzole. However, the present data do not rule out the possibility that higher concentrations of riluzole inhibit the excitatory response to glutamate application by interacting with excitatory amino acid receptors (Debono *et al*., 1993).

Conclusions

An enhanced glutamatergic transmission associated with an increased level of intracellular sodium and calcium plays an important role in the pathophysiology of epileptogenesis, the ischaemia/anoxic neuronal damage and neurodegenerative diseases (Grenamyre & Young, 1989; Siesjo & Bengston, 1989; Meldrum & Garthwaite, 1990; McNamara, 1994; Bradford, 1995). In fact, EAA, sodium and calcium antagonistic drugs have been proven to exert neuroprotective and anticonvulsant actions (Meldrum, 1990; Rothstein & Kuncl, 1995; Schachter, 1995; Taylor & Meldrum, 1995). Since riluzole depresses excitatory synaptic transmission in the cerebral cortex very likely through the inhibition of both voltage-activated sodium and calcium channels, the pharmacological properties described in the present study render this drug of interest for the treatment of neurodegenerative and epileptic diseases.

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