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Protective effect of riluzole on excitatory amino acid-mediated neurotoxicity in motoneuron-enriched cultures

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Abstract

Excitatory amino acid-mediated neurotoxicity was investigated in motoneuron-enriched cultures from fetal rats at 12–14 days of gestation. The cultures were mainly composed of differentiated motoneurons identified by choline acetyl transferase and calcitonin gene-related peptide (CGRP) immunoreactivity. Addition of glutamate (600 μM) to the conditioned medium induced no acute neuronal swelling. However, it was followed by a widespread neuronal degeneration over the next 24 h, accounting for 77% of the total cell number. Glutamate toxicity was dose dependent, with an EC_{50} around 300 μM. Treatment for 24 h with the agonists, *N*-methyl-D-aspartate (NMDA, 100 μM), kainate (500 μM) or *RS*-α-amino-3-hydroxy-5-methyl-4-isoxalopropionate (AMPA, 10 μM), also induced a significant cell loss. Riluzole (2 amino 6-trifluoromethoxybenzothiazole), a compound known to interfere with glutamatergic transmission pre- and postsynaptically, significantly reduced glutamate and NMDA neurotoxicity in a dose-dependent manner. These results suggest that a prolonged activation of one or more subtypes of ionotropic excitatory amino acid receptors can lead to motoneuron degeneration in vitro, and provide direct experimental evidence supporting the neuroprotective effect of riluzole in cultured motoneurons.

Keywords: Neurotoxicity; Excitatory amino acid; Glutamate; Glutamate receptor subtype; Motoneuron; Cultured neuron

1. Introduction

The neurotoxicity derived from abnormal excitatory amino acid transmission in the central nervous system has been proposed to contribute to the neuronal loss in a variety of acute and chronic neurological disorders in man (Choi, 1988). Among the different types of central neurons known to be sensitive to glutamate-induced neurotoxicity, spinal motoneurons appear to be particularly vulnerable to excitotoxic insults (Faden and Simon, 1988; Faden et al., 1988). Indeed, motoneuron degeneration has been linked to a number of pathological conditions which may involve prolonged activation of glutamate receptors resulting from abnormal metabolism and increased extracellular levels of glutamate (Plaitakis et al., 1988) and decreased high-affinity glutamate uptake by synaptosomes from spinal cord and motor cortex (Rothstein et al., 1992) in patients suffering from amyotrophic lateral sclerosis. It has been also shown under in vitro conditions that long-lasting blockade of glutamate uptake systems in cultured organotypic spinal cord slices induces a specific degeneration of motoneurons (Rothstein et al., 1993). Moreover, it has been suggested that motoneuron degeneration may be mediated by an endogenous excitotoxin recently identified in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis (Couratier et al., 1993). This neurotoxicity was blocked by these authors using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor antagonist but not by MK 801, an NMDA receptor antagonist. Glutamate toxicity in the spinal cord may involve different pathophysiological mechanisms and a distinct participation of the excitatory amino acid receptors. For example, motoneuron degeneration linked to chronic inhibition of glutamate uptake was prevented by non-NMDA receptor antagonists but was unaffected by NMDA receptor blockers

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(Rothstein et al., 1993). In contrast, the NMDA receptor antagonist, MK 801, has been shown to prevent motoneuron degeneration after traumatic injury of the spinal cord (Mentis et al., 1993) while it was not effective in wobbler mice, a genetic model of motoneuron disease (Krieger et al., 1992). Similarly dextrometorphan, which has been shown to block the NMDA receptor channel in spinal motoneurones (Lodge and Johnson, 1990) has been studied in amyotrophic lateral sclerosis. No positive activity was demonstrated on either of the parameters tested (Norris score, maximum isometric strength; Asmark et al., 1993). In the same way, lamotrigine, a Na^+ channel blocker and consequently a glutamate release inhibitor, was devoid of effect in patients with amyotrophic lateral sclerosis when functional scores or survival rate were studied (Eisen et al., 1993). Thus blockade of glutamate receptors might be a potential way of rescuing motoneurons, though the receptor subtype to be targeted is still a matter of debate.

It has been recently reported that riluzole (2-amino-6-trifluoromethoxy-benzothiazole), a drug known to interfere with glutamatergic neurotransmission (Mizoule et al., 1985; Benavides et al., 1985; Debono et al., 1993), appears to slow the progression of amyotrophic lateral sclerosis and may improve survival in patients with disease of bulbar onset (Bensimon et al., 1994). In the present study we have investigated the neuroprotective effects of riluzole on motoneurons using motoneuron-enriched cultures derived from fetal rat spinal cord. According to previous reports, these cultures are mainly composed of well-differentiated motoneurons (Martinou et al., 1989) and thus may be considered as a useful model to study glutamate neurotoxicity at the cellular level and test the protection exerted by compounds that interfere with glutamatergic transmission. Accordingly, we now describe the vulnerability of motoneurons to long-term exposure to glutamate and specific excitatory amino acid receptor agonists, and the neuroprotective effects of riluzole in this model.

2. Material and methods

2.1. Cell culture

Motoneuron-enriched cultures were prepared from E12-14 rat embryos. Briefly, spinal cords were dissected under sterile conditions, stripped of meninges and dorsal ganglia and collected in ice-cold Dulbecco's modified Eagle's medium (DMEM). After incubation in phosphate buffer saline (PBS) containing glucose (5 mM) and trypsin (0.0025%) for 30 min at 37° C, 1 ml of fetal calf serum was added and cell dissociation was completed mechanically using a 5-ml plastic pipette. Finally, the cells were collected by centrifugation and resuspended in DMEM. The cell suspension was centrifuged again on a 9% metrizamide cushion in DMEM for isolation of motoneurons according to Schnaar and Schaffener (1981). Cells retained in the upper fraction of the metrizamide cushion were plated (10 000 cells/cm^2) on 18 mm side coverslips or microtiter plates (96 wells, 6 mm diameter) precoated with poly-L-lysine (0.1 mg/ml, molecular weight > 300 000). The culture medium was minimal essential medium (MEM) supplemented with glucose (33 mM final concentration), insulin (5 μg/ml), muscle extract (200 μg of protein/ml), fetal calf serum (10%) and horse serum (10%). Muscle extract was prepared from neonatal limb muscle according to Smith and Appel (1983). Cultures were incubated at 37° C in 5% CO_2/95% air and saturating humidity.

2.2. Drug treatments

Excitotoxins and riluzole were dissolved in PBS and NaCl 0.9%-HCl 0.01 N respectively, diluted in fresh culture medium to 100-fold the final concentration and sterilized by filtration. Treatments were made by addition of small aliquots of these solutions to the conditioned medium. In all cases, treatments were applied for 20 h, 24–36 h after plating.

2.3. Assessment of cell viability

The viability of the cultures after treatment with excitotoxins was estimated by colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosmann, 1983; Skaper et al., 1991). MTT is cleaved by living cells with functional mitochondria to generate formazan which specifically labels viable cells with a blue precipitate. Briefly, the culture medium was replaced by Hepes-buffered salt solution containing 1 mg/ml of MTT and further incubated for 2 h at 37° C. Then, the number of blue-stained cells was counted in 3–5 representative fields per plate using an inverted microscope. Finally, MTT-containing saline solution was removed and the precipitated formazan was solubilized in 2-propanol (50 μl) and quantified in a microwell plate reader (EIA, USA) at 570 nm. Preliminary experiments showed that the amount of formazan generated was directly proportional to the number of viable cells stained with MTT. Thus, cell viability was expressed as the percentage of the optical density of untreated control sister cultures.

2.4. Materials

Riluzole was provided by Rhône-Poulenc Rorer. Media and sera were generously provided by Gibco-BRL. Insulin was obtained from Boehringer-Mann-

heim and plastic culture plates were from Corning. All other chemicals were purchased from Sigma.

2.5. Data analysis

All experiments were performed in triplicate and each experiment was repeated at least 3 times. The data are reported as means ± S.E.M. The statistical significance of differences between means was determined by applying the two-tailed Student t-test. Differences were considered significant when $P < 0.05$.

3. Results

Motoneuron-enriched cultures grown for 24-48 h in the presence of muscle extract were mainly composed of neurons (15–30 μM), with few and relatively long neurites, grouped around glial cells that appeared dispersed in the culture (Fig. 1A). The glial cells represented 5–10% of the total cell number. In agreement with previous reports showing the dependence of motoneuron-enriched cultures on trophic factors present in muscle extracts (Arakawa et al., 1990; Bloch-Gallego et al., 1991), impaired cell growth in cultures grown in the absence of muscle extract was found.

Addition of glutamate to the conditioned medium (600 μM final concentration) resulted in a widespread neuronal degeneration that was only apparent several hours after the beginning of the stimulus (Fig. 1C). Twenty hours after glutamate addition, most neurons were replaced by debris while an important proportion of the remaining cells presented signs of degeneration such as dark and granular cell bodies or lysis of neurites. Only a small number of neurons did not show marked morphological abnormalities. No neuronal

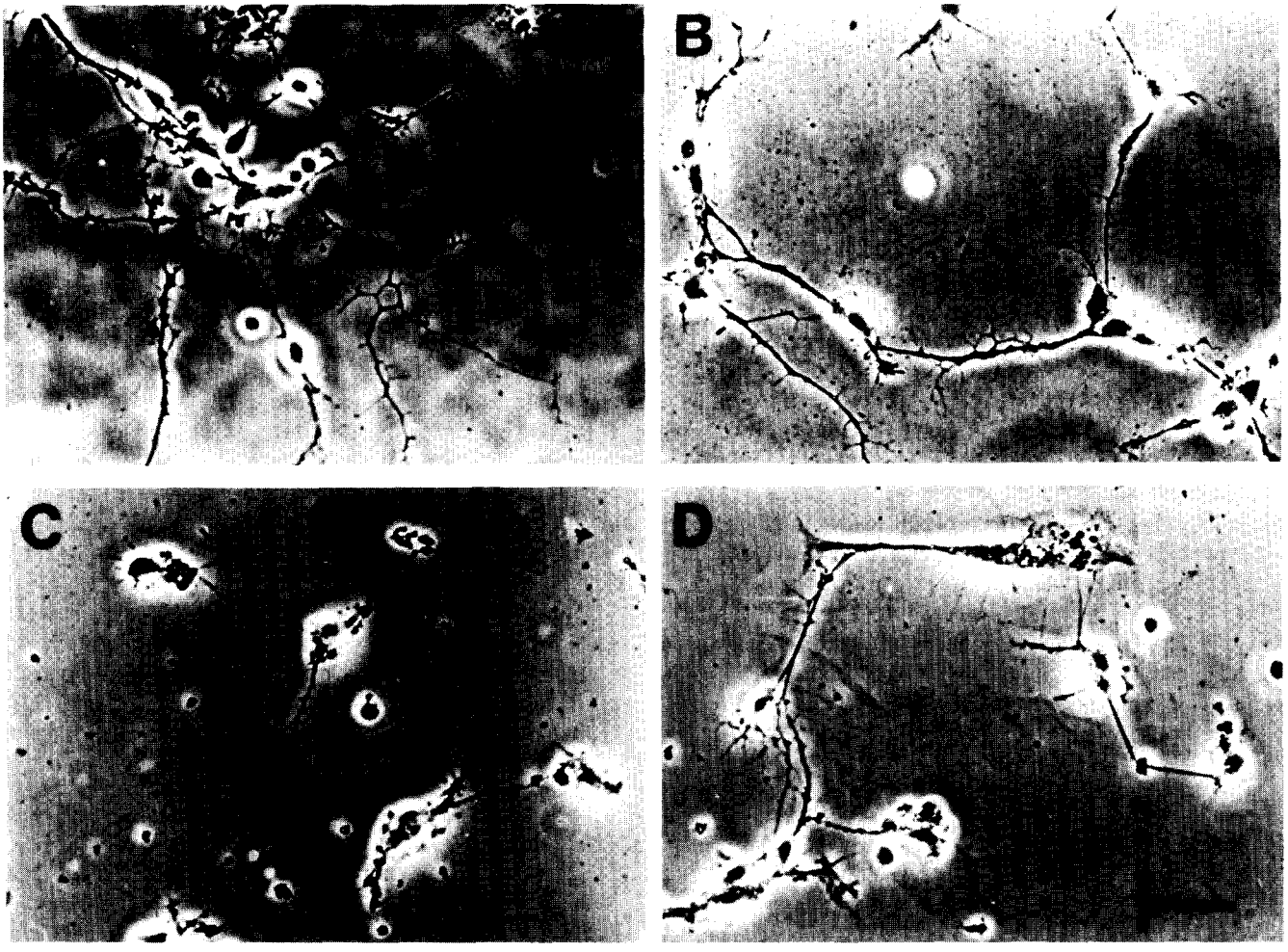

Fig. 1. Representative phase-contrast microphotographs of motoneuron-enriched cultures at 2 days in vitro (DIV). (A) Untreated cultures; (B) after exposure to 600 μM glutamate; (C) 1 μM riluzole; (D) 600 μM glutamate plus 1 μM riluzole. In untreated cultures, healthy differentiated motoneurons are grouped in small aggregates; (B) exposure to glutamate resulted in an extensive neuronal degeneration evidenced by a low number of cells and the appearance of cellular debris; (C) riluzole induced no apparent morphological changes; (D) riluzole partially prevented the effects of glutamate. Scale bar = 50 μM.

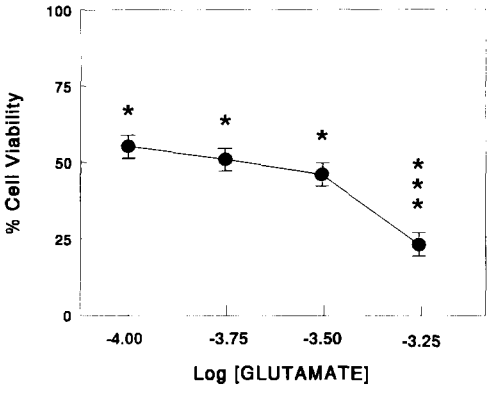

Fig. 2. Dose-response relationship for glutamate neurotoxicity. Sister motoneuron-enriched cultures were exposed to different concentrations of glutamate and cell viability was determined by the MTT colorimetric assay 24 h later. Cell viability in the control group corresponded to 100%. On the abscissa, −4, −3.75, −3.5 and −3.25 corresponded to 100, 200, 400 and 600 μM as a final concentration of glutamate. *Significantly different from control ($P < 0.0001$); ***significantly different from glutamate 100 μM ($P < 0.001$). Values are the means ± S.E.M. from 3–6 experiments run in triplicate.

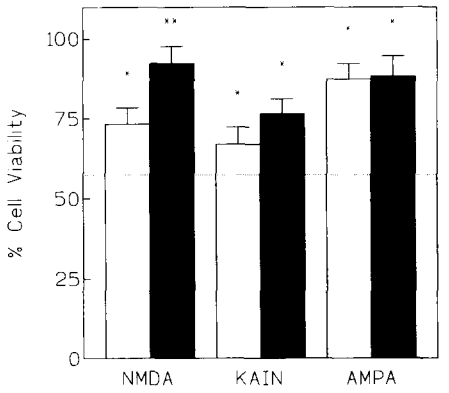

Fig. 3. Excitatory amino acid (EAA)-mediated neurotoxicity in motoneuron-enriched cultures. Cultures grown for 24 h were exposed to 100 μM NMDA, 500 μM kainate or 10 μM AMPA in the absence (empty columns) or in the presence of 1 μM riluzole (filled columns). Cell viability was assessed 24 h later by the MTT colorimetric assay. Values are expressed as percents of the untreated culture values and are the mean ± S.E.M. from 3 different experiments run in triplicate. For comparison, the effect of glutamate (100 μM) on cell viability is shown as a dotted line. *Significantly different from untreated culture values, $P < 0.03$; **significantly different from NMDA, $P < 0.02$.

swelling was observed immediately after glutamate addition and for the next 2 h. Fig. 1B and D shows that while the addition of riluzole (1 μM) to the culture medium did not induce any apparent cellular change in the cultures, the compound was partially able to prevent the morphological effects induced by high concentrations of glutamate.

Fig. 2 shows a quantitative determination of motoneuron viability estimated by MTT labelling versus various glutamate concentrations added to the conditioned medium. Glutamate toxicity in motoneuron-enriched cultures increased with increasing extracellular concentrations of the amino acid. However, the curve obtained showed a non-linear relationship between the two variables, as might have been expected for the narrow range of glutamate concentrations used. Thus, while 100 μM glutamate induced a 42.9 ± 2.9% cell loss, 200 μM and 400 μM concentrations of the amino acid did not induce any further significant cell loss (47.5 ± 4.7% and 52.8 ± 4.9% respectively). In contrast, 600 μM glutamate induced a more extensive neuronal degeneration involving 77.4 ± 2.6% of the cells (Fig. 2).

Riluzole, when added simultaneously with glutamate to the conditioned medium, markedly reduced glutamate neurotoxicity in a concentration-dependent manner (Table 1). Low concentrations of riluzole (0.1 μM) partially prevented the cell loss induced by 100 μM glutamate but not the neurotoxicity induced by 600 μM glutamate. Higher concentrations of riluzole (10 μM) significantly prevented the cell loss induced by 600 μM glutamate. However, it was observed that treatment with riluzole (1 and 10 μM) for 24–48 h resulted in a significant decrease in the number of viable cells as estimated by the MTT method (Table 1), with no morphological effects on the remaining neurons.

The effects of NMDA, kainate and AMPA were also assayed in order to determine whether glutamate neurotoxicity was related to activation of a specific excitatory amino acid receptor subtype. Both NMDA (100 μM) and kainate (500 μM) induced a moderate

Table 1
Effect of riluzole on glutamate neurotoxicity in motoneuron-enriched cultures

Condition	Control	Riluzole		
		0.1 μM	1 μM	10 μM
None	100.0 ± 3.5	94.4 ± 4.4	84.0 ± 4.9 [a]	72.5 ± 1 [a]
Glutamate 100 μM	57.8 ± 2.9 [a]	77.7 ± 5.2 [a,b]	71.2 ± 4.6 [a,b]	ND
Glutamate 600 μM	22.6 ± 2.6 [a]	27.4 ± 2.5 [a]	37.2 ± 4.4 [a,c]	67.3 ± 4.3 [a,c]

Values are the means ± S.E.M. from 3–6 experiments run in triplicate and are expressed as percents of cell viability of untreated cultures. [a] $P < 0.03$ vs. control; [b] $P < 0.02$ vs. glutamate 100 μM; [c] $P < 0.01$ vs. glutamate 600 μM. ND, not determined.

but significant decrease in cell viability with respect to control values (26.9 ± 5% and 34.1 ± 6% respectively, $P < 0.01$). Likewise, AMPA (10 μM) induced a small but significant neuronal loss (12.7 ± 8%, $P < 0.03$). Riluzole (1 μM) significantly reduced the neurotoxic effects of NMDA ($P < 0.02$) and did not show any clear protective effect against kainate and AMPA (Fig. 3).

4. Discussion

The present study used motoneuron-enriched cultures to demonstrate the direct neurotoxic effect of glutamate on isolated motoneurons and the protective effects of riluzole. A slight decrease in the number of motoneurons in cultures treated with riluzole (1 and 10 μM) for 24 h was observed. Although there is no clear explanation for this effect, we speculate that, by decreasing cell excitability and the spontaneous membrane electrical activity of motoneurons, riluzole could affect cell differentiation and survival under culture conditions. Such an effect of neuronal activity on motoneuron differentiation has been reported by Kalb and Hockfield (1992).

Previous studies have shown that motoneuron-enriched cultures are mainly composed of differentiated neurons expressing specific neurochemical markers such as choline acetyl transferase activity and CGRP immunoreactivity (Henderson et al., 1993; Juurlink et al., 1990). In agreement with these reports, most cells recovered a well-defined morphology as soon as one day after plating in the presence of muscle extract. Furthermore, cultured motoneurons become vulnerable to glutamate shortly after plating, suggesting that these neurons also express a critical number of functional excitatory amino acid receptors capable of triggering excitotoxic damage.

It is known that the vulnerability to excitatory amino acids appears after several days or weeks in cultured neurons, e.g. from spinal cord (Regan and Choi, 1991) or cerebral cortex (Choi et al., 1987; Keilhoff and Erdö, 1991; Kho et al., 1991), suggesting that excitotoxicity requires a certain degree of neuronal differentiation and probably the establishment of synaptic contacts between cultured neurons (Choi et al., 1987). We now report an ED_{50} of about 300 μM glutamate in motoneuron-enriched cultures grown for 24 h and treated with the amino acid for 20 h, a value that is higher than those previously reported for spinal cord or cerebrocortical mature primary cultures (Regan and Choi, 1991; Choi et al., 1987). Such differences in glutamate sensitivity may be accounted for by some distinct characteristics of motoneuron-enriched cultures. Firstly, motoneurons are relatively isolated from other spinal cord cell types. Secondly, although cultured motoneurons are clearly differentiated, it is not likely that they could establish functional synaptic contacts shortly after plating. Thirdly, motoneurons are grown in a culture medium containing substantial amounts of muscle extract trophic factors, such as basic fibroblast growth factor and cholinergic differentiating factor (Henderson et al., 1993; Martinou et al., 1992) that could be protecting neurons from excitotoxic degeneration as has been demonstrated in other preparations (Frim et al., 1993).

The low sensitivity of motoneuron-enriched cultures to glutamate seems to be an intrinsic characteristic of the preparation. Because of the low density of neurons and the almost complete absence of astrocytes found in the cultures it is unlikely that the glutamate uptake systems provide protection to motoneurons by decreasing the extracellular concentration of the amino acid. Moreover, cultured motoneurons also showed a very low sensitivity to excitatory amino acid receptor agonists when compared with values reported in the literature for other neuronal types (Frandsen et al., 1989).

The present experiments also provide further evidence that excitotoxic motoneuron damage requires a prolonged activation of excitatory amino acid receptors. In fact, acute exposures to glutamate do not induce significant cell loss in these cultures (results not shown). Interestingly, neurotoxic concentrations of glutamate do not induce acute neuronal swelling in cultured motoneurons as is the case for other neuronal cultures (Rothman, 1985; Choi et al., 1987; Regan and Choi, 1991). This suggests that acute excitotoxic mechanisms may not be important for mediating glutamate neurotoxicity in motoneurons. This observation is in agreement with recent reports showing that long-lasting activation of excitatory amino acid receptors can lead to motoneuron degeneration in spinal cord explants (Rothstein et al., 1992).

The pattern of motoneuron degeneration in response to increasing extracellular glutamate levels (Fig. 2) demonstrates that cultured motoneurons show a heterogenous response to neurotoxic glutamate concentrations. For instance, more than 40% of the cells degenerate at low concentrations of the amino acid (≤ 100 μM) while another 35% of the cells required glutamate concentrations > 400 μM. These results are consistent with the existence of at least two neuronal populations in motoneuron-enriched cultures displaying high and low vulnerability to glutamate, respectively. It should be determined whether these neuronal types correspond to different classes of motoneurons in vivo, or to different stages of differentiation in vitro.

It has been shown previously that excitotoxic neuronal damage is mediated by the pathological activation of one or more excitatory amino acid receptor subtypes (Frandsen et al., 1989). In the present study we have attempted to estimate the involvement of

ionotrophic glutamate receptors in motoneuron degeneration by assessing the effect of neurotoxic concentrations of NMDA, kainate and AMPA (Frandsen et al., 1989). The results show that all three agonists induced a significant motoneuron loss, suggesting that glutamate neurotoxicity in motoneurons may be mediated by the cooperative participation of NMDA and non-NMDA receptors.

In the present study, we show that riluzole exerts partial protection against glutamate toxicity in motoneurons. Riluzole is a compound capable of interfering pre- and postsynaptically with glutamatergic neurotransmission (Benavides et al., 1985; Girdlestone et al., 1989; Chéramy et al., 1992) by a complex mechanism of action involving the blockade of voltage-sensitive Na^+ channels (Benoît and Escande, 1991; Hebert et al., 1994), ionic flux through NMDA channels (Debono et al., 1993) and possibly also by interaction with G proteins (Doble et al., 1992). Accordingly, riluzole blocks glutamate release (Chéramy et al., 1992), decreases the excitatory amino acid-evoked firing of rat facial motoneurons (Girdlestone et al., 1989) and exerts neuroprotective effects in experimental models of chronic or acute neurodegenerative diseases including ischemia (Malgouris et al., 1989; Pratt et al., 1992; Stutzmann and Doble, 1994). The present data show that low concentrations of riluzole in motoneuron-enriched cultures (0.1 and 1 μM) significantly prevent cell damage induced by glutamate and NMDA but fail to prevent kainate and AMPA neurotoxicity. This is in agreement with the finding that riluzole specifically blocks NMDA-evoked firing in rat facial motoneurons in vivo and that NMDA currents in receptors expressed in *Xenopus* oocytes were more sensitive than on AMPA currents (Debono et al., 1993). Thus a direct AMPA blockade, whose efficacy was demonstrated by Couratier et al. (1993), appears not to be the sole mechanism able to confer protection of motoneurones from excitotoxicity. We have shown here for the first time the protective effect of riluzole on excitatory amino acid-mediated neurotoxicity in motoneuron-enriched cultures. Nevertheless, the question of which mechanism of action of riluzole is involved in this neuroprotective effect remains to be clarified.

In conclusion, the present study demonstrated that prolonged activation of one or more subtypes of excitatory amino acid receptors can lead to motoneuron degeneration in vitro. In addition, evidence is provided for a neuroprotective role of riluzole on cultured motoneurons and for its potential use in the treatment of neurodegenerative disorders.

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