Antagonism by riluzole of entry of calcium evoked by NMDA and veratridine in rat cultured granule cells: evidence for a dual mechanism of action

¹J.P. Hubert, *J.C. Delumeau, *J. Glowinski, *J. Prémont & A. Doble

Rhône-Poulenc-Rorer, CRVA 13 quai J Guesde, 94403 Vitry-sur-Seine and *Chaire de Neuropharmacologie U114, Collège de France, 11, place M. Berthelot, 75005 Paris, France

1 Intracellular calcium levels were measured in cultured cerebellar granule cells of the rat by use of the fluorescent dye, indo-1/AM.

2 Intracellular calcium levels were increased by depolarizing stimuli such as N-methyl-D-aspartate (NMDA) (100 μ M), glutamic acid (20 μ M), and veratridine (10 μ M). This increase was essentially due to entry of external calcium.

3 Riluzole (10 μ M) blocked responses to all the depolarizing agents.

4 Riluzole could still block the increase in intracellular calcium evoked by NMDA or glutamic acid when sodium channels were blocked by tetrodotoxin, suggesting that this effect is not mediated by a direct action of riluzole on the voltage-dependent sodium channel.

5 Pretreatment of the cells with pertussis toxin (0.1 μ g ml⁻¹) did not modify the increases in intracellular calcium evoked by NMDA, glutamic acid or veratridine.

6 In pertussis toxin-treated cells, riluzole could no longer block responses to excitatory amino acids, but still blocked responses to veratridine.

7 It is concluded that riluzole has a dual action on cerebellar granule cells, both blocking voltage-dependent sodium channels and interfering with NMDA receptor-mediated responses via a pertussis toxin-sensitive mechanism. Furthermore, these two processes have been shown to be independent.

Keywords: Cerebellar granule cell culture; glutamic acid; indo-1/AM; intracellular calcium; NMDA; riluzole; veratridine

Introduction

Riluzole (2-amino-6-trifluoromethoxybenzothiazole, PK 261-24) is an anticonvulsant drug with an unusual anticonvulsant profile which suggests that it might interfere preferentially with excitatory amino acid-mediated transmission (Mizoule *et al.*, 1985). A number of subsequent studies have shown that riluzole can indeed block excitatory amino acid-evoked events in the central nervous system. These observations were first reported for glutamic acid-evoked guanosine 3':5'-cyclic monophosphate (cyclic GMP) production in cerebellar slices (Benavides *et al.*, 1985), and have since been extended to excitatory amino acid-evoked neuronal firing in the rat facial nucleus (Girdlestone *et al.*, 1989), glutamic acid-evoked calcium entry in cultured neurones (Frandsen *et al.*, 1989), and excitatory amino acid-evoked neurotransmitter release both *in vivo* (Chéramy *et al.*, 1986; 1992; Becquet *et al.*, 1989; 1990) and *in vitro* from brain slices (Benavides *et al.*, 1985; Martin *et al.*, 1993) and cultured neurones (Drejer *et al.*, 1986; 1988; Hubert & Doble, 1989).

However, radioligand binding studies have failed to provide the slightest evidence that riluzole interacts with any of the known binding sites on any excitatory amino acid receptor. Furthermore, riluzole does not antagonize effects of glutamic acid at the metabotropic glutamate receptor coupled to phospholipase C in the striatum or cerebellum (Doble & Perrier, 1989a,b). An interaction of riluzole with an allosteric site on the N-methyl-D-aspartate (NMDA) receptors has however been suggested by recent studies on excitatory amino acid receptors expressed in *Xenopus* oocytes, (Debono *et al.*, 1993). However, it is also possible that indirect effects of riluzole contribute to the blockade of excitatory amino acid-mediated events.

Tsai *et al.* (1987) were the first to suggest that blockade of voltage-dependent sodium channels and subsequent suppression of action potential generation may underlie certain effects of riluzole. This hypothesis was recently confirmed directly by Benoît & Escande (1991), who showed that, in frog sciatic nerve, riluzole stabilizes the inactivated state of the voltage-dependent sodium channel. It would, however, seem unlikely that an effect on the sodium channel underlies all of the effects of riluzole on glutamic acid-evoked events, since certain of these are observed in the presence of tetrodotoxin, (Chéramy *et al.*, 1986; Hubert & Doble, 1989; Martin *et al.*, 1993). In parallel with these studies on sodium channels, we have also shown that the inhibition of glutamic acid-evoked transmitter release from cultured cerebellar granule cells is abolished upon pretreatment of the cells with pertussis toxin (Doble *et al.*, 1992). It was concluded that, in this case, the effect of riluzole involves activation of a G-protein-dependent process.

In the present study, we have examined the actions of riluzole on a system where possible interactions with both voltage-dependent sodium channels and excitatory amino acid receptors can be compared. The experimental paradigm chosen was the elevation of intracellular calcium concentrations ([Ca²⁺]_i) in cultured neurones upon depolarization. Cytosolic calcium levels were monitored with the fluorescent dye indo-1 (Grynkiewicz *et al.*, 1985; Delumeau *et al.*, 1991). The results shows that riluzole does indeed interact with both sodium channels and G-protein-dependent processes, that these two actions are independent and that it is mainly the latter that contributes to the inhibition of glutamic acid-evoked calcium entry in these neurones. These results confirm and extend data that we have obtained previously on mouse cultured mesencephalic neurones (Hubert *et al.*, 1992).

¹ Author for correspondence.

Methods

Cell culture

Granule cell cultures were prepared as described by Gallo *et al.* (1982). Cerebella were removed from 7-day old rat pups (Iffa-Credo, Lyon, France), and dissociated enzymatically. The cell suspensions were plated (1.3×10^5 cells cm^{-2}) onto glass slides 45×70 mm coated successively with poly-L-ornithine ($1.5 \mu\text{g ml}^{-1}$) and laminin ($2 \mu\text{g ml}^{-1}$) in 10 cm Petri dishes in Dulbecco's modified Eagle medium containing 4.5 g l^{-1} glucose supplemented with heat-inactivated foetal calf serum (3%), glutamine (1 mM) and Mixed Hormone mixture (7%). The cells were used between 4 and 6 days in a non depolarizing (5.4 mM KCl) culture condition.

Loading with indo-1/AM

Before each experiment, the nutrient medium was replaced with a modified Hank's solution containing (mM): NaCl 130, KCl 5.4, HEPES 20, glucose 5.5, CaCl_2 1.25, MgCl_2 1 and buffered to pH 7.4. The cells were pre-loaded with indo 1/AM by incubation in 4 ml Hank's solution containing $30 \mu\text{g}$ indo-1/AM, $50 \mu\text{l}$ 2.5% BSA and $2 \mu\text{l}$ dimethylsulphoxide (DMSO) for 1 h at 37°C , then washed and maintained at room temperature in the same modified Hank's solution prior to the fluorescence measurements.

Compound application

The cells on their glass slices were placed in a cylindrical Perspex perfusion chamber (diameter 10.6 cm, depth, 3.4 cm) on the stage of a microscope (Nikon Diaphot). The chamber was perfused horizontally at a rate of 2 ml min^{-1} with modified Hank's solution. Individual cells were identified optically and the microperfusor placed directly above them. Compounds were applied directly to the cell from a gravity

fed remote controlled mutli-barralled microsuperfusion system. The diameter of the tip of the perfusion barrels was 0.2 mm, and the tip was positioned perpendicular to the flow of medium across the chamber. The material ejected from the pipettes was removed by aspiration from the other side of the cell. Normally, depolarizing agents were applied to the cell for 10 s.

Fluorescence measurements

Cytosolic calcium measurements were carried out by dual-emission microfluorometry with the indo-1/AM probe. The cells, loaded with the fluorescent probe, were excited through a $40 \times$ oil immersion objective using a 100 W xenon light, neutrally attenuated to avoid bleaching, and filtered at 360 nm. Excitation and emission beams were separated by a 380 nm dichroic filter. Emission spectra were then divided into two halves by a 455 nm dichroic filter. From the two halves of the indo-1 emission spectra; two signals were selected by interference filters at 405 nm and 480 nm. These signals were recorded by photometers and passed to an amplifier. The fluorescence ratio F_{405}/F_{480} , which is independent of the probe concentration, was directly calculated from both signals. All optics and photometers for indo-1 were obtained from Nikon-France.

Calibration

Cytosolic calcium concentrations were calculated from the ratio according to the following equation described by Grynkiewicz *et al.* (1985) in which K_D is the dissociation constant of indo-1 for ionized calcium ions ($2.5 \times 10^{-7} \text{ M}$), $F_{480(f)}$ is the fluorescence of free indo-1 (1.833 ± 0.182 , $n = 15$), $F_{480(b)}$ the fluorescence of indo-1 bound to calcium (0.436 ± 0.106 , $n = 15$) and $R_{\min}$ (0.244 ± 0.057 , $n = 15$) and $R_{\max}$ (2.132 ± 0.277 , $n = 15$) were determined in the presence of ionomycin

Figure 1 Effect of different depolarizing agents in the increase in intracellular calcium. Data represent a typical recording from a single cell exhibiting a response pattern similar to and representative of those obtained in 12 cells: (a) responses to increasing concentrations of veratridine (3, 10, 30, 100 μM); (b) responses to glutamic acid (20 μM), and *trans* 1S,3R ACPD (300 μM); (c) responses to increasing concentrations of NMDA (10, 30, 100, 300 μM); (d) responses to increasing concentrations of glutamic acid (3, 10, 30, 100 μM); (e) responses to glutamic acid (20 μM) with or without EGTA (3 mM).

(10^{-5} M) and either EGTA (5×10^{-3} M) or CaCl_2 (5×10^{-3} M) respectively.

$$[\text{Ca}^{2+}]_i = K_D \times \frac{F_{480(f)}}{F_{480(b)}} \times \frac{R - R_{\min}}{R_{\max} - R}$$

Reagents

Riluzole was synthesized by Dr P. Jimonet at Rhône-Poulenc-Rorer, Vitry, France. Pertussis toxin was purchased from List Biologicals Inc, San Diego, U.S.A. Tetrodotoxin, D-serine, lanthanum chloride, glutamic acid, NMDA (N-methyl-D-aspartic acid), excitatory amino acids and veratridine were from Sigma Chemical Co, St. Louis, U.S.A.; quisqualic acid, (1S,3R) ACPD (1 aminocyclopentane-1S, 3R-dicarboxylic acid), CNQX (6-cyano-7-nitroquinoline-2,3-dione), and MK 801 ((+)-5-methyl-10, 11-dihydro-5H-dibenzol [a, d] cycloheptene-5-10-imine) from Research Biochemicals Inc, Natick, U.S.A. Cell culture media were purchased from GIBCO, Glasgow, UK, except the NU-Serum which was from Collaborative Research. Indo-1/AM was obtained from Molecular Probes Inc, Eugene, U.S.A.

Results

Rat cerebellar granule cells grown in primary culture had basal intracellular calcium levels of 142 ± 5 nM ($n = 104$). Superfusion of these cells with L-glutamic acid ($20 \mu\text{M}$) led to a rapid rise in intracellular calcium levels to 906 ± 34 nM ($n = 60$), which quickly returned to basal upon removal of glutamic acid (Figure 1). In the presence of D-serine ($10 \mu\text{M}$) and LaCl_3 ($1 \mu\text{M}$) in nominally magnesium-free medium, basal calcium levels were 169 ± 6 nM ($n = 106$), which increased to 827 ± 50 nM ($n = 63$) in the presence of NMDA ($100 \mu\text{M}$, Figure 2). Quisqualic acid ($300 \mu\text{M}$) had a small stimulatory effect on intracellular calcium 347 ± 42 nM on some, but not all of the cells tested (13/20). (1S,3R) ACPD

($300 \mu\text{M}$) was inactive ($n = 15$). Veratridine ($10 \mu\text{M}$) increased intracellular calcium levels to 1079 ± 66 nM ($n = 30$, Figure 1). Depolarization with KCl (20 mM) also raised intracellular calcium levels (694 ± 86 ; $n = 12$). The EC_{50} values for the different depolarizing agents are summarized in Table 1, and typical dose-response curves in Figure 1. Stimulations by the different depolarizing agents could be reiterated up to six times without observing tachyphylaxis or drift of the basal intracellular calcium concentrations.

Tetrodotoxin (TTX, $1 \mu\text{M}$) completely abolished the response to veratridine ($10 \mu\text{M}$) in these cells (Figure 2). In contrast, the response to glutamic acid ($20 \mu\text{M}$) was unaffected by tetrodotoxin. The response to glutamic acid could, however, be markedly reduced by the NMDA receptor antagonist MK 801 ($1 \mu\text{M}$, Figure 3). The NMDA-evoked response was also antagonized by MK 801. The relevant IC_{50} values are given in Table 2. The stimulatory effects of glutamic acid were not modified by inclusion of LaCl_3 in the incubation medium, whereas those of veratridine were abolished (data not shown). When external calcium was

Table 1 Increases in intracellular calcium evoked by excitatory amino acids and veratridine

Agonists	$\text{EC}_{50}(\mu\text{M})$
<i>trans</i> -1S,3R ACPD	NA
Glutamic acid	8 ± 0.6
Quisqualic acid	NA
NMDA	18 ± 1
Veratridine	8.3 ± 0.8

Four-point cumulative concentration-response curves were obtained for each cell and the EC_{50} values determined by computer-assisted iterative non-linear regression analysis. Data are expressed as the mean $\pm$ s.e.mean of EC_{50} values obtained from 12 individual cells.

Figure 2 Top: effect of tetrodotoxin ($1 \mu\text{M}$) on the increase in cytosolic calcium evoked by glutamic acid ($20 \mu\text{M}$; a), NMDA ($100 \mu\text{M}$; b), or veratridine ($10 \mu\text{M}$; c). Bottom: effect of riluzole ($1 \mu\text{M}$ versus glutamic acid (d); $100 \mu\text{M}$ versus NMDA (e); or $10 \mu\text{M}$ versus veratridine (f)); the sodium channel blocker TTX ($1 \mu\text{M}$) was added after the third stimulation. Data represent a typical recording from a single cell exhibiting a response pattern similar to and representative of those obtained in 12 cells from three different preparations.

removed and buffered with EGTA (3 mM), the rise in intracellular calcium evoked by glutamic acid (20 μ M) was completely abolished (Figure 1).

Riluzole inhibited the elevation of intracellular calcium evoked by glutamic acid and by veratridine with IC_{50} values of 0.30 ± 0.07 , 0.31 ± 0.02 respectively (Figure 3, Table 2). A similar inhibition of the NMDA response was observed at somewhat higher concentrations (Figure 3, Table 2), but K^+ -evoked $[Ca^{2+}]_i$ was unaffected (data not shown). The inhibitory effect of riluzole on the glutamic acid response was not modified in the presence of TTX (Figure 2). It was, of course, not possible to study veratridine-evoked responses in the presence of TTX. MK 801 blocked the responses to glutamic acid ($IC_{50} = 0.11 \pm 0.01$ μ M) and NMDA ($IC_{50} = 0.074 \pm 0.020$ μ M), but not to veratridine (Figure 3, Table 2). When the cells were pretreated for 24 h with pertussis toxin (0.1 μ g ml^{-1}), no changes were observed in either basal calcium levels in standard conditions 144 ± 9 nM ($n = 24$) or in nominally magnesium-free medium 158 ± 13 nM ($n = 24$) res-

Table 2 Inhibition of depolarization-evoked calcium responses by riluzole and MK 801

	Glutamic acid (12)	NMDA (12)	Veratridine (12)
Riluzole	0.30 ± 0.07	12.8 ± 2.4	0.31 ± 0.02
MK 801	0.11 ± 0.01	0.074 ± 0.020	NA

The values are expressed as the mean $\pm$ s.e.mean (μ M) of at least 12 observations from cells taken from three separate preparations. Individual cells were stimulated three times with a fixed concentration of glutamic acid (20 μ M), NMDA (100 μ M), or veratridine (10 μ M). Antagonists were added during the second stimulation. Three concentrations of antagonist were tested on separate populations of 12 cells. The percentage inhibition of the agonist response was calculated and the IC_{50} values determined by regression analysis as a function of the logarithm of the antagonist concentration performed on the total population of observations.

Figure 3 Inhibition of calcium responses to depolarizing agents by riluzole and MK 801. Data are expressed as the mean $\pm$ s.e.mean of the percentage inhibition of responses to glutamic acid (20 μ M; $\square$), NMDA (100 μ M; $\square$) or veratridine (10 μ M; $\square$), and were obtained from 12 cells from at least three different preparations at each concentration. (a) Inhibition by MK 801; (b) inhibition by riluzole.

Figure 4 Effect of pertussis toxin on inhibition by riluzole of calcium responses to depolarizing agent. Data are expressed as $[Ca^{2+}]_i$; the dotted horizontal line represents the basal intracellular calcium levels in these cells. Each column represents the mean $\pm$ s.e.mean of data from 12 cells from three different preparations. The cells were stimulated three times with glutamic acid (20 μ M; (1)), NMDA (100 μ M; (3)) or veratridine (10 μ M; (5)) following the protocol illustrated in Figure 5, and riluzole added during the second application: (2) glutamic acid + 1 μ M riluzole; (4) NMDA + 100 μ M riluzole; (6) veratridine + 10 μ M riluzole. A significant ($P \leq 0.005$; Wilcoxon's test) inhibition by riluzole is indicated by the asterisk.

Figure 5 Effect of pretreatment with pertussis toxin on glutamic acid-evoked responses. Pretreatment of granule cells with pertussis toxin (PTX) ($0.1 \mu\text{g ml}^{-1}$) for 20 h before the fluorescence measurement did not modify the increase in cytosolic calcium evoked by L-glutamic acid ($20 \mu\text{M}$), ((a) control and (c) PTX). Such a treatment did however, completely abolish the inhibition by riluzole ($1 \mu\text{M}$), (b) control, and (d) PTX). Data represent a typical recording from a single cell exhibiting a response pattern similar to and representative of those obtained from three different cell preparations and represent 12 cells.

pectively, or in glutamic acid $822 \pm 58 \text{ nM}$ ($n = 12$), or in veratridine $995 \pm 81 \text{ nM}$ ($n = 12$), and in NMDA $783 \pm 62 \text{ nM}$ ($n = 24$) evoked $[\text{Ca}^{2+}]_i$. Such a treatment completely abolished the inhibitory effect of riluzole on the responses evoked by glutamic acid (Figures 4 and 5) and NMDA (Figure 4). The inhibitory effect of MK 801 persisted in the cells pretreated with pertussis toxin ($87 \pm 3\%$ inhibition at 10^{-6} M ; data not shown). On the other hand, responses to veratridine in pretreated cells could still be blocked by riluzole.

Discussion

The feasibility of using a dual-emission microfluorimetric system for the measurement of intracellular calcium in single cerebellar granule cells has been demonstrated by Irving *et al.* (1992). This study extends the use of the technique from previous applications in, for example pituitary cells (Mollard *et al.*, 1989), cultured rat spinal neurones (Schieren & MacDermot, 1988) and astrocytes (Delumeau *et al.*, 1991). We have applied this technique to study the effect of riluzole on depolarization-evoked $[\text{Ca}^{2+}]_i$ in single identified neurones. In these cells, $[\text{Ca}^{2+}]_i$ can be observed after depolarizing the cells with either excitatory amino acids, potassium ions or the sodium-channel activator, veratridine.

The responses to excitatory amino acids seem to be mediated essentially by receptors of the NMDA subtype, since the metabotropic receptor agonist 1S,3R *t* ACPD and the mixed metabotropic and AMPA receptor agonist, quisqualic acid, did not lead to pronounced increases in intracel-

lular calcium. Furthermore, the highly selective NMDA antagonist, MK 801, completely abolished responses to both glutamic acid and NMDA with indistinguishable IC_{50} values. These findings are partially in agreement with those of Pearson *et al.* (1992) using the fura-2 technique, who found that the dominant $[\text{Ca}^{2+}]_i$ response to excitatory amino acids in those cells cultured under these conditions was mediated by NMDA receptors. However, in contrast to Zhao & Peng (1993), we did not observe responses to metabotropic receptor agonists, even though the cells were grown in non-depolarizing conditions. On the other hand, Nicoletti *et al.* (1993) did not observe expression of metabotropic receptors coupled to phospholipase C in cerebellar granule cells cultured in low potassium medium. It is also likely that the rises in intracellular calcium are due to calcium entry via the NMDA receptor channel, since they are not modified by blocking voltage-sensitive calcium channels with lanthanum chloride and are no longer seen in the absence of extracellular calcium. This is supported by recent studies by Simpson *et al.* (1993), who have demonstrated that the rise in intracellular free calcium levels evoked by NMDA receptor activation is essentially due to calcium entry. Moreover, Frandsen *et al.* (1989) demonstrated that glutamic acid directly stimulated $^{45}\text{Ca}^{2+}$ entry into cultured cortical neurones. Riluzole was able to antagonize the increase in cytosolic calcium evoked by glutamic acid and NMDA at concentrations previously shown to block the effects of excitatory amino acids in other systems (Benavides *et al.*, 1985; Drejer *et al.*, 1986; Hubert & Doble, 1989) and at which blockade of NMDA responses has been observed in *Xenopus* oocytes (Debono *et al.*, 1993). The IC_{50} value of riluzole against

responses to NMDA was higher than that against responses to glutamic acid or veratridine. If the antagonism of NMDA responses by riluzole reported in the studies of Benavides *et al.* (1985) and Debono *et al.* (1993) were to be competitive, this might be explained in part by the concentration of agonist used in the NMDA experiments (100 μM), which was 5.5 times the EC_{50} value, compared to that used in the glutamic acid experiments (20 μM), which was 2.5 times the EC_{50} value. However, a competitive action of riluzole at the NMDA receptor would be difficult to reconcile with the lack of activity of riluzole in binding studies. Further electrophysiological experiments would be necessary to identify the kinetic mechanisms of the interaction of riluzole with the NMDA receptor. We are currently unable to give a satisfactory explanation for difference in IC_{50} values seen with NMDA and glutamic acid. In order to determine whether the effect of riluzole on NMDA receptor-mediated calcium entry was direct, or, as suggested by Tsai *et al.* (1987), indirect, the effect of riluzole was examined in the presence of either the sodium-channel blocker tetrodotoxin or pertussis toxin, an inactivator of certain G-proteins. Riluzole still blocked responses to glutamic acid and NMDA in the presence of concentrations of tetrodotoxin that totally abolished veratridine-induced depolarization. This suggests that this effect of riluzole is not mediated by a sodium channel blocking effect of this drug. On the other hand, in the presence of pertussis toxin, the activity of riluzole on NMDA and glutamic acid-evoked responses was lost. This is in agreement with our previous work on the release of D-[^3H]-aspartate from cerebellar granule cells, where, again, the inhibitory effect of riluzole was abolished by pertussis toxin (Doble *et al.*, 1992). These data suggest that the interaction between riluzole and excitatory amino acid-mediated events may involve activation of a pertussis toxin-sensitive G-protein, perhaps by directly activating a coupled receptor (such an interaction has been observed between adenosine and α -adrenoceptors on astrocytes by Delumeau *et al.* (1991)).

References

- BECQUET, D., FAUDON, M. & HERY, F. (1989). Effect of thalamic parafascicularis nucleus stimulation in regulation of serotonergic transmission in the cat caudate nucleus: involvement of autoreceptors in the dorsalis raphe nucleus. *Neuroscience*, **33**, 293–300.
- BECQUET, D., FAUDON, M. & HERY, F. (1990). In vivo evidence for an inhibitory glutamatergic control of serotonin release in the cat caudate nucleus: involvement of GABA neurones. *Brain Res.*, **519**, 82–88.
- BENAVIDES, J., CAMELIN, J.C., MITRANI, N., FLAMAND, F., UZAN, A., LEGRAND, J.J., GUÉRÉMY, C. & LE FUR, G. (1985). 2-Amino-6-trifluoromethoxy benzothiazole. A possible antagonist of excitatory amino acid neurotransmission II. *Neuropharmacology*, **24**, 1085–1092.
- BEN-ARI, Y., ANIKSZTEJN, L. & BREGESTOVSKI, P. (1992). Protein kinase C modulation of NMDA currents: an important link for LTP induction. *Trends Neurosci.*, **15**, 333–339.
- BENSIMON, G., LACOMBLEZ, L., MENINGER, V. & THE ALS/RILUZOLE STUDY GROUP. (1994). A controlled trial of riluzole in Amyotrophic Lateral Sclerosis. *N. Engl. J. Med.*, **330**, 585–591.
- BENOÎT, E. & ESCANDE, D. (1991). Riluzole specifically blocks inactivated Na channels in myelinated nerve fibre. *Pflügers Arch.*, **361**, 1–7.
- CHÉRAMY, A., BARBEITO, L., GODEHEU, G. & GLOWINSKI, J. (1992). Riluzole inhibits the release of glutamate in the caudate nucleus of the cat in vivo. *Neurosci. Lett.*, **147**, 209–212.
- CHÉRMAY, A., ROMO, R., GODEHEU, G., BARUCH, P. & GLOWINSKI, J. (1986). In vivo presynaptic control of dopamine release in the cat caudate nucleus II. Facilitatory or inhibitory influence of L-glutamate. *Neuroscience*, **9**, 1081–1090.
- DEBONO, M.W., LE GUERN, J., CANTON, T., DOBLE, A. & PRADIER, L. (1993). Inhibition by riluzole of electrophysiological responses mediated by rat kainate and NMDA receptors expressed in *Xenopus* oocytes. *Eur. J. Pharmacol.*, **235**, 283–289.
- It is somewhat surprising that pertussis toxin should abolish the effect of riluzole on a receptor ion channel such as the NMDA receptor, since these are not generally thought to be regulated by or coupled to G-proteins. However, it has been shown recently by Ben-Ari *et al.* (1992), that NMDA receptor function can be modified by the phosphorylation state of the receptor protein. Furthermore, Murphy *et al.* (1994) propose that phosphorylation by PKC exerts not only a potentiating but also a permissive effect on the NMDA-evoked elevation in cytosolic calcium in striatal neurones. Such a mechanism might underly the non-competitive block of the NMDA receptor by riluzole, if this compound were to activate a G-protein dependent intracellular event which would lead to the dephosphorylation of the NMDA receptor. On the other hand, the ability of riluzole to block the increase in intracellular calcium evoked by veratridine is unaffected by pretreatment of the cells with pertussis toxin. This suggests that this activity does not involve G-proteins, and may reflect a direct action of riluzole on the voltage-dependent sodium channel, as has been demonstrated by Benoit & Escande (1991) in the frog sciatic nerve. It should be pointed out that it is unlikely that riluzole interferes with the mechanism of $[\text{Ca}^{2+}]_i$ itself, since this compound does not affect the elevation of intracellular calcium seen upon depolarization of the cells with potassium ions.
- In conclusion, these studies demonstrate that riluzole has two distinct mechanisms of action in mammalian neurones. Firstly, this molecule interacts with the voltage-dependent sodium channel, probably by stabilizing the inactivated conformation, as first shown by Benoit & Escande (1991). Secondly, by activation of a G-protein-dependent process, riluzole counteracts the activation by excitatory amino acids of the NMDA receptor. Which of these two mechanisms underlies the anticonvulsant and neuroprotective activities of riluzole seen in animal studies (Mizoule *et al.*, 1985; Malgouris *et al.*, 1989) or in the clinic (Bensimon *et al.*, 1994) remains to be demonstrated.
- DELUMEAU, J.C., TENCÉ, M., MARIN, P., CORDIER, J., GLOWINSKI, J. & PREMONT, J. (1991). Synergistic regulation of cytosolic calcium concentration by adenosine and α 1-adrenergic agonists in mouse striatal astrocytes. *Eur. J. Neurosci.*, **3**, 539–550.
- DOBLE, A., HUBERT, J.P. & BLANCHARD, J.C. (1992). Pertussis toxin pretreatment abolishes the inhibitory effect of riluzole and carbachol on D-[^3H]-aspartate release from cultured cerebellar granule cells. *Neurosci. Lett.*, **140**, 251–254.
- DOBLE, A. & PERRIER, M.L. (1989a). Pharmacology of excitatory amino acid receptors coupled to inositol phosphate metabolism in neonatal rat striatum. *Neurochem. Int.*, **15**, 1–8.
- DOBLE, A. & PERRIER, M.L. (1989b). Kainic acid-preferring receptors stimulate inositol phosphate formation in cultured cerebellar granule cells. *Br. J. Pharmacol.*, **96**, 316P.
- DREJER, J., HONORÉ, T., MEIER, E. & SCHOUSBOE, A. (1986). Pharmacologically distinct glutamate receptors on cerebellar granule cells. *Life Sci.*, **38**, 2077–2085.
- DREJER, J., HONORÉ, T. & SCHOUSBOE, A. (1988). Excitatory amino acid-induced release of ^3H -GABA from cultured mouse cerebral cortex interneurons. *J. Neurosci.*, **7**, 2910–2916.
- FRANDSEN, A., DREJER, J. & SCHOUSBOE, A. (1989). Glutamate induced $^{45}\text{Ca}^{++}$ uptake into immature cerebral cortex neurones shows a distinct pharmacological profile. *J. Neurochem.*, **53**, 1959–1962.
- GALLO, V., CIOTTI, M.T., COLETTI, A., ALOSI, F. & LEVY, G. (1982). Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 7919–7923.
- GIRDLESTONE, D., DUPUY, A., ROY-CONTANCIN, L. & ESCANDE, D. (1989). Riluzole antagonises excitatory amino acid evoked firing in rat facial motoneurons. *Br. J. Pharmacol.*, **97**, 383P.
- GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

- HUBERT, J.P., DELUMEAU, J.C., PRÉMONT, J., DOBLE, A. & GLOWINSKI, J. (1992). Mobilisation du calcium intracellulaire dans des neurones de mésencéphale de rat en culture induite par une dépolarisation. Antagonisme par le riluzole. *Colloque de la Société des Neurosciences*, Strasbourg, France, **83**, A79.
- HUBERT, J.P. & DOBLE, A. (1989). Ibotenic acid stimulates D-[^3H]aspartate release from cultured cerebellar granule cells. *Neurosci. Lett.*, **96**, 345–350.
- IRVING, A.J., COLLINGRIDGE, G.L. & SCHOFIELD, J.G. (1992). Interactions between calcium mobilizing mechanisms cultured rat cerebellar granule cells. *J. Physiol.*, **456**, 667–680.
- MALGOURIS, C., BARDOT, F., DANIEL, M., PELLIS, F., RATAUD, J., UZAN, A., BLANCHARD, J.C. & LADURON, P.M. (1989). Riluzole, a novel antigitamate, prevents memory loss and hippocampal neuronal damage in ischemic gerbils. *J. Neurosci.*, **9**, 3720–3727.
- MARTIN, D., THOMPSON, M.A. & NADLER, V. (1993). The neuroprotective agent riluzole inhibits release of L-glutamate and L-aspartate from slices of the hippocampal area CA1. *Eur. J. Pharmacol.*, **250**, 473–476.
- MIZOULE, J., MELDRUM, B., MAZADIER, M., CROUCHER, M., OLLAT, C., UZAN, A., LEGRAND, J.J., GUÉRÉMY, C. & LE FUR, G. (1985). 2-amino-6-trifluoromethoxy benzothiazole, a possible antagonist of excitatory amino acid neurotransmission I. *Neuropharmacology*, **24**, 767–773.
- MOLLARD, P., GUERINEAU, N., AUDIN, J. & DUFY, B. (1989). Measurement of Ca^{2+} transients using simultaneous dual-emission microspectrofluorimetry and electrophysiology in individual pituitary cells. *Biochem. Biophys. Res. Commun.*, **164**, 1045–1052.
- MURPHY, N.P., CORDIER, J., GLOWINSKI, J. & PRÉMONT, J. (1994). Does protein kinase C exert a permissive effect on the N-Methyl-D-Aspartate-evoked rise in cytosolic calcium in striatal neurones? *Eur. J. Neurosci.* (in press).
- NICOLETTI, F., ARONICA, E., DELL'ALBANI, P., CONDORELLI, D.F., HACK, N. & BALAZS, R. (1993). Mechanisms underlying developmental changes in the expression of metabotropic glutamate receptors. *J. Neurochem.*, **61**, S123D.
- PEARSON, H., GRAHAM, M.E. & BURGOYNE, R.D. (1992). N-methyl-D-aspartate responses in rat cerebellar granule cells are modified by chronic depolarisation in culture. *Neurosci. Lett.*, **142**, 27–30.
- SCHIEREN, I. & MACDERMOTT, A. (1988). Flow cytometric identification and purification of cells by ligand-induced changes in intracellular calcium. *J. Neurosci. Methods*, **26**, 35–44.
- SIMPSON, P.B., CHALLISS, R.A.J. & NAHORSKI, S.R. (1993). Involvement of intracellular stores in the calcium responses to N-methyl-D-aspartate and depolarization in cerebellar granule cells. *J. Neurochem.*, **61**, 760–763.
- TSAI, C., STEEL, D.J., MCPHERSON, S., TAYLOR, C.A., WOOD, P.L. & LEHMANN, J. (1987). PK 26124 is not a competitive antagonist at NMDA-type receptors. In *Excitatory Amino Acid Transmission*. ed. Hicks, P., Lodge, D. & McClennan, H. pp. 79–82. London: A.R. Liss.
- ZHAO, Z. & PENG, L. (1993). Glutamate effects on calcium homeostasis in cerebellar granule cells in primary cultures grown under depolarizing and non-depolarizing conditions. *Synapse*, **13**, 315–321.

(Received January 6, 1994

Revised April 22, 1994

Accepted May 5, 1994)