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Effects of depolarizing stimuli on calcium homeostasis in cultured rat motoneurones

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1 Intracellular calcium concentrations in individual rat motoneurones in enriched primary cultures were measured by Indo-1 fluorimetry.

2 Motoneurones in the cultures were characterized morphometrically and by cholineacetyltransferase immunocytochemistry.

3 Depolarization of the cells with glutamic acid or veratridine increased intracellular calcium levels, which returned to baseline only slowly after removal of the depolarizing agent.

4 The use of selective agonists (*N*-methyl-D-aspartic acid, AMPA, kainic acid, quisqualic acid and 1*R*-3*S*-ACPD) and antagonists (MK 801 and CNQX) showed that the excitatory amino acid-evoked responses were mediated by AMPA/kainate receptors rather than by NMDA receptors.

5 Depolarization-evoked calcium transients in motoneurones are blocked by the neuroprotective drug riluzole

6 Calcium transients reflected entry of calcium from without the cell, and their blockade by nitrendipine and lanthanum chloride suggested that this entry took place primarily through voltage-dependent calcium channels.

7 These findings may be relevant for understanding the selective vulnerability of motoneurones to excitotoxicity in amyotrophic lateral sclerosis, and the therapeutic activity of riluzole in the treatment of this disease.

Keywords: ALS; motoneurone culture; glutamic acid; Indo-1/AM; intracellular calcium; NMDA; AMPA; kainate; riluzole; veratridine

Introduction

The concept of excitotoxicity was first proposed by Olney to account for the ability of agents that activate excitatory amino acid receptors to kill neurones *in vivo* and *in vitro* (Olney, 1978). It appears that prolonged or excessive stimulation of these receptors initiates an irreversible series of events within the cell that lead to neuronal death. Of the many intracellular processes that have been suggested to be involved (including e.g. free radical damage, metabolic failure and apoptosis), a central role is undoubtedly played by disturbances in intracellular calcium homeostasis (Choi, 1988a).

Calcium concentrations within neurones rise transiently following activation of excitatory amino acid receptors principally due to calcium entry through the *N*-methyl-D-aspartate (NMDA) receptor/ion-channel. Entry of calcium through voltage-dependent calcium channels (Choi, 1988b), or certain calcium-permeable AMPA/kainate receptors (Iino *et al*., 1990) as well as mobilization of intracellular calcium pools following activation of metabotropic excitatory amino acid receptors (Murphy & Miller, 1988), or secondary to calcium influx (Mody & Macdonald, 1995), may also be important in certain neuronal populations, or under certain conditions. In addition, prolonged elevations in intracellular calcium levels may be a consequence of impaired activity of the membrane sodium-calcium exchanger due to alterations in the electrochemical gradient for sodium following membrane depolarization (Mattson *et al*., 1989; Koch & Barish, 1994).

High levels of intracellular calcium for extended periods of time are prejudicial to cell survival for a number of reasons (Lipton & Rosenberg, 1994). Direct activation of calcium-dependent lytic enzymes (proteases, lipases and nuclases) will destroy cell structure. Accumulation of calcium phosphate within mitochondria will disrupt these organelles and thus impair ATP production. Moreover, apoptotic programmes may be triggered, or free radical damage produced by activation of nitric oxide synthesis.

Although the evidence is essentially circumstantial, it has been suggested that excitotoxic processes may play a part in the initiation or progression of neuronal loss in neurodegenerative disease in man (Choi, 1988a; Lipton & Rosenberg, 1994). Given the abundance of glutamic acid in the mammalian central nervous system, release of this amino acid from dead or degenerating neurones may help to spread or aggravate foci of neuronal death whatever the nature of the primary aetiological event.

One of the neurodegenerative diseases for which there is considerable evidence that excitotoxicity may contribute to the disease process is amyotrophic lateral sclerosis (ALS). This disease is characterized by progressive muscular weakness leading to paralysis and eventually death, usually due to respiratory failure (Williams & Windebank, 1991). Although the course of the disease is quite variable, death usually occurs after between 2–5 years after diagnosis, making it one of the most lethal of all neurological diseases. Histopathologically, ALS is characterized by a selective loss

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of both upper (corticospinal) and lower (spinomuscular) motoneurones.

The evidence for a role for excitotoxicity in the development or progression of ALS has been presented in a number of recent reviews (e.g. Shaw & Ince, 1997; Plaitakis *et al*., 1996; Rothstein, 1995; Shaw, 1994). Originally, this excitotoxic hypothesis for ALS was based primarily on data showing changes in glutamic acid levels in plasma (Plaitakis & Carascio, 1987), cerebrospinal fluid (Rothstein *et al*., 1990) and post mortem brain tissue (Perry *et al*., 1987) from ALS patients. Cerebrospinal fluid from ALS patients has also been demonstrated to provoke AMPA/kainate receptor-mediated cell death in primary cultures of rat cortical neurones (Couratier *et al*., 1993).

More recently, a more precise idea of the nature of the proposed glutamatergic defect in ALS has come from the work of Rothstein and co-workers. They first showed that glutamic acid uptake was impaired in vesicular preparations from post mortem spinal cord and motor cortex (Rothstein *et al*., 1992), and then went on to suggest that this deficit could be explained by the selective loss (demonstrated immunohistochemically) of one of the subtypes of glutamic acid transporter, EAAT-2 (GLT-1) that is expressed uniquely in glial cells (Rothstein *et al*., 1995).

Further support for the idea that excitotoxicity may contribute to the development or progression of the disease has come from clinical trials with the antiglutamate drug riluzole (Wokke, 1996). Riluzole appears to act by both presynaptic and postsynaptic mechanisms to attenuate glutamatergic neurotransmission (Bryson *et al*., 1996; Doble, 1996). We have previously published data showing that riluzole can block depolarization-evoked calcium transients in cultured rat cerebellar granule cells (Hubert *et al*., 1994) and mesencephalic neurones (Hubert *et al*., 1992), and suggested that this may be pertinent to the neuroprotective effects of this drug.

The present study was undertaken in order to study the same phenomena on a neuronal population more relevant to ALS, motoneurones. We have used calcium imaging technology with Indo-1 to visualize the effects of depolarizing agents on intracellular calcium handling in primary cultures of rat spinal motoneurones. We wished to observe whether these cells responded in the same way to pharmacological manipulation as neurones from higher up the neuroaxis.

Methods

Animals

Pregnant female Sprague-Dawley rats [Ico:OFA-SD(IOPS Caw) IFFA-CREDO, Domaine des Oncins, 69120 L'Arbresle, France] were used. They were kept for at least 5 days before their utilization in our animal quarters according to the principles of good animal care. Throughout this period, they were housed alone and allowed free access to food (M20, Ets L. Piètrement, Sainte Colombe, 77650 Longueville, France) and appropriately filtered drinking water. The animal house was maintained on a timer-regulated 12-h light-dark cycle, an ambient temperature of $22 \pm 2^{\circ}C$ and hygrometry of $55 \pm 20\%$.

Drugs and chemicals

Glutamic acid, NMDA (*N*-methyl-D-aspartic acid), kainic acid, tetrodotoxin, lanthanum chloride, and veratridine were from Sigma Chemical Co, St. Louis, U.S.A.; quisqualic acid, (1S,3R) ACPD (1-aminocyclopentane-1*S*, 3*R*-dicarboxylic acid), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and nitrendipine from Tocris Cookson Ltd, Langford, U.K., and MK 801 ((+)5-methyl-10,11-dihydro-5*H*-dibenzol[a,d]cyclo-heptine-5-10-imine) from Research Biochemicals Inc, Natick, U.S.A. Riluzole was synthesized by Dr P. Jimonet at Rhône-Poulenc Rorer, Vitry, France. Cell culture media and enzymes were purchased from GIBCO, Glasgow, U.K. Indo-1/AM was obtained from Molecular Probes Inc., Eugene, U.S.A. The primary antibody against ChAT (AB 143) was from Chemicon International Inc, Temecula, U.S.A., and the monoclonal anti-p75 NGF receptor antibody (IgG 192 clone) from Boehringer Mannheim, U.S.A. The secondary biotinylated antibody and the streptavidin-peroxidase complex from Amersham International plc, Amersham, U.K. 3-Amino-9-ethylcarbazole, was from Biogenex, San Ramon, U.S.A.

Preparation of primary cultures of rat motoneurones

The method used was adapted from that described for chick motoneurones by Dohrmann *et al*. (1986). When the embryos were 15 days old, the pregnant dam was anaesthetized with ether and the embryos removed. The anaesthetized dam was then promptly killed by cervical dislocation. The spinal cords were carefully dissected out of the embryos and dissociated enzymatically with trypsin (0.125%; 4 ml) for 15 min at 37°C in 4 ml of phosphate buffered saline (calcium and magnesium free) containing glucose (0.3%). They were then separated mechanically in Leibovitz L15 culture medium (2 ml) containing DNase ($0.1\ mg\ ml^{-1}$) by repeated passage through a burnished pasteur pipette. This cell suspension (2 ml) was placed in a centrifuge tube on a cushion (2 ml) of L15 culture medium containing bovine serum albumin (4%) and foetal calf serum (3%) and centrifuged for 10 min at $200 \times g$. The resulting pellet was resuspended in L15 medium containing foetal calf serum (3%) and DNase ($10\ mg\ ml^{-1}$) and the volume adjusted to obtain 1 ml for four embryos. This cell suspension (1 ml) was centrifuged for 15 min at $400 \times g$ on a cushion of L15 medium (2 ml) containing metrizamide (6.6%) and foetal calf serum (3%), buffered with sodium bicarbonate 7.5% ($35\ \mu l\ ml^{-1}$). The centrifuge was allowed to stop without braking. Motoneurones retained at the interface were carefully removed in a volume of 300 μl and adjusted to a final volume of 6 ml with L15 medium containing foetal calf serum (3%). This resulting cell suspension was centrifuged for 10 min at $200 \times g$ on a cushion (2 ml) of L15 culture medium containing bovine serum albumin (4%) and foetal calf serum (3%). The final pellet was resuspending in L15 medium containing FCS (3%).

The cell density was adjusted to 0.4 ml of culture medium per embryo. Aliquots (3 ml) were plated onto glass slides (45×70 mm), which after 30 min were placed into Petri dishes (90 mm diameter) and immersed in L15 cell culture medium (7 ml) containing foetal calf serum (3%), mixed hormones N2 (7%) and muscle extract ($100\ \mu g\ ml^{-1}$) and sodium bicarbonate 7.5% ($35\ \mu l\ ml^{-1}$). This muscle extract was prepared by centrifugation at $40{,}000 \times g$ for 1 h as described by Henderson *et al*. (1983). The cells were incubated at 37°C in 5% CO_2/95% air and saturating humidity and used between 24 and 72 h after plating.

Characterization of motoneurones

The motoneurones in the cell culture were characterized both morphologically and immunohistochemically. For immunohistochemistry, the cultures were fixed with paraformaldehyde

(4%) and permeabilized with Triton X-100 (0.1%). After blocking of non-specific sites (30 min at room temperature with 10% FCS), the cells were incubated successively with the primary antibody against cholineacetyltransferase (1 : 500, 18 h at 4°C), the secondary biotinylated anti rabbit-IgG antibody (1 : 300; 3 h at 20°C) and, finally, the streptavidin-peroxidase complex (1 : 300; 1 h t 20°C). The specificity of the choline acetyltransferase antibody has been demonstrated by Western blot (Grosman *et al*., 1995), and was validated in our laboratory using a variety of cell types before undertaking the present experiments. Peroxidase staining was revealed with 3-amino-9-ethylcarbazole and the cells counter-stained with Mayer's haematoxylin. Some cultures were also processed for double immunofluorescence staining: cultures were incubated with a monoclonal anti-p75 NGF receptor antibody (10 μg ml^{-1}; Boehringer Mannheim) and with the rabbit anti-cholineacetyltransferase antibody. The anti-p75 antibody was detected by a goat anti-mouse antibody coupled to fluroscein isothiocyanate and the anti-cholineacetyltransferase antibody was revealed by a goat anti-rabbit antibody conjugated to tetramethylrhodamine isothiocyanate. These immunohistochemical studies having shown that the largest cells were essentially all cholineacetyltransferase and p75 positive (see Results), cells with a cell diameter greater than 10 μm were considered to be motoneurones for the calcium imaging experiments.

Preparation of primary cultures of rat cerebellar granule cells

Granule cell cultures were prepared as previously described (Gallo *et al*., 1982; Hubert *et al*., 1994). Briefly, cerebella were removed from 7-day old rat pups 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 μg ml^{-1}) and laminin (2 μg ml^{-1}) and placed 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 after between 4 and 6 days in culture.

Calcium imaging

Before each experiment, the nutrient medium was replaced with a modified Hank's solution containing NaCl (130 mM), KCl (5.4 mM), HEPES (20 mM), glucose (5.5 mM), $CaCl_2$ (1.25 mM), $MgCl_2$ (1 mM), and buffered to pH 7.4. The cells were pre-loaded with Indo-1/AM by addition of indo-1/AM (5 μM) 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.

The cells on their glass slides, loaded with the fluorescent probe, 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. Compounds were applied into the perfusion medium. Normally, depolarizing agents were added to the perfusion medium for 20 s. The chamber was observed through a video-camera coupled to an ARGUS 50/CA image processor (Hamamatsu). Windows corresponding to cell bodies of identified motoneurones were defined for image analysis. The cells were excited through a 40× 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 and 480 nm. These signals were recorded directly by the image analyser and converted to the fluorescence ratio F_{405}/F_{480}, which is independent of the probe concentration.

Data handling

Cytosolic calcium concentrations were calculated from the fluorescence 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 (2.5×10^{-7} M), $F_{480(f)}$ is the fluorescence of free indo-1 (54.2 ± 5.0, $n = 23$), $F_{480(b)}$ the fluorescence of indo-1 bound to calcium (3.87 ± 0.91, $n = 23$) and R_{min} (0.30 ± 0.02, $n = 23$) and R_{max} (5.85 ± 0.28, $n = 23$) were determined in the presence of ionomycin (10^{-5} M) and either EGTA (10^{-3} M) or $CaCl_2$ (10^{-3} M) respectively.

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

EC_{50} and IC_{50} values were calculated by computer-assisted iterative non-linear regression analysis of concentration-response curves, fitted to a simple Langmuir isotherm.

Results

Characterization of motoneurones in primary culture

The primary cultures of motoneurones obtained were characterized morphometrically and immunocytochemically. Most of the neurones (>95%) had a large cell body (diameter >10 μM) with two to three long asymmetric processes. The average cell diameter of these large cells was 17.62 μm (Table 1), and all of them stained positively for cholineacetyltransferase and p75 (Figure 1A,B). Only these large neurones were used for calcium imaging studies. The minority of small

Table 1 Glutamic acid-evoked calcium transients in primary cultures of rat motoneurones and cerebellar granule cells

	Motoneurones (n)	*Granule cells* (n)
Diameter (μm)	17.62 ± 0.59 (67)	7.62 ± 0.24 (41)
Basal $[Ca^{2+}]_i$ (nM)	95.7 ± 6.4 (66)	87.6 ± 11.07 (38)
Peak $[Ca^{2+}]_i$ (nM)	137.3 ± 16.2 (66)	583.5 ± 41.34 (38)
$[Ca^{2+}]_i$ peak duration (s)	102 ± 4 (103)	46 ± 3 (187)
Integrated $[Ca^{2+}]_i$ (nmol pixel^{-1})	444.94 ± 33.59 (23)	246.08 ± 17.96 (16)

The diameter of the cells was measured at their widest point. Basal calcium levels were measured before exposure to glutamic acid. Cells were then stimulated three times with a fixed concentration of glutamic acid (100 μM), using a protocol identical to that in Figure 2. The average of the maximal intracellular calcium concentration (peak $[Ca^{2+}]_i$) reached for the three stimulations was measured. The total calcium entry (integrated $[Ca^{2+}]_i$ was calculated as described in the Methods. The values are expressed as the mean ± s.e.mean (%) of at least nine observations from individual cells taken from at least three separate preparations.

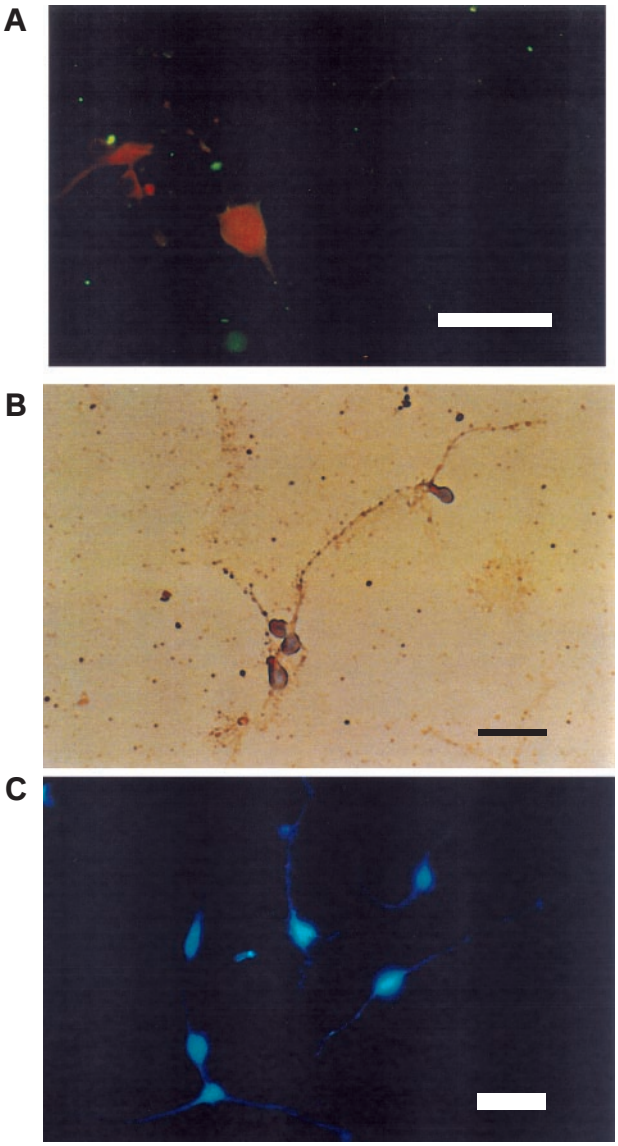

Figure 1 Rat motoneurones growing in primary culture. (A) Fluorescence photomicrograph showing double staining of a motoneurone growing in primary culture with antibodies against choline acetyltransferase (red fluorescence) and p75 (green fluorescence). A typical large ChAT-positive motoneurone with a log arborized neurite staining for p75 can be seen. In the bottom of the field, the green nucleus of a ChAT/p75 negative cell can be seen. Scale bar: 50 μm. (B) Light-field photomicrograph showing peroxidase staining of motoneurones used for a calcium imaging experiment labelled with an anti-ChAT antibody. Several large ChAT-positive cells with processes can be seen. To the right, a ChAT-negative glial cell can be seen. Scale bar: 50 μm. (C) Fluorescence photomicrograph of cultured motoneurones incubated with Indo-1/AM. Fluorescence can be seen in both the cell bodies and processes of these cells. Scale bar: 50 μm.

neurones did not stain for cholineacetyltransferase and were not characterized further. A small number of GFAP-staining astrocytes were also found in the culture.

Calcium transients evoked in motoneurones by glutamic acid

Intracellular calcium concentrations in primary cultures of rat motoneurones could be measured by Indo-1 fluorimetry (Figure 1C). The basal calcium concentrations of these cells was 95.7 ± 6.4 nM ($n = 66$). Application of glutamic acid (100 μM) led to an increase of intracellular calcium to a peak concentration of 137 ± 16 nM (Table 1). This elevation of calcium levels returned slowly to normal on removal of the glutamic acid (mean duration 102 ± 4 s, $n = 103$), and oscillations on the curve were sometimes seen. An estimation of the total exposure of the cell to elevated intracellular calcium (ΣCa) was calculated from the average local calcium concentration in nmol per pixel measured at each image sampling time (0.1 s) corrected for the duration of the calcium transient:

$$\Sigma\text{Ca} = \text{duration time} \times (\text{mean[Ca]/pixel/sample time})/\text{sample frequency}$$

The total calcium exposure was thus estimated to be 444.94 ± 33.59 nmol pixel^{-1}s ($n = 23$). A typical experiment is shown in Figure 2A.

Calcium transients evoked in cerebellar granule cells by glutamic acid

For comparison, intracellular calcium concentrations in primary cultures of rat cerebellar granule cells were also measured. The basal calcium concentrations of these cells was 87.6 ± 11.1 nM ($n = 38$). The average cell diameter of these cells was 7.62 μm (Table 1). The peak concentration of intracellular calcium reached after application of glutamic acid (100 μM) was 583.5 ± 41.3 (Table 1). These calcium transients returned very rapidly to normal on removal of the glutamic acid (mean duration 46 ± 3 s, $n = 187$), and oscillations were not seen. The total exposure of the cell to elevated intracellular calcium was estimated as 246.1 ± 17.9 nmol.pixel.s. A typical experiment is shown in Figure 2B.

Agonist pharmacology of glutamic acid-evoked calcium transients

A variety of excitatory amino acid receptor agonists with different receptor specificities were evaluated for their ability to modify intracellular calcium concentrations in rat motoneurones (Table 2). The non-NMDA receptor agonists AMPA and kainic acid (both 100 μM) increased intracellular calcium concentrations to a similar extent to glutamic acid itself. NMDA (100 μM) and the metabotropic receptor agonist 1*S*, 3*R*-ACPD (10 μM) were both inactive. For the experiments with NMDA, magnesium-free buffer was used, and D-serine (10 mM) added to the culture medium. Quisqualic acid, which is an agonist at both non-NMDA receptors and metabotropic receptors, produced a small rise in intracellular calcium concentrations.

Antagonist pharmacology of glutamic acid-evoked calcium transients

Increases in intracellular calcium concentrations evoked by glutamic acid (100 μM) could be blocked completely by the non-NMDA receptor antagonist CNQX (100 μM). In contrast, the non-competitive NMDA receptor antagonist MK 801 (100 μM) had relatively little effect (Table 3). Riluzole also inhibited this increase in intracellular calcium concentrations with an IC_{50} value of 21.6 ± 6.5 μM (Figure 3).

Effect of inhibitors of calcium entry

Chelation of extracellular calcium with EGTA (1 mM) abolished completely the ability of glutamic acid (100 μM) to increase intracellular calcium concentrations in rat motoneur-

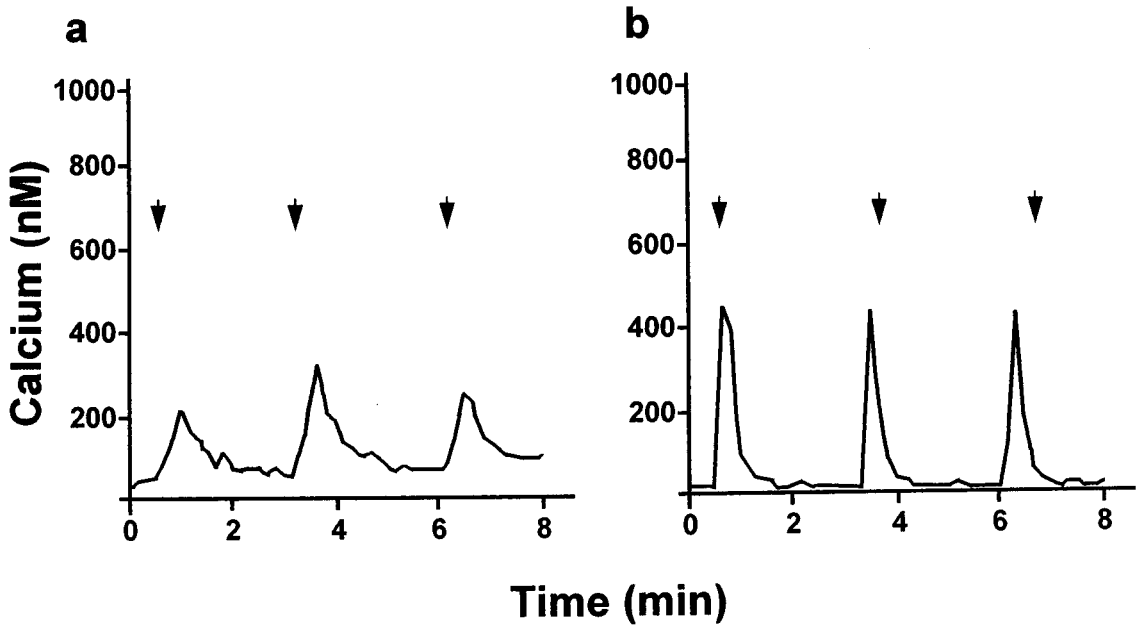

Figure 2 Calcium transients evoked by glutamic acid in primary cultures of rat motoneurones and rat cerebellar granule cells. (A) Evolution of intracellular calcium levels in an individual motoneurone. Glutamic acid (100 μM) was applied to the cell as indicated by the arrows. The recording is similar to and representative of those obtained in 12 individual cells from three different preparations. (B) Evolution of intracellular calcium levels in an individual cerebellar granule cell. Glutamic acid (100 μM) was applied to the cell as indicated by the arrows. The recording is similar to and representative of those obtained in 12 individual cells from three different preparations.

Table 2 Agonist pharmacology of depolarization-evoked calcium transients

Agonist	$[Ca^{2+}]_i$ (nM) mean ± s.e.m	(n)
Glutamic acid 100 μM	137.3 ± 16.2	(66)
NMDA† 100 μM	10.9 ± 6.8	(14)
AMPA 100 μM	75.7 ± 14.6	(9)
Quisqualic acid 10 μM	31.2 ± 6.3	(14)
Kainic acid 100 μM	144.8 ± 28.2	(14)
1*S*, 3*R*-ACPD 10 μM	5.6 ± 2.4	(13)
Veratridine 30 μM	776.7 ± 83.1	(46)

Individual cells were stimulated five times with a fixed concentration of the different excitatory amino acids (10 or 100 μM). The mean peak calcium concentrations for the five stimulations was calculated. The values are expressed as the mean ± s.e.m. (%) of at observations made in the indicated (*n*) number of individual cells. †NMDA was applied in the presence of D-serine (10 mM) and in the absence of magnesium in order to optimize the chances of demonstrating an effect.

Table 3 Antagonist pharmacology of depolarization-evoked calcium transients

	Inhibition (%)	
Antagonist	*Glutamic acid*	*Veratridine*
MK 801 100 μM	21.0 ± 7.3	12.77 ± 6.02
CNQX 100 μM	91.0 ± 4.4*	26.28 ± 5.58*
Tetrodotoxin 1 μM	ND	98.63 ± 1.1*
Riluzole 100 μM	84.7 ± 2.5*	96.5 ± 1.4*

Individual cells were stimulated three times with a fixed concentration of glutamic acid (100 μM) or veratridine (30 μM). Antagonists were added during the second stimulation. The percentage inhibition of the agonist response was calculated with respect to the average of the preceding and following control stimulation. The values are expressed as the mean ± s.e.m. (%) of at least eight observations from individual cells taken from three separate preparations. ND: not determined. The asterisk indicates a statistically significant ($P<0.05$) difference from control values (Wilcoxon test).

ones (Table 4), without effecting basal calcium levels (102.7 ± 15.1 nM, $n=18$). Similarly, the effect of glutamic acid could also be prevented with two blockers of voltage-sensitive calcium channels, lanthanum chloride (1 μM) and nitrendipine (10 μM). Lanthanum chloride had no effect on basal calcium levels (107.3 ± 10.9 nM, $n=20$)

Calcium transients evoked in motoneurones by veratridine

Calcium transients could also be evoked in cultured rat motoneurones by veratridine, an alkaloid that activates voltage-dependent sodium channels. The peak concentration of intracellular calcium reached was 776.7 ± 83.1 nM ($n=46$). These transients were blocked by tetrodotoxin and riluzole, but not by MK 801 (Table 3). A small, but statistically significant, inhibition of the veratridine response was seen with CNQX. The IC_{50} value for riluzole against veratridine was 1.30 ± 0.50 μM (Figure 3).

Discussion

This study demonstrated the feasibility of measuring intracellular calcium levels in primary cultures of rat motoneurones by Indo-1 fluorimetry. Calcium transients could be evoked in these cells by application of glutamic acid or veratridine. There were a number of differences between the responses in motoneurones and those observed in other cell types, such as the cerebellar granule cell. Firstly, the peak concentration of intracellular calcium reached was lower in motoneurones than in granule cells. This may reflect the

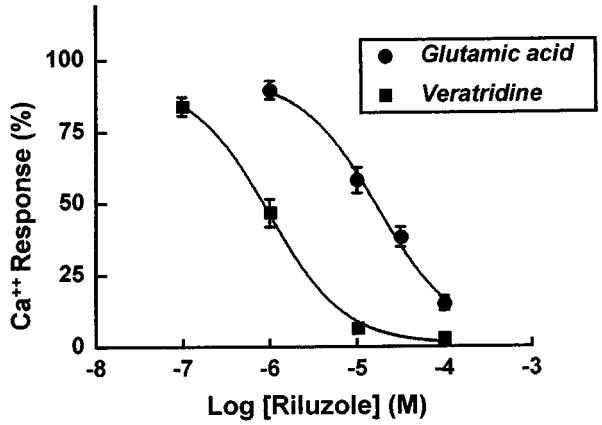

Figure 3 Antagonism of depolarization-evoked calcium transients by riluzole. Individual cells were stimulated three times with a fixed concentration of glutamic acid (100 μM) or veratridine (30 μM). Different concentrations of riluzole were added for 1 min before and during the second stimulation. The percentage inhibition of the agonist response was calculated with respect to the average of the preceding and following control stimulations. The values are expressed as the mean $\pm$ s.e.m. of at least 23 observations from individual cells taken from three separate preparations. The relationship between the mean percentage inhibition and the logarithm of the riluzole concentration was determined by computer-assisted iterative non-linear regression to a simple Langmuir isotherm.

Table 4 Calcium dependence of depolarization-evoked calcium transients

	Inhibition (%)	(n)
EGTA 1 mM	98.8 $\pm$ 0.8*	(17)
$LaCl_3$ 1 μM	99.1 $\pm$ 0.7*	(15)
Nitrendipine 10 μM	85.5 $\pm$ 7.6*	(8)

Individual cells were stimulated three times with a fixed concentration of glutamic acid (100 μM). Compounds were added during the second stimulation. The percentage inhibition of the agonist response was calculated with respect to the average of the preceding and following control stimulation. The values are expressed as the mean $\pm$ s.e.m. (%) of at least eight observations from individual cells taken from two separate preparations. The asterisk indicates a statistically significant ($P<0.05$) difference from control values (Wilcoxon test).

different geometry of the cells – motoneurones, being much larger than granule cells, would require a comparatively larger total quantity of calcium to enter the cell to reach an equivalent intracellular calcium concentration.

More surprisingly, the transients in motoneurones returned to basal rather slowly after removal of the glutamic acid, sometimes in an oscillatory fashion. The average recovery time was 102 s compared to 46 s in cerebellar granule cells. We have never seen such slowly recuperating transients before in any other neuronal cell type, although Limbrick *et al.* (1995) have described prolonged increases in intracellular calcium following exposure of hippocampal neurones to glutamate. These authors have correlated the inability of these cells to restore intracellular calcium levels to their susceptibility to excitotoxic cell death.

Our results may suggest that motoneurones have difficulty in removing calcium from the cytosol. In this context, it is interesting to note that Ince *et al.* (1993) have shown that motoneurones lack the calcium buffering proteins calbindin D-28K and parvalbumin, present in most other neuronal populations. These authors suggested that motoneurones would thus be less able to buffer intracellular calcium, and that this might be pertinent for the selective vulnerability of motoneurones in ALS. Our results suggest that motoneurones are indeed less competent at restoring intracellular calcium homeostasis after an excitatory challenge, and are thus exposed to a higher calcium load than other neuronal types. However, it should be born in mind that the large size of the motoneurone may influence the kinetics of the intracellular calcium response, and that conclusions as to the mechanisms underlying the responses observed should be treated with caution.

The calcium transients evoked by glutamic acid can also be generated by AMPA, kainic acid, and to a lesser degree by quisqualic acid, suggesting that AMPA/kainate receptors are responsible. This is supported by the finding that the responses are antagonized by the AMPA/kainate receptor antagonist CNQX, but not by the NMDA receptor antagonist MK 801. Again, this contrasts with findings in other cell types, where such responses are mediated predominantly by NMDA receptors, for example in cerebellar granule cells (Parks *et al.*, 1991; Hubert *et al.*, 1994), hippocampal neurones (Kudo & Ogura, 1986; Michaels & Rothman, 1990), cortical neurones (Bugard & Hablitz, 1995) and striatal neurones (Murphy *et al.*, 1987). However, the finding that AMPA/kainate receptors are involved in the calcium responses is of great interest, since excitotoxicity in motoneurones is unusual in being mediated not by NMDA receptors, but by AMPA/kainate receptors (Rothstein *et al.*, 1993; Rothstein & Kuncl, 1995; Regan, 1996). Carriedo *et al.* (1996) have recently shown that kainic acid toxicity in cultured rat motoneurones is calcium dependent.

If the calcium transients are triggered by activation of AMPA/kainate receptors, the question arises as to the origin of the calcium. Chelation of calcium in the incubation medium with EGTA abolishes the calcium responses, indicating that they are likely to correspond to calcium entry, rather than mobilization of intracellular calcium reserves. Although AMPA/kainate receptors are not generally permeable to calcium, there are certain combinations of GluR receptor subunits, notably those that do not contain an edited $GluR_2$ subunit, that do form receptors of AMPA/kainate pharmacology that are permeable to calcium ion (Hollmann *et al.*, 1991; Geiger *et al.*, 1995). Pruss *et al.* (1991) proposed an interesting method whereby to identify neurones bearing AMPA/kainate receptors permeable to calcium, using the uptake and subsequent cytochemical visualization of cobalt ion. In their recent paper, Carriedo *et al.* (1996) have applied this technique to primary cultures of rat spinal cord and shown that motoneurones do indeed take up cobalt ion in response to kainic acid, suggesting that these cells have calcium-permeable AMPA/kainate receptors. Moreover, Williams *et al.* (1997) have shown that human motoneurones do not express mRNA for the $GluR_2$ subunit of the AMPA/kainate receptor, which would be compatible with the oligomeric receptors being permeable to calcium. However, to our knowledge, no direct electrophysiological determination of the calcium permeability of AMPA/kainate receptors in motoneurones has been performed. In the rat, motoneurones do, however, appear to express $GluR_2$ (Tölle *et al.*, 1993). Our data are not, however, compatible with the observations of Estevez *et al.* (1995), using similar motoneuronal cultures, who found both NMDA and kainic acid to be excitotoxic.

Nonetheless, in our experiments, the calcium transients evoked by glutamic acid were abolished by lanthanum chloride

and by nitrendipine, suggesting *prima facie* that most of the calcium is entering the cell through voltage-dependent calcium channels. It is not known, however, whether nitrendipine or lanthanum ion can block calcium flux through the AMPA/kainate receptor ion channel (although blockade of NMDA receptors by nitrendipine has been described; Skeen *et al*., 1993). If voltage-dependent calcium channels are indeed the primary route of calcium entry into the motoneurones, this finding is difficult to reconcile with the observation that only cells bearing calcium-permeable AMPA/kainate receptors are sensitive to AMPA/kainate receptor-mediated excitotoxicity (Brorson *et al*., 1995; Turetsky *et al*., 1994). In this context, it is interesting to recall that Weiss *et al*. (1990) showed that the slow toxicity of kainic acid to cultured mouse cortical neurones could be blocked by nifedipine, and that kainic acid-evoked calcium influx into cultural striatal neurones is, at least in part, sensitive to nitrendipine (Murphy & Miller, 1989).

As in other neuronal types studied previously, the calcium transients evoked by depolarization either with glutamic acid or with veratridine can be blocked with riluzole. The IC_{50} value for riluzole towards glutamic acid-evoked responses was higher than that observed previously in cerebellar granule cells (21.6 μM *versus* 0.3 μM), consistent with the known selectivity of this drug for NMDA receptor and AMPA/kainate receptor-mediated responses (Debono *et al*., 1993). Similar concentrations of riluzole are needed to protect cultured rat motoneurones against the excitotoxicity of glutamic acid (Estevez *et al*., 1995). Inhibition of depolarization-evoked calcium transients in motoneurones may thus be the neuropharmacological substrate whereby riluzole protects these cells against excitotoxic damage and hence exerts its therapeutic effect in ALS.

In conclusion, this study has demonstrated depolarization-evoked calcium transients in primary cultures of rat motoneurones and characterized the excitatory amino acid receptor pharmacology thereof. Glutamic acid-evoked calcium transients recover more slowly than in other cell types, and are generated by the activation of AMPA/kainate, rather than NMDA, receptors. These two observations may be relevant to understanding the selective vulnerability of motoneurones to the excitotoxic insult that may contribute to the aetiopathology of ALS. Depolarization-evoked calcium transients in motoneurones are blocked by riluzole, thus providing a mechanistic basis for the activity of this drug in the treatment of ALS.

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