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Contribution of Ca^{2+} calmodulin-dependent protein kinase II and mitogen-activated protein kinase kinase to neural activity-induced neurite outgrowth and survival of cerebellar granule cells

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Abstract

In this report we describe our studies on intracellular signals that mediate neurite outgrowth and long-term survival of cerebellar granule cells. The effect of voltage-gated calcium channel activation on neurite complexity was evaluated in cultured cerebellar granule cells grown for 48 h at low density; the parameter measured was the fractal dimension of the cell. We explored the contribution of two intracellular pathways, Ca^{2+} calmodulin-dependent protein kinase II and mitogen-activated protein kinase kinase (MEK1), to the effects of high $[K^+]_e$ under serum-free conditions. We found that 25 mM KCl (25K) induced an increase in calcium influx through L subtype channels. In neurones grown for 24–48 h under low-density conditions, the activation of these channels induced neurite outgrowth through the activation of Ca^{2+} calmodulin-dependent protein kinase II. This also produced an increase in long-term neuronal survival with a partial contribution from the MEK1 pathway. We also found that the addition of 25K increased the levels of the phosphorylated forms of Ca^{2+} calmodulin-dependent protein kinase II and of the extracellular signal-regulated kinases 1 and 2. Neuronal survival under resting conditions is supported by the MEK1 pathway. We conclude that intracellular calcium oscillations can triggered different biological effects depending on the stage of maturation of the neuronal phenotype. Ca^{2+} calmodulin-dependent protein kinase II activation determines the growth of neurites and the development of neuronal complexity.

Keywords: CaMKII and MEK1 pathways, cerebellar granule cells, fractal dimension, neural activity, neurite outgrowth, neuronal survival.

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Neural activity can modulate several characteristics of neural development such as neurogenesis, differentiation and survival (Cone 1980; Gallo *et al.* 1987; Franklin *et al.* 1995; Borodinsky and Fiszman 1998; Fiszman *et al.* 1999). Activity, thus, can be considered as a regulatory factor of neuronal fate and function. Neural activity increases $[Ca^{2+}]_i$ either by activation of voltage-gated calcium channels (VGCC) or ionotropic glutamate receptors. In the developing CNS, intracellular calcium oscillations modulate axon guidance, neurite extension and neurite retraction (Davenport *et al.* 1996; Mattson *et al.* 1988; Gomez *et al.* 1995; Rajnicek and McCaig 1997). In mature neurones, calcium is a messenger in events involving neuronal plasticity and survival (Franklin *et al.* 1995; Gallo *et al.* 1987; Hanson and Schulman 1992; Wu *et al.* 1996; Mao *et al.* 1999).

Intracellular calcium oscillations due to neural activity can activate several intracellular pathways such as ras/mitogen-activated protein kinase (MAPK), Ca^{2+} calmodulin-dependent kinase type II (CaMKII) or p38/MEF2 (Curtis and Finkbeiner 1999; Rosen *et al.* 1994; Ghosh and Greenberg 1995; De Koninck and Schulman 1998; Mao *et al.* 1999). However, the pathways that induce differentiation have not yet been identified.

Cerebellar granule cells (CGC) grown *in vitro* disclose characteristics of the mature phenotype *in vivo*: they are

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Abbreviations used: BSA, bovine serum albumin; $[Ca^{2+}]_I$, intracellular calcium; CaMKII, Ca^{2+} calmodulin-dependent protein kinase II; CGC, cerebellar granule cells; D, neuronal fractal dimension; Erk1/2, extracellular signal-regulated kinase 1 and 2; FRC-HRP, tetanus toxin fragment C–horseradish peroxidase; 25K, 25 mM KCl; 5K, 5 mM KCl; $[K^+]_e$, extracellular potassium concentration; MAPK, mitogen-activated protein kinase; MEK1, MAPK kinase; VGCC, voltage-gated calcium channels; $VGCC_L$, VGCC of the L subtype.

glutamatergic and show rounded soma and an extensive neuritic network. Cultured CGC have extensively been used to study amino acid receptor pharmacology and the mechanisms of neuronal survival and programmed cell death (Gallo *et al*. 1987; Miller and Johnson 1996; Carlson *et al*. 1998). This cell preparation offers several advantages. Survival of 80% of the cell population can be maintained by increases in $[Ca^{2+}]_i$ through the addition of depolarizing potassium concentrations, even in the absence of serum (Miller and Johnson 1996), or by the addition of glutamate or ionotropic glutamate receptor agonists (Gallo *et al*. 1987; Balázs *et al*. 1990). The preparation is phenotypically homogeneous, 99% of the cells in the culture are CGC and therefore all glutamatergic. These two characteristics confirm the CGC in culture as an ideal model to evaluate the biological effects of neural activity with a single neurotransmitter, glutamate, being released upon stimulation.

We have studied in this work the effect of VGCC activation on neurite outgrowth and survival of CGC grown *in vitro* under serum-free conditions. We also investigated the role of CaMKII in neurite outgrowth and survival as it has been implicated in the control of neuronal growth and synaptogenesis as well as in activity-dependent synaptic plasticity (Zou and Cline 1996). We found that in immature CGC, VGCC activation induces neuritogenesis that is mediated by CaMKII activation. A depolarization-induced increase in neuronal survival is independent of the presence of serum and is mediated by CaMKII and MAPK kinase (MEK1) activation. In agreement with these results, the depolarization induced an increase in the levels of the active forms of CaMKII and Erk1/2.

In this report we provide evidences that Ca^{2+} influx triggered by VGCC activation induces different biological effects through specific pathways, depending on the stage of maturation of the neural cell.

Experimental procedures

Cell cultures

The CGC were obtained from 6- to 8-day old (P6–8) Sprague–Dawley rats. All efforts were made to minimize animal suffering. Cerebella were excised and cut in small pieces and placed in Krebs solution containing 0.035% $MgSO_4$, 0.6% glucose and 0.3% bovine serum albumin (BSA). Tissue slices were incubated for 12 min at 37°C in the same buffer containing 0.02% trypsin, dissociated with fire-polished Pasteur pipettes of decreasing diameters in a Krebs solution with 0.01% DNAse (Boheringer Mannheim, Mannheim, Germany), 0.05% trypsin inhibitor, and centrifuged for 4 min at 150 *g*. The cells were seeded in serum-free medium (neurobasal) supplemented either with B27 or with N2 (all from Gibco, Rockville, MD, USA) containing 5 mM KCl (5K) in plates precoated with laminin or poly-L-lysine at a density of 6875 cells/cm^2 for morphometrical studies and 259 875 cells/cm^2 for calcium imaging and survival experiments.

Single cell $[Ca^{2+}]_i$ measurements

The cells were grown for 1, 2 or 6 days *in vitro* (1 DIV, 2 DIV, 6 DIV, respectively) in serum-free medium, which was then removed and replaced by Locke media containing Fura-2 and Pluronic acid for 30 min. The cultures were then washed twice with Locke media. Calcium imaging was performed using an inverted Zeiss microscope with a 40 × fluorescence objective under continuous superfusion at a flow of 1 mL/min. The experimental solutions were removed with a microaspirator connected to a vacuum pump. The cells were illuminated using a xenon lamp with quartz collector lenses. A shutter and a filter wheel containing the two different interference filters (340 nm and 380 nm) were controlled by a computer. Emitted light was passed through a 400-nm dichroic mirror, filtered at 490 nm and collected by a CCD camera connected to a light intensifier. The images were digitized in an image processor (Atto Bioscience, Rockville, MD, USA) connected to a computer equipped with Attofluor software. An internal calibration curve was performed; R_{max} and R_{min} are the ratios at saturating and zero $[Ca^{2+}]_i$, respectively, and were obtained by perfusing cells with a salt solution containing 10 mM $CaCl_2$, 10 μM ionomycin and subsequently with a Ca^{2+}-free salt solution containing 10 mM EGTA. Ninety-nine cells were analysed simultaneously for each experiment.

Tetanus toxin fragment C cell staining

Tetanus toxin fragment C–horseradish peroxidase conjugate (FRC-HRP, List Biological Laboratories, Inc., Campbell, CA, USA), was used as previously described (Borodinsky and Fiszman 1998) at a final concentration of 5 μg/mL in HEPES/BSA (bovine serum albumin): NaCl 145 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, HEPES 10 mM and glucose 10 mM; BSA (final concentration 0.1%). The cultures were rinsed three times with Hank's balanced salt solution (HBSS)/BSA, and incubated for 30 min at room temperature (22°C) in FRC-HRP. After rinsing with HBSS/BSA three times, the cells were fixed for 30 min in 4% paraformaldehyde. The samples were then washed three times in 0.01 M phosphate-buffered saline (PBS) and incubated in 3-3′ diaminobenzidine 0.75 mg/mL in Tris buffer 50 mM pH 7.4 containing 0.01% H_2O_2 (substrate) for 30 min. The reaction was stopped with four washes of 50 mM Tris–HCl.

Measurement of fractal dimension

Neuronal complexity was determined by measuring the fractal dimension (*D*) of 2 DIV neurones grown at low density and stained with FRC-HRP. Fractal dimension was calculated as previously described (Borodinsky and Fiszman 2001). Briefly, binary silhouettes were obtained using ScionImage software (Scion Corp., Frederick, MD, USA) and *D* was calculated using the dilation method included in an appropriate macro (Smith *et al*. 1996). The perimeter of the border of the cell was determined by replacing each border pixel with a disc that varied in size from 4 to 128 pixels. The equivalent perimeter length after each dilation was plotted against the diameter of the dilating disc in a log–log scale. The points were fitted by least squares regression and the fractal dimension was calculated from the linear slope (S) by the formula $D = 1–S$. Neuronal fractal dimension range is between 1 and 2, with higher *D* indicating greater complexity. The fractal dimension of neurone increases with the ruggedness of the cellular border, the degree of

branching and with space-filling capacity (Smith *et al*. 1989; Neale *et al*. 1993).

Drug treatments [25 mM KCl (25K), nifedipine, PD098059, KN93, $MgCl_2$] were performed 24 h before image processing analyses.

Cell survival

MTT assay

Cerebellar granule cells were seeded in 96-multiwell (300 000/well) in serum-free medium in a humidified atmosphere (37°C, 5% CO_2). Drugs were added after 48 h *in vitro*, and kept in culture for 5 days. A colourimetric viability assay [3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma, St Louis, MO, USA] was carried out as previously described (Borodinsky and Fiszman 1998). Briefly, 10 µL of the MTT-labelling reagent were added to each well and the microtiter plate was incubated for 4 h in a humidified atmosphere (37°C, 5% CO_2), 100 µL of the solubilization solution were added into each well and the plates were allowed to stand overnight in the incubator in a humidified atmosphere (37°C, 5% CO_2). The absorbance was measured using an ELISA reader at a wavelength of 570 nm, and expressed as the ratio: (experimental–control)/control values.

Cell counting

Cerebellar granule cells were seeded in 24-multiwell (300 000/well) in neurobasal supplemented in 10% fetal bovine serum or in serum-free medium. After 48 h *in vitro,* the neurones were identified as phase-bright cells with one or more processes, that stained for TTC (Borodinsky and Fiszman 1998) and counted in three fields of quadruplicate samples (1 mm^2) using an ocular with a grid, under phase-contrast optics at 400 × magnification. The drugs were added at day 2 and remained with medium for 5 days after which the cells were counted. The ratio between 7- and 2-DIV neurones was calculated for each individual dish. This survival index was expressed as the ratio of (experimental–control)/control values.

Western blot analysis

To assess the levels of phosphorylated MAP kinase (Erk 1/2) and phosphorylated CaMKII, CGC kept in culture for 2 or 7 DIV were treated with 25K for 5, 15 or 30 min at 37°C. After this treatment, the neurones were washed with ice-cold PBS and harvested in a lysis buffer containing 25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X100, 0.5 mM glycerolphosphate, 0.1 mM sodium vanadate, 2 µg/mL leupeptin, 0.6 mM phenylmethyl sulfonyl fluoride (PMSF) and 0.5 mM dithiothreitol (DTT). The samples were centrifuged and the pellet was discarded. The amount of protein present in supernatants was measured in each sample as described by Lowry *et al.* (1951). Approximately 200 µg aliquots were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrilamide gels. After electrophoretic separation, proteins were transferred to nitrocellulose membranes (0.45 µm; Bio-Rad, Hercules, CA, USA), blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20, and were probed with specific antibodies (antiphosphorylated ERK, 1 : 500, Santa Cruz Biothechnology, Santa Cruz, CA, USA; antiphosphorylated CaMKII, 1 : 5000, Promega, Madison, WI, USA). Immunoreactive bands were visualized using a chemiluminescence method and were quantified by NIH IMAGE software (NIH, Bethesda, MD, USA).

Drug treatments

All treatments: $MgCl_2$, nifedipine (all from Sigma), PD098059 (New England Biolabs Inc., Beverly, MA, USA), KN93 (Seikagaku Corporation, Falmouth, MA, USA), were performed 1 h before the addition of 25K.

Results

Fura-2 calcium imaging experiments demonstrated that 25K induced an increase in $[Ca^{2+}]_i$ on CGC grown *in vitro* for 1, 2 or 6 days (Fig. 1). This increase was significantly higher in 6-day-old cultures (33 times, $\Delta[Ca^{2+}]_i = 1745 \pm 300$ nM) as compared with 1- or 2-day-old-samples (nine times, $\Delta[Ca^{2+}]_i = 445 \pm 30$ nM, Fig. 1). The rise in $[Ca^{2+}]_i$ was blocked by 1 µM nifedipine (Fig. 1). The average basal $[Ca^{2+}]_i$ was 55 ± 5 nM (mean ± SEM, $n = 20$).

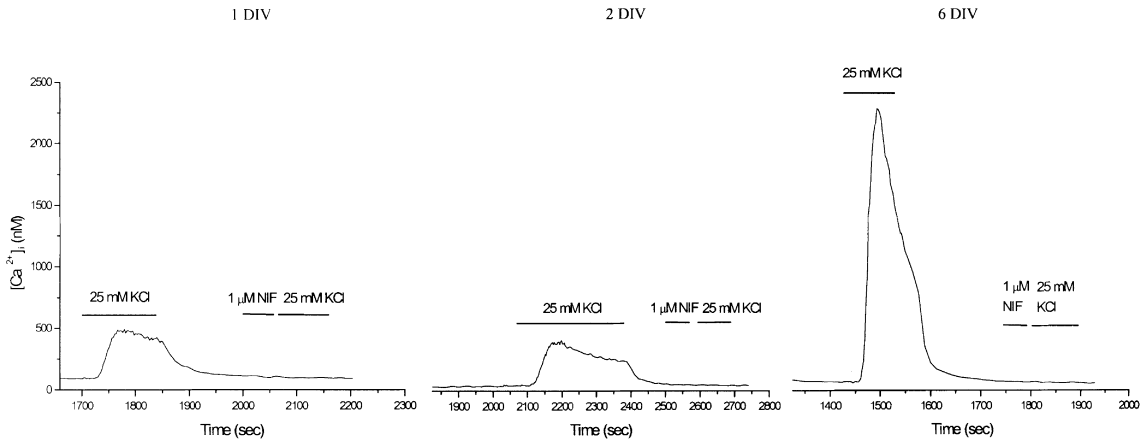

Fig. 1 Fura-2 measurements of intracellular calcium levels in response to 25 mM KCl. Representative traces recorded from 83, 81 and 84 CGC (for 1, 2 and 6 DIV, respectively) registered in a single experiment. Bars at the top of the traces indicate the period of drug application.

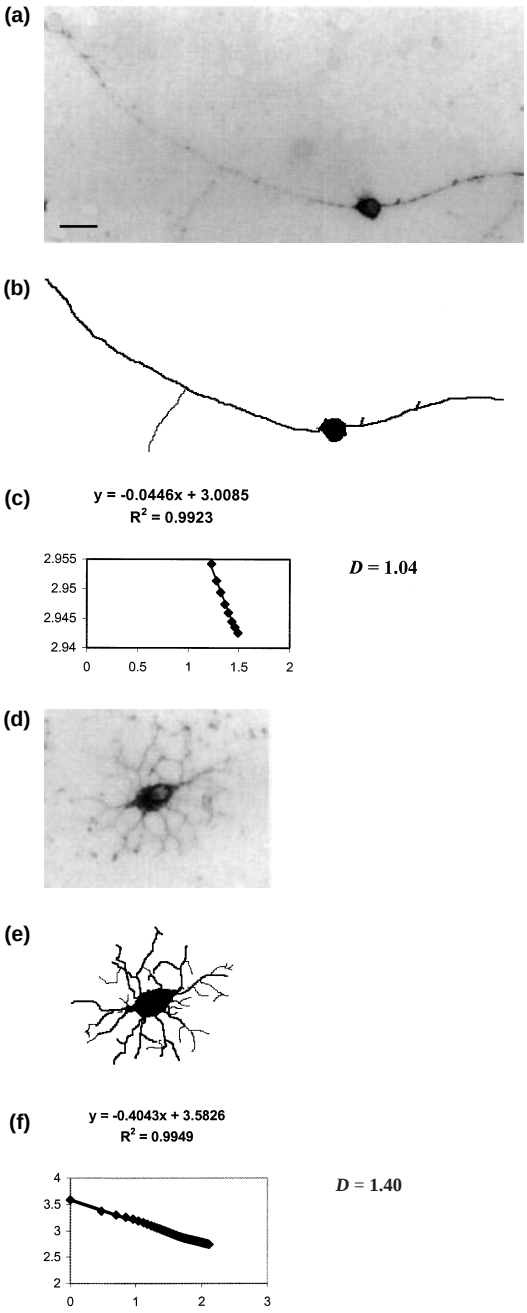

Fig. 2 Image analyses for cultured cerebellar granule cells fractal dimension in 5 and 25 mM KCl. Cerebellar granule cells were inoculated at low density (5500 cells/cm^2) in serum-free medium (5K; a, b, c) and 25K was added after 24 h *in vitro* (d, e, f). FRC-HRP staining was performed 48 h after inoculation (a,d). Binary silhouettes were obtained using Scion Image software (b,e). Fractal dimension (*D*) was calculated using the dilation method (c,f). The log of the perimeter of the neuron is plotted against the log of the disc diameter used to measure the perimeter. A graph resulting in a straight line indicates that the object is fractal, with a *D* equal to 1 minus the slope of the line. The *D* for neuron (a) is $D = 1.05$, for neuron (d), $D = 1.40$. Scale bar in (a): 5 μm.

Neurite outgrowth

Cells grown for 48 h under serum-free and low-density conditions become differentiated phenotypes and they are intensively stained with FRC-HRP, a specific post-mitotic neuronal marker (Fig. 2a,d). The neuronal fractal dimension (*D*) for isolated neurones grown in 5K is close to the unit, corresponding to the less complex (bipolar) neuronal morphology (Fig. 2a–c). The addition of 25K after 24 h *in vitro* induced an increase in the number of primary neurites and correspondingly in the *D* (Fig. 2d–f). Figure 3(a) shows three examples of binary silhouettes obtained from cells treated as described in the left panel. Treatment with 25K induced an increase in either the number of primary and/or secondary neurites corresponding to a threefold increase on the complexity of neuronal morphology ($D_{5K} = 1.081 \pm 0.009$; $D_{25K} = 1.25 \pm 0.01$, $p < 0.0001$; Fig. 3b). This increase in complexity was blocked by Ca^{2+} influx blockers such as 10 mM $MgCl_2$ or by 1 μM nifedipine, an L-type VGCC blocker (Fig. 3).

To evaluate the intracellular pathway involved in the increase on neuronal complexity induced by Ca^{2+} influx, we studied the effect of PD098059, a MEK1 inhibitor, and KN93, a CaMKII inhibitor. The addition of 75 μM PD098059 failed to block 25K induced-increase on *D* (Fig. 3) and cell morphology was similar to that seen with 25K alone (Fig. 3a). On the contrary, 10 μM KN93 completely reversed the 25K effect (Fig. 3).

Neuronal survival

Figure 4 shows CGC cultures grown for 7 days in 5K or 25K under serum-free optimized formula (neurobasal + B27). Serum-free formula allowed an enriched neuronal culture to be obtained, 99% of the cells stained with the neuronal marker (Fig. 4).

Samples grown in 25K showed a larger number of neurones as compared with 5K cultures. Large fasciculating networks stained with FRC-HRP developed in 25K samples grown for 7 days (Fig. 4d). In contrast, the staining of neurones grown in 5K was weaker and the neurites were shorter as compared with cells grown in 25K (Fig. 4b).

Neuronal survival was similarly increased by 25K in serum-free and serum-containing media, suggesting that this phenomenon was independent of the presence of serum (Table 1). The effect of 25K was independent of the serum-free additives used, as there were no differences between survival in cells cultured with optimized B27-supplemented medium (Table 1) or N2 (% survival in 5K: 0.4 ± 0.1; in 25K: 0.79 ± 0.15, $n = 3$), a supplement not as enriched as the former.

As previously reported for serum-supplemented conditions, the increase in neuronal survival induced by 25K was completely blocked by 10 mM $MgCl_2$ and 10 μM nifedipine (Fig. 5a), and neither 10 mM $MgCl_2$ nor 10 μM nifedipine affected neuronal survival in 5K cultures (Fig. 5a).

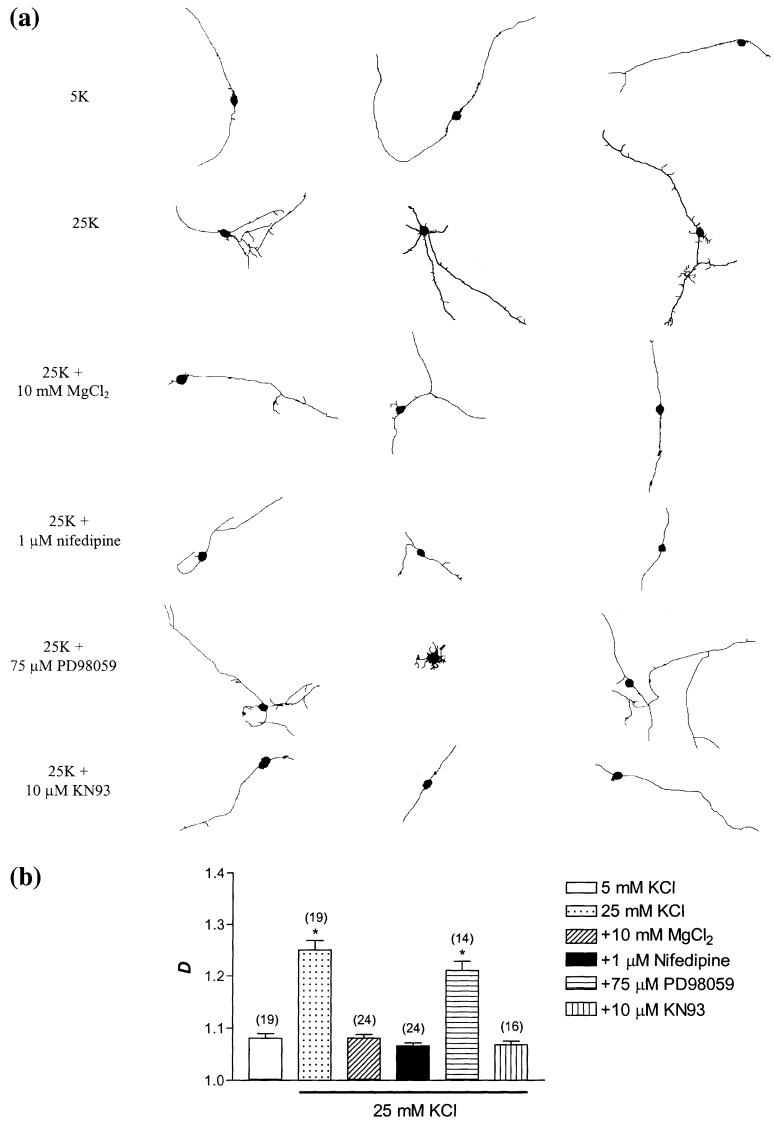

Fig. 3 Effect of different treatments on cerebellar granule cells fractal dimension. (a) Binary silhouettes of typical examples of neuronal morphologies obtained in control and treated samples. (b) Pooled data of *D* calculated for CGC grown under different treatments. Parenthesis, number of neurones analysed. $p < 0.0001$ versus 5K samples (*t*-test).

The blockade of 25K-induced increase in neuronal survival was not completely blocked, even at supramaximal effective concentrations (Alessi *et al*. 1995; Villalba *et al*. 1997) with PD098059 (20–75 μM; Fig. 5b). In 5K cultures, the MEK1 inhibitor induced a concentration-dependent decrease on neuronal survival (Fig. 5b). KN93 completely blocked 25K-induced increase in neuronal survival in a concentration-dependent manner, without affecting the survival of cells plated in 5K (Fig. 5c).

Kinase activity in CGCs

Because the results described above suggested that 25K-induced neuritogenesis and CGC survival involved the activity of CaMKII and/or MEK1 cascades, it seemed worthwhile to assess the activity of CaMKII and Erk1/2.

Figure 6(a,b) show that in 2 DIV as well as in 7 DIV CGC cultures, there was a two- to fivefold increase in CaMKII activity during the first 30 min after 25K addition, and that such an effect was already evident as early as 5 min. The data in Fig. 6(c,d) show a two- to fourfold increase in the active phosphorylated form of Erk1/2 after the addition of 25K in 2 and 7 DIV cultures, which was not due to an increase in the total levels of the kinase, and correlates with the kinase activity detected by immunoprecipitation of endogenous Erk1/2 and phosphorylation of a substrate *in vitro* (data not shown).

Discussion

Activity-dependent increase in complexity of neuronal morphology

Neuronal function relays on the ability of cells to make specialized connections, that are a function of an appropriate neuronal morphology. Thus, the quantification of neuronal morphology might probe useful in the study of neuronal

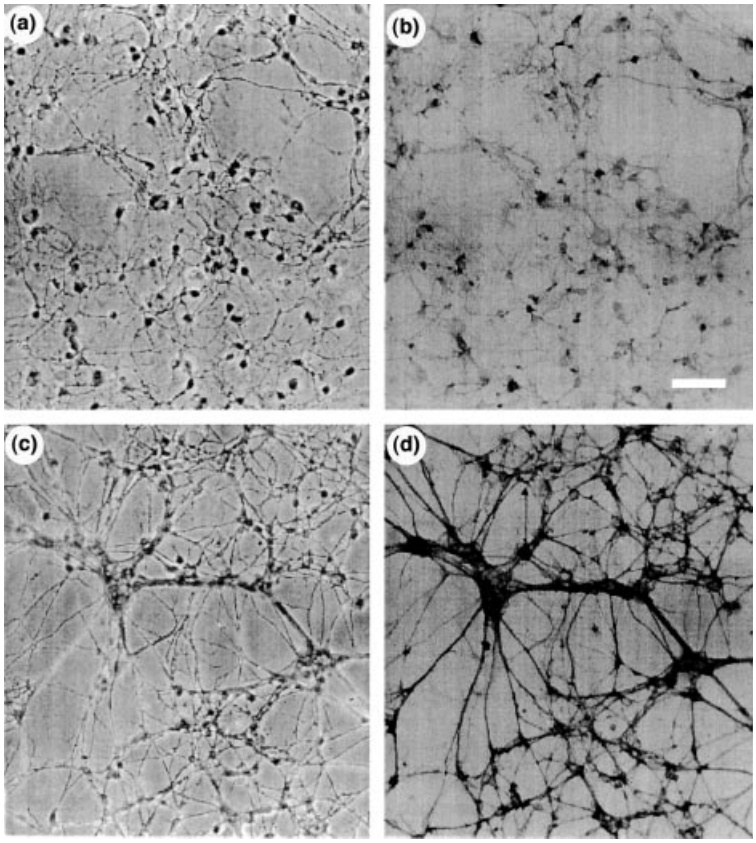

Fig. 4 Long-term cerebellar granule cells cultures grown under serum-free conditions. Phase-contrast (a,c) and bright field photomicrographs of FRC-HRP staining (b,d) of cerebellar granule cell culture grown for 7 days in neurobasal + B27 medium containing 5 (a,b) or 25 mM KCl (c,d). Similar data were obtained when cultures were grown for 7 days in neurobasal + N2. Scale bar in (b): 30 μm.

Table 1 The effect of 25 mM KCl on cerebellar granule cells survival under different culture conditions

	Cell counting		MTT assay
	BME + 10% FBS	Neurobasal + B27	Neurobasal + B27
5K	0.42 ± 0.06 (6)	0.33 ± 0.05 (4)	0.49 ± 0.04 (7)
25K	0.84 ± 0.08 (9)[a]	0.81 ± 0.12 (4)[a]	0.89 ± 0.06 (7)[a]

Cerebellar granule cells were inoculated on serum-free or serum-supplemented medium for two days in 5 mM KCl (5K). The addition of 25 mM KCl (25K) was performed at day 2 and data (mean ± SEM) were collected at day 7 as described in Experimental procedures. Between parenthesis are the number of experiments. [a]$p < 0.0001$ versus 5K samples (*t*-test).

differentiation. Fractal dimension, *D*, has been considered an appropriate parameter for measuring the complexity of neuronal morphology (Smith *et al*. 1989, 1991, 1996; Borodinsky and Fiszman 2001).

In the present study we found that control neurones grown in resting conditions exhibited a *D* close to 1, a value for fractal dimension that corresponds to the simplest neuronal morphology. The exposure of isolated neurones to 25K, as a way of generating depolarizing responses, induced a significant increase in *D* that was dependent on $VGCC_L$ activation. There are many reports in the literature on the effect of VGCC activation on neurite outgrowth with similar, opposite or no effect. For instance, Ca^{2+} influx induced an inhibition of growth cone motility in dorsal root ganglion neurones (Fields *et al*. 1993), while in diencephalic neurones it promoted growth cone extension (Connor 1986). In rat sympathetic neurones, depolarization does not influence neurite outgrowth (Franklin *et al*. 1995), while in cultured hippocampal neurones, it strongly influences the number of dendritic spines without affecting the complexity of the dendritic arbour (Kossel *et al*. 1997). Therefore, different phenotypes seem to respond in different ways to the same stimuli (for review see Neely and Nicholls 1995).

The intracellular mechanism triggered by calcium that induces changes in neuronal morphology is not known. Our data obtained with kinases blockers suggest that neural activity induces the increase in *D* in CGC via CaMKII activation and not via Erk1/2, although both enzymes were activated by depolarization. As was previously reported using time-lapse video observations in the developing optical tectum of the *Xenopus* before and during synaptogenesis, neurones go through a period of rapid dendritic elaboration that slows when synapses are well established. The establishment of synapses coincides with an increase in CaMKII expression (Wu and Cline 1998; Wu *et al*. 1999). In the present study, in immature CGC grown in isolation, CaMKII seems to play a stimulatory role in neurite outgrowth as it

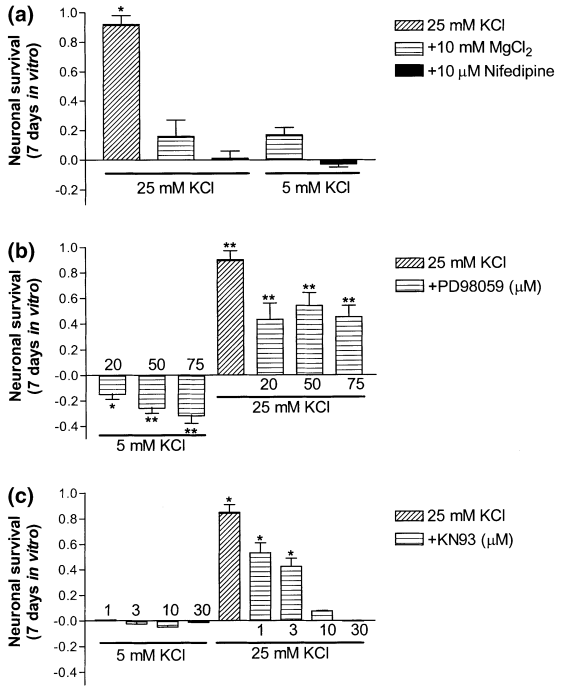

Fig. 5 The influence of voltage-gated calcium channels and kinases inhibitors in 25 mM KCl-induced increased granule cell survival under serum-free conditions. Cerebellar granule cells were inoculated in serum-free medium. After 2 days *in vitro*, 25 mM KCl were added. When used, $MgCl_2$, nifedipine, PD098059 and KN93 were incubated 1 h before the addition of 25K. MTT assay was performed 7 days after inoculation. Data are expressed as the mean of the ratio (experimental–control)/control of four experiments. *$p < 0.01$ versus 5 mM KCl samples (*t*-test); **$p < 0.0001$ versus 5 mM KCl samples (*t*-test).

does in the pre-synaptogenic period of the *Xenopus*. Future experiments will define the role of CaMKII during synaptogenesis in CGC.

Activity-dependent survival versus resting neuronal survival

Many factors have been reported to increase *in vitro* long-term CGC survival including IGF-1, NT3/4 and brain-derived neurotrophic factor (BDNF; D'Mello *et al*. 1997; Villalba *et al*. 1997; Skaper *et al*. 1998; Bonni *et al*. 1999). Neural activity, mimicked either by incubating cell cultures with depolarizing KCl concentrations (Galli *et al*. 1995; Gallo *et al*. 1987; Mao *et al*. 1999) or by glutamate or glutamate receptors agonists (Hack *et al*. 1993; Bhave *et al*. 1999), is also reported to play a role as a survival factor for this cell phenotype. The receptors for a number of these neurotrophic factors have intrinsic tyrosine kinase activity, whereas neural activity-dependent survival is mediated by increased Ca^{2+} influx.

Many of the studies exploring the effects of high $[K^+]_e$ in this cell preparation were carried out in the presence of serum, which may contain trophic substances capable to triggering tyrosine kinases cascades. In the present report it was found that the magnitude of the increase in survival of CGC due to VGCC activation is similar in samples grown with or without serum, suggesting that neural activity is sufficient to support trophic effects.

In CGC cultured under serum-free conditions, calcium influx promotes survival through a CaMKII-dependent mechanism, as KN93 completely blocked depolarization-induced survival. This confirms previous observations on 25K-induced increase in CGC survival performed by others (Hack *et al*. 1993). However, whereas our data show that resting survival is not dependent on CaMKII activation, data reported by Hack *et al*. (1993) showed that KN62 (CaMKII blocker) decreased basal cell survival. This discrepancy may be due to the absence of serum in our culture media.

Erk1/2 activation has been already linked to the promotion of cell proliferation or the prevention of cell death in neurones and other cell types (Cuvillier *et al*. 1996; Borodinsky and Fiszman 1998; Ajenjo *et al*. 2000). We have previously described that the blockade of the Erk cascade in CGC inhibits cell proliferation induced by 25K in early cultures (Borodinsky and Fiszman 1998) and has a significant detrimental effect on the survival promoted by 25K after 7 DIV (Fig. 5). Western blot assays revealed that 25K was able to increase Erk1/2 phosphorylation in 7-DIV cultures, and neuronal survival was partially dependent on MEK1 activation. MEK1 can be activated through a direct depolarization as reported in PC12 cells (Rosen *et al*. 1994), or through neurotrophins (Ghosh and Greenberg 1995). In this report, PD098059 blocked survival of CGC plated in 5K in a concentration-dependent manner, suggesting that resting survival may be dependent on MEK1 activity. This was in agreement with the results obtained in western blots that revealed a high basal level of ERK1/2 in its phosphorylated form in control cultures. There are several possible explanations for MEK1-mediated resting survival: autocrine factors may activate MEK1 pathway; intracellular Ca^{2+} stores may contribute to the phosphorylation of this pathway under resting conditions. In agreement with this last hypothesis, Pende *et al*. (1997) postulated, for oligodendrocyte progenitors, that resting levels of Ca^{2+} are essential as a co-factor of some regulatory elements of the MAPK pathway, as they found that chelation of cytoplasmic Ca^{2+} affects the basal phosphorylation of MAPK.

In conclusion, in CGC, Ca^{2+} influx can trigger different effects depending on the stage of cell maturation. In the round-shaped neuroblast, calcium induces proliferation and then neurite extension finally supports survival of mature neurones. Our data show that neuritogenesis and neuronal survival are mediated by CaMKII activity. Because this enzyme was reported to play a critical role in synaptic establishment (Wu *et al*. 1996), the CaMKII-induced increase in survival may be related to the maintenance of

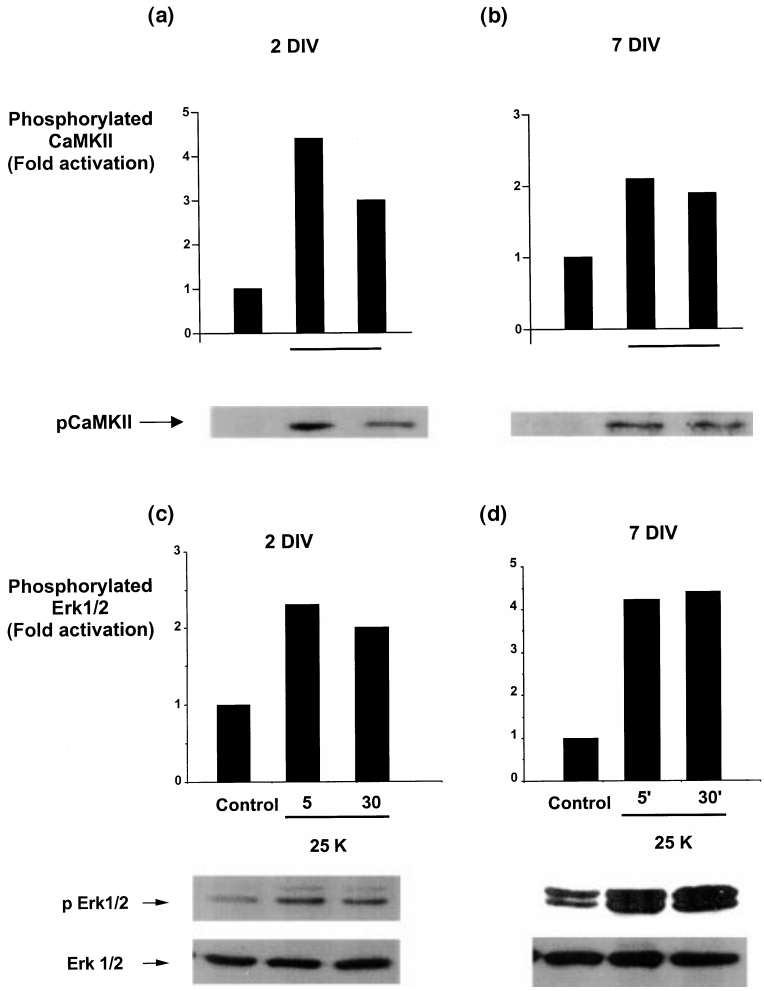

Fig. 6 Effect of 25 mM KCl on phosphorylation of Erk1/2 and CaMKII. CGC were prepared and grown in serum-free medium, as described in Experimental procedures. On 2 (a,c) or 7 (b,d) DIV, the cells were treated with 25K for 5 or 30 min. Cytosols were extracted and immunobloting for phosphorylated CaMKII (a,b) or for phosphorylated Erk1/2 (c,d) as described in Experimental procedures. Bar graphs show the densitometric analysis of the immunoreactive bands using NIH Image software and considering the control lane in each immunoblot as 1.

neural networks, suggesting that CaMKII acts as the intracellular mediator between neural activity and a variety of events that take place during neural development.

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