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Apoptosis in Cerebellar Granule Cells Is Blocked by High KCl, Forskolin, and IGF-1 Through Distinct Mechanisms of Action: The Involvement of Intracellular Calcium and RNA Synthesis

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Cerebellar granule cells deprived of depolarizing concentration of extracellular potassium, $[K^+]_o$, undergo apoptosis. We here report that this apoptotic process is associated with an immediate and permanent decrease in the levels of free intracellular calcium, $[Ca^{2+}]_i$. Although forskolin and IGF-1 are both able to prevent apoptosis, only forskolin is able to counteract the instantaneous decrease of $[Ca^{2+}]_i$. However, the early effect of forskolin on $[Ca^{2+}]_i$ is lost after longer incubation in low $[K^+]_o$.

The calcium antagonist nifedipine is able to inhibit the survival effect of high $[K^+]_o$, while not affecting forskolin and IGF-1 promoted survival, as assessed by viability and genomic DNA analysis. Accordingly, the L-type calcium channels agonist Bay K8644 significantly enhanced the survival of low KCl treated neurons. To temporally characterize the signal transduction events and the essential transcriptional step in cerebellar granule cells apoptosis, we determined the time course of the rescue capacity of high $[K^+]_o$, forskolin, IGF-1, and actinomycin D. Addition of high KCl, forskolin, or IGF-1 6 hr after the initial KCl deprivation saves 50% of cells. Remarkably, 50% of neurons loss the potential to be rescued by actinomycin D after only 1 hr in low $[K^+]_o$. Finally, we show that the survival promoting activities of high $[K^+]_o$, forskolin, and IGF-1 do not require RNA synthesis.

We conclude that $[Ca^{2+}]_i$ is involved in the survival promoting activity exerted by high $[K^+]_o$ but not in those of forskolin and IGF-1, and that all three agents, although rescuing neurons from apoptosis through distinct mechanisms of action, do not necessitate RNA transcription.

[Key words: apoptosis, cerebellar, neurons, calcium, RNA, forskolin, IGF-1]

Programmed cell death in the nervous system is a process which leads entire populations of neurons to degenerate and die during development. The function of apoptosis in neuronal development seems to be the removal of excess neurons and the establishment of correct synaptic connections (Catsicas et al., 1987; Oppenheim, 1991). Though much less is known, a link between apoptosis and neuronal degeneration has been hypothesized in Alzheimer's disease (May et al., 1991; Forloni et al., 1993; Loo et al., 1993).

Sympathetic neurons deprived of NGF represent the most commonly used *in vitro* model for the study of neuronal apoptosis (Martin et al., 1988). This process has an *in vivo* correlate in axotomized cervical ganglia which can be rescued from degeneration by administration of NGF (Hendry and Campbell, 1976). Parameters such as the apoptotic time course, commitment point, dependence on protein synthesis (Martin et al., 1988; Edwards et al., 1991), relationship with intracellular calcium concentration (Koike and Tanaka, 1991) and gene expression (D'Mello and Galli, 1993) have been thoroughly investigated in sympathetic neurons.

We have recently identified the first *in vitro* model for apoptosis in the central nervous system, demonstrating that cerebellar granule cells, deprived of depolarizing levels of extracellular potassium, undergo apoptosis (D'Mello et al., 1993). We have demonstrated that apoptotic death, triggered in mature cerebellar granule cells by lowering extracellular KCl concentration, $[K^+]_o$, from 25 mM to 5 mM, can be inhibited by forskolin, IGF-1, and, as in most apoptotic models, by inhibitors of macromolecule synthesis (D'Mello et al., 1993). Wood et al. (1993) have recently found the *in vivo* correlate of cerebellar granule cells apoptosis, showing naturally occurring DNA fragmentation in the granular layer of newborn rat cerebellum.

The signaling pathways leading to apoptosis and the mechanism of action by which certain agents prevent apoptosis are largely unknown. Intracellular calcium has been postulated to play a key role in the regulation of apoptosis (Franklin and Johnson, 1992); in fact neuronal survival has been correlated to sustained levels of cytosolic calcium in several neuronal models such as rat sympathetic neurons (Koike et al., 1989), chick ciliary ganglia (Collins et al., 1991), dorsal root ganglion neurons (Eichler et al., 1992), and developing cerebellar granule cells (Gallo et al., 1987; Koike, 1991; Pearson et al., 1992). Since depolarizing KCl induces opening of voltage operated calcium channels (Carboni and Wojicik, 1988; Courtney et al., 1990), it must be expected that, in cerebellar granule cells, changes in intracellular calcium concentration, $[Ca^{2+}]_i$, are associated with the induction of apoptosis by repolarization.

Much less is known about the relationship between $[Ca^{2+}]_i$ and the action of forskolin and IGF-1. Trophic factors and

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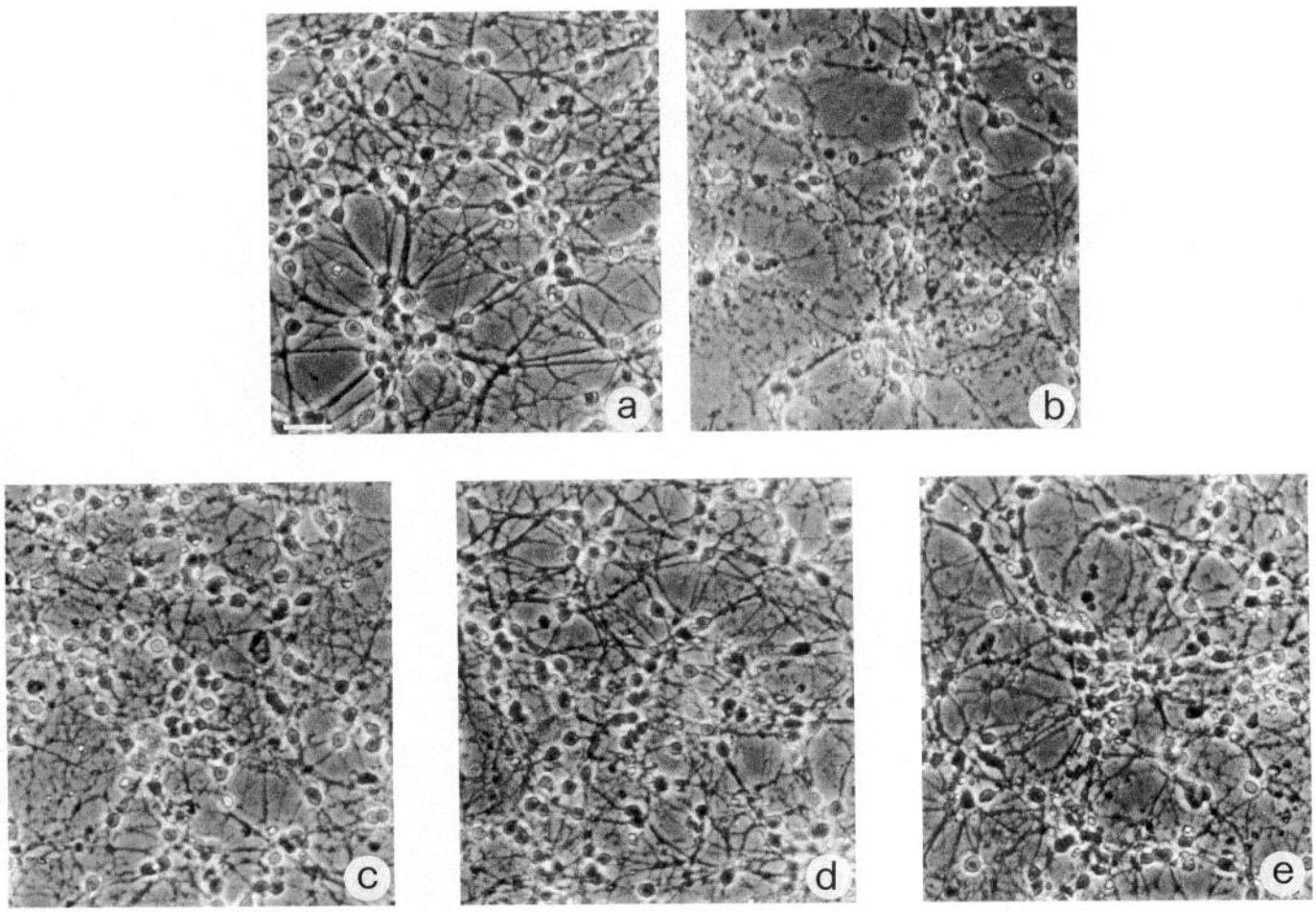

Figure 1. Low KCl-induced apoptosis can be inhibited by various agents. Six DIV neurons were shifted from high to low KCl (5 mM) serum-free culture medium and photographed 48 hr later using Nikon phase contrast microscope. Cultures *a* were maintained in 25 mM, cultures *b–e* were kept at 5 mM KCl. Neurons in *c–e* were added with 10 μM forskolin, 25 ng/ml IGF-1, and 1 μg/ml actinomycin D, respectively. Scale bar, 15 μm).

depolarizing $[K^+]_o$ possess a certain degree of interchangeability in the promotion of *in vitro* neuronal development and survival (Wakade et al., 1983; Collins and Lile, 1989; Koike et al., 1989). Whether or not the intracellular messages of these stimuli act on a common target is yet unknown. Although brain-derived neurotrophic factor and neurotrophin-3 induce increase of $[Ca^{2+}]_i$ in hippocampal neurons (Berninger et al., 1993), there is no direct evidence for a role of intracellular calcium in the survival promoting activity of growth factors (Franklin and Johnson, 1992).

The triggering of apoptosis is transcription dependent, a finding which allowed the hypothesis that neosynthesized "suicide proteins" are responsible for apoptotic degeneration (Martin et al., 1992; D'Mello and Galli, 1993; Johnson and Deckwerth, 1993). Furthermore, in NGF-deprived sympathetic neurons, it has been demonstrated that survival agents such as cAMP, high KCl, and NGF, block the suicide program of apoptosis by unknown posttranslational events (Edwards et al., 1991), which should neutralize the effect of suicide proteins. It was therefore interesting to characterize the role of $[Ca^{2+}]_i$ and macromolecule synthesis in the apoptotic death of mature cerebellar granule cells with particular attention to the action of the agents able to interfere with the apoptotic process.

In this study we have correlated measurements of $[Ca^{2+}]_i$ at single cell level to the survival promoting action of high KCl, forskolin, and IGF-1, and found significant differences between the mechanisms of action of these three agents. We further show that the protective effect of high KCl, forskolin, and IGF-1 is independent of neosynthesis of RNA.

Materials and Methods

Neuronal cultures. Primary cultures of cerebellar granule neurons were obtained from dissociated cerebella of 8 d old Wistar rats (Charles River Breeding Laboratories) (Levi et al., 1984). Cells were plated in basal medium Eagle (BME, GIBCO) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine (GIBCO), and 100 μg/ml gentamicin (GIBCO) on poly-(L-lysine) precoated dishes (NUNC). At plating, cells were 3×10^5 per cm^2 (2.5×10^6 cells per 35 mm dish or 7×10^6 per 60 mm dish). 10 μM cytosine-β-D arabinofuranoside was added to the culture medium 18–22 hr after plating to prevent proliferation of non-neuronal cells.

Treatment of cultures. Six to seven days *in vitro* (DIV) cultures were washed twice and maintained in serum-free BME (normally containing 5 mM KCl) supplemented with glutamine, gentamicin, and cytosine-β-D-arabinofuranoside at the concentrations indicated above. Control cells were washed identically and maintained in the same medium supplemented with KCl at the final concentration of 25 mM.

Single cell intracellular $[Ca^{2+}]_i$ measurements. Cells were plated on 25 mm clean N.1 glass coverslips previously coated with 10 μg/ml poly-(L-lysine) placed in a 35 mm dish. Culture medium of 6–7 DIV neurons was replaced by 2 ml of Locke's solution containing (mM) 154 NaCl, 5

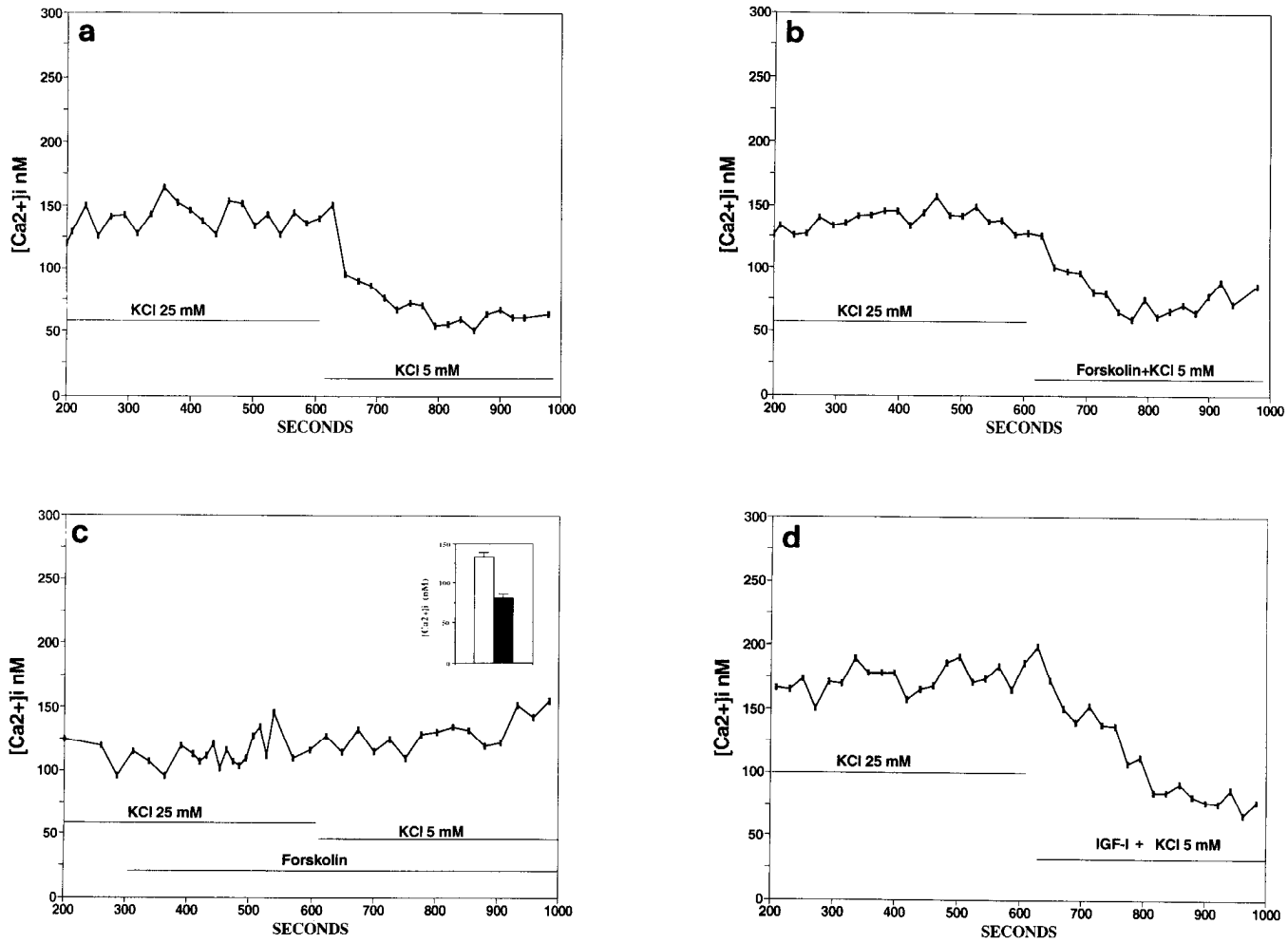

Figure 2. Rapid change in intracellular $[Ca^{2+}]_i$ induced by lowering extracellular KCl. Neurons, that had been grown for 6–7 DIV in standard culture conditions were kept in 25 mM KCl during the entire procedure of washing, loading with fura-2, and equilibrating (see also Materials and Methods). Neurons were shifted to 5 mM KCl with no addition (*a*), 10 μM forskolin (*b*, *c*), 25 ng/ml IGF-1 (*d*). In *c*, cells were preincubated with 10 μM forskolin for 5 min prior the shift to low KCl. Traces correspond to four cells from different cultures and each one is representative of 45–60 neurons. *c inset*, As in *c* with the addition of PKA inhibitor KT5720 1 μM simultaneously to forskolin, values represent means with SEM of 20 cells.

or 25 KCl, 3.6 $NaHCO_3$, 5 HEPES, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 5.6 glucose, pH 7.4, and incubated for 10 min at room temperature. Cells were subsequently incubated for 20 min in 2 ml of Locke's solution containing 4 μM fura-2 acetoxymethyl ester (Calbiochem, San Diego, CA) and then washed in 2 ml of Locke's solution for 10 min before starting the experiment. Coverslips were mounted on a coverslip chamber (Medical System Co., Greenvale, NY) for fluorescence measurements. All measurements were made at room temperature (23°C). Cells were continuously superfused using a peristaltic pump (Gilson, France) set at a flow rate of 450 μl/min and the removal of experimental solutions was achieved using a microaspirator (Medical System Co., Greenvale, NY) connected to a vacuum pump. Fura-2 fluorescence was imaged with an inverted Nikon Diaphot microscope using a Nikon 40×/1.3 NA Fluor DL objective lens. Cells were illuminated with a xenon lamp (Osram, Germany) with quartz collector lenses. A shutter and a filter wheel containing the two different interference filters (340 nm and 380 nm) were controlled by 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. Images were digitized and averaged (four frames for each data point) in an image processor connected to a computer equipped with TARDIS software. For the calibration of fluorescence signals we used cells loaded with fura-2; R_{max} and R_{min} are ratios at saturating and zero $[Ca^{2+}]_i$ respectively and were obtained by perfusing cells with a salt solution containing 10 mM $CaCl_2$, 2.5 μM digitonin, 2 μM ionomycin and subsequently with a Ca^{2+}-free salt solution containing 10 mM EGTA. The values of the obtained R_{max} and R_{min}, expressed as gray level mean, were used to calculate the calibration curve using the TARDIS software. Intracellular calcium concentration was determined according to the equation of Grynkiewicz et al. (1985).

Neuronal survival. The culture medium was removed and neurons were lysed with a detergent-containing solution (Soto and Sonnenschein, 1985; Volonté et al., 1994) which dissolves cell membranes. Intact nuclei were counted in a hemacytometer. As apoptosis is a process which primarily affects the nucleus this method shows higher sensitivity in detecting apoptotic damaged cells than methods based on cytoplasmic enzymatic activity.

Human recombinant IGF-1 was from Boehringer Mannheim, KT5720 was from Kamiya Biochemical (Thousand Oaks, CA), nifedipine was from RBI (Natick, MA), and Bay K8644 was from Calbiochem (La Jolla, CA). All others chemicals were from Sigma.

Results

As already reported (D'Mello et al., 1993) and shown in Figure 1, *a* and *b*, apoptosis is triggered in cerebellar granule cells by lowering $[K^+]_o$ from 25 to 5 mM. The apoptotic process can be inhibited by forskolin, IGF-1, and actinomycin D (Fig. 1*c*–). To investigate the role of free intracellular calcium as second messenger in this process, we characterized the single cell variations of $[Ca^{2+}]_i$ in response to apoptosis inducing or inhibiting treatments.

The decrease of $[Ca^{2+}]_i$ by lowering $[K^+]_o$ is prevented by forskolin but not by IGF-1

The $[Ca^{2+}]_i$ was monitored for 20 min following repolarization by lowering $[K^+]_o$ from 25 mM (K25) to 5 mM (K5). We observed an instantaneous (within 5 sec) drop of $[Ca^{2+}]_i$ from 158 ± 37 nM (SD) (n = 187) to 78 + 15 nM (SD) (n = 187) (Fig. 2*a*), after which $[Ca^{2+}]_i$ remained stable. We noted that cells were not homogenous with respect to their $[Ca^{2+}]_i$ which may explain the differential susceptibility to treatments influencing this intracellular parameter (see Discussion). However, apoptosis induced by lowering $[K^+]_o$ was always associated with a 50% drop of $[Ca^{2+}]_i$.

In order to assess the possibility that survival promoting agents interfere with the change of $[Ca^{2+}]_i$, we lowered $[K^+]_o$ in presence of either forskolin or IGF-1.

Forskolin (10 μM) had no effect on $[Ca^{2+}]_i$ when added to the perfusion medium simultaneously to the potassium deprivation (Fig. 2*b*). In contrast, when cells were preincubated in K25 plus forskolin for 5 min prior to potassium withdrawal, forskolin was able to prevent the decrease of $[Ca^{2+}]_i$, as shown in Figure 2*c*. The effect of forskolin was prevented by the specific protein kinase A (PKA) inhibitor, KT5720 (Kase et al., 1987) (Fig. 2*c* inset) [K25 = 132 ± 6 nM (SEM) (n = 20) (open column); K5 + forskolin + KT5720 = 81 ± nM (SEM) (n = 20) (solid column)].

As demonstrated in Figure 2*d*, IGF-1 did not significantly affect the low $[K^+]_o$-induced decrease of $[Ca^{2+}]_i$ which, in these neurons, had similar kinetics to control cells.

The induction of apoptosis by low $[K^+]_o$ in mature cerebellar granule cells is therefore associated with a reduction of free cytosolic calcium, a phenomenon which may be prevented by forskolin but not by IGF-1, although both agents rescue neurons from apoptosis (Fig. 1*c,d*).

Depolarizing $[K^+]_o$, but neither forskolin nor IGF-1, keeps long term high $[Ca^{2+}]_i$

Since apoptosis is a long term process, we measured the basal level of $[Ca^{2+}]_i$ at different time up to 48 hr following KCl deprivation. The control neurons were kept in serum-free culture medium with 25 mM KCl and their basal intracellular calcium level remained stable at around 122 ± 4 nM (SEM) (n = 42) from 2 to 48 hr. This value is 20% lower than the one reported above for untreated cells and can most easily be explained by the shift from conditioned serum-containing medium to fresh serum-free medium. As shown in Figure 3, the basal $[Ca^{2+}]_i$ of K5 neurons was stably 50% lower than control up to 48 hr.

After 2 hr of forskolin treatment cells showed a $[Ca^{2+}]_i$ 35% lower than in control K25 cells [81 ± 4 nM (SEM) (n = 42)]. In the following 46 hr the basal $[Ca^{2+}]_i$ decreased linearly to the value of K5 neurons (Fig. 3). Nevertheless forskolin-treated cells were perfectly viable 48 hr after the treatment as shown in Figure 1*c*. Therefore, the forskolin survival activity can not be attributed to long term maintenance of a high level of $[Ca^{2+}]_i$.

Although IGF-1 did not influence $[Ca^{2+}]_i$ in the first 20 min of treatment, it was able to maintain long term survival of neurons kept at 5 mM KCl (Fig. 1*d*). Since growth factors are able to influence neuronal excitability either by rapid posttranslational modifications of ionic channels (Magni et al., 1991; Matsuki et al., 1992; Selinfreund and Blair, 1992) or by increasing transcription of ionic channels mRNA (Ginty et al., 1992),

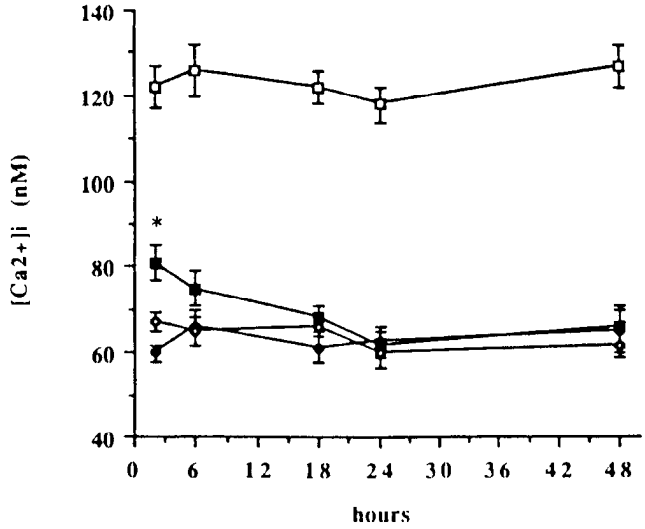

Figure 3. Time course of intracellular $[Ca^{2+}]$ measurement in mature cerebellar granule neurons undergoing apoptosis. Effect of treatment with forskolin and IGF-1. Cultures were washed twice with serum-free BME (5 mM KCl) and incubated for the indicated time with the same medium alone (*solid diamonds*), 10 μM forskolin (*solid squares*), 25 ng/ml IGF-1 (*open diamonds*). Control neurons were kept in serum free BME added with 25 mM KCl (*open squares*) (treatments were allowed to continue during intracellular calcium measurements). $[Ca^{2+}]_i$ was measured at the indicated times and plotted as the mean ± SEM for 60–75 neurons per experimental point. Values corresponding to K5 and K5 + forskolin at 2 hr were found to be statistically different using ANOVA plus Neuman-Keuls test. *, $p < 0.01$.

IGF-1 might modify calcium homeostasis by this second mechanism, thus rescuing neurons from apoptosis. We therefore measured the basal $[Ca^{2+}]_i$ of IGF-1 treated neurons at different times up to 48 hr. As illustrated in Figure 3, neurons treated with IGF-1 displayed a $[Ca^{2+}]_i$ of 65 ± 3 nM (SEM) (n = 21) throughout the experiment, a value corresponding to the basal $[Ca^{2+}]_i$ of K5-treated neurons. We conclude that survival promoted by IGF-1 in low KCl does not correlate to modifications of $[Ca^{2+}]_i$.

Nifedipine reduces neuronal survival induced by high $[K^+]_o$, but does not affect the trophic action of forskolin and IGF-1

In order to evaluate the functional relevance of the correlations between $[Ca^{2+}]_i$ and the survival induced by the agents described above, we quantified the survival of neurons treated with each of the three agents in presence of calcium channels antagonists. Neurons were treated with high $[K^+]_o$, forskolin or IGF-1 both in presence or in absence of 1 μM nifedipine and survival was assessed 48 hr later. As shown in Figure 4 the blockade of Ca^{2+} influx through L-type Ca^{2+} channels by this antagonist, is able to inhibit 40% of the survival promoted by high $[K^+]_o$; moreover, neuronal death induced by nifedipine is associated with DNA fragmentation (data not shown), identifying the death process as apoptotic.

Nifedipine inhibits only a fraction of the depolarization-stimulated Ca^{2+} influx (data not shown; Carboni and Wojcik, 1988; Courtney et al., 1990) and it likely causes death only of those neurons which, having a lower intracellular calcium level (reported above), are more susceptible to a further decrease of $[Ca^{2+}]_i$. Voltage operated nifedipine-insensitive Ca^{2+} channels are present in cerebellar granule cells (Randall et al., 1993; Zhang et al., 1993) and their involvement in the depolarization-pro-

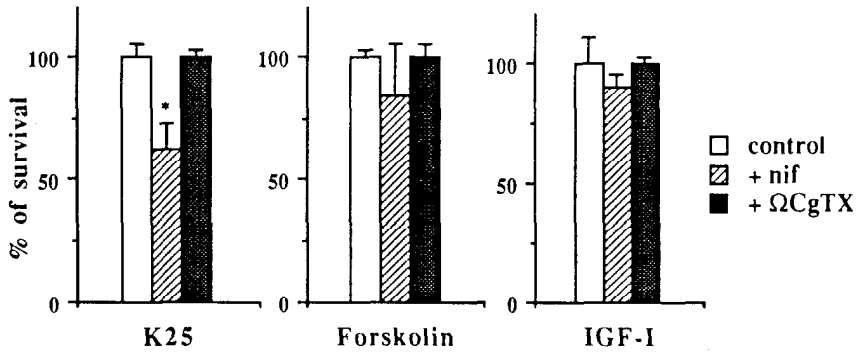

Figure 4. Effect of nifedipine and ω- conotoxin GVIA on the survival promoted by K25, forskolin and IGF-1 in mature cerebellar granule cells. Six to seven DIV cultures were washed twice with serum free BME (5mM KCl) and incubated 48 hr with the same medium added with 25 mM KCl (final concentration), 10 μM forskolin, 25 ng/ml IGF-1, in absence or in presence of 1 μM nifedipine (*nif*) or 1 μM ω-conotoxin GVIA (*ΩCgTX*). Neuronal viability was evaluated by counting intact nuclei after 48 hr from the initial KCl deprivation. Values obtained in presence of calcium channels blockers are expressed as percentage of the viability resulting from treatment with the respective survival agent alone. Values are compiled from three separate experiments performed in duplicate and are means ± SD. K25 + nif significantly different from its control using Student's *t* test; *, $p < 0.05$.

moted survival remains to be shown. However, inhibition of the Ca^{2+} influx through N-type Ca^{2+} channel by ω-conotoxin GVIA did not affect the survival promoted by any of the three examined survival agents (Fig. 4).

As demonstrated in Figure 4, the viability of forskolin-treated neurons was not affected by nifedipine, suggesting that L-type Ca^{2+} channels are not involved in the survival promoting activity of forskolin. Unfortunately we could not assess the effect of KT 5720 on forskolin-promoted survival as the compound exerted a toxic effect on forskolin-treated and control neurons, which was morphologically clearly distinct from apoptosis. Also, the survival promoted by IGF-1 was not influenced by the addition of nifedipine (Fig. 4) confirming, as suggested by $[Ca^{2+}]_i$ measurement, that IGF-1 can rescue cells from apoptosis in spite of their low intracellular calcium concentration.

Bay K8644 increases depolarization-mediated survival

To further confirm the importance of Ca^{2+} influx in the survival promoted by high potassium we quantified survival of mature cerebellar granule neurons exposed for 48 hr to increasing concentration of extracellular KCl both in presence and in absence of the L-type Ca^{2+} channels agonist Bay K8644. As shown in Figure 5 the addition of Bay K8644 decreases the neuronal requirement for extracellular potassium (from 25 mM to 15 mM). Furthermore, at 25 mM KCl, Bay K8644 did not have a significant additive effect, suggesting that, at this level of membrane depolarization, although Bay K8644 can further increase Ca^{2+} influx through L-type channels (Schettini et al., 1991), the $[Ca^{2+}]_i$ has already attained the required level in order to obtain maximal survival.

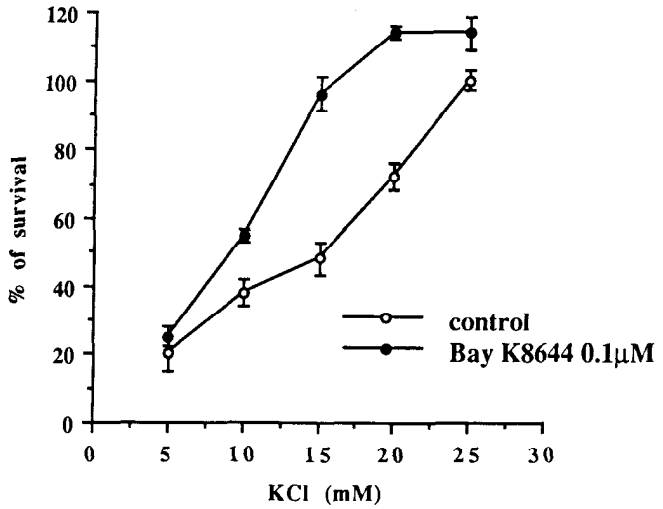

Figure 5. Effect of Bay K8644 on survival of cerebellar granule cells. Six to seven DIV cultures were incubated for 48 hr in serum free BME (5 mM KCl) containing increasing concentration of KCl in absence or in presence of 0.1 μM Bay K8644. Neuronal viability was evaluated by counting intact nuclei. Values are expressed as percentage of the survival obtained with 25 mM KCl. Data are compiled from three separate experiments performed in duplicate and are means ± SD.

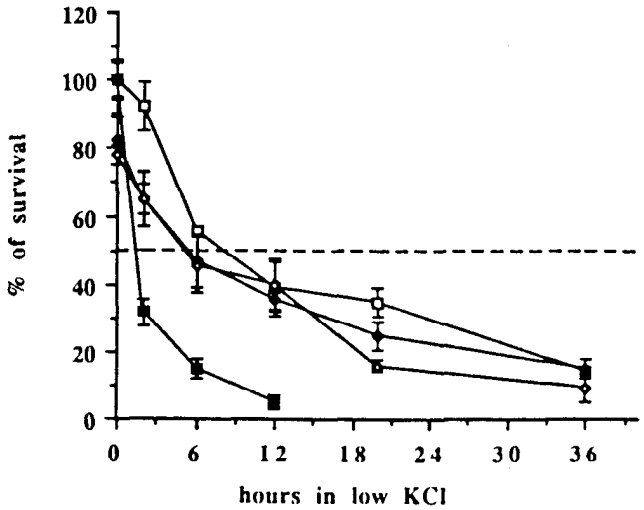

Figure 6. Commitment point to death in cerebellar granule neurons. Apoptosis was triggered by lowering extracellular KCl from 25 mM to 5 mM as described above. Rescue treatments were done by adding high KCl (*open squares*), 10 μM forskolin (*solid diamonds*), 25 ng/ml IGF-1 (*open diamonds*), 1 μg/ml actinomycin D (*solid squares*) at the indicated times. Neuronal viability was evaluated by counting intact nuclei after 48 hr from the initial KCl deprivation. Here 100% correspond to the number of neurons susceptible to death after 48 hr in K5. Values are expressed as mean ± SD of data from three separate experiments performed in duplicate.

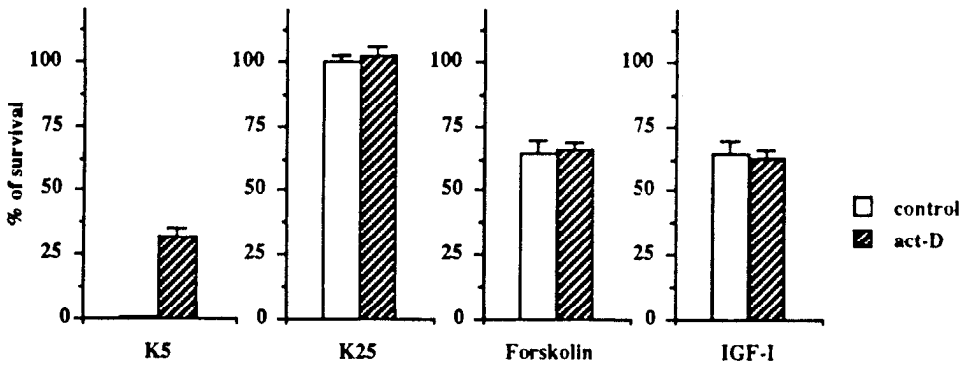

Figure 7. Survival promoted by high KCl, forskolin and IGF-1 is independent of RNA synthesis. Neurons were shifted in low KCl for 2 hr and treated for the following 46 hr with either high KCl, 10 μM forskolin, or 25 ng/ml IGF-1 in the absence or presence of 1 μg/ml actinomycin D. All values are expressed as percentage of survival obtained incubating cells in K25 for 48 hr. Values are compiled from three separate experiments performed in duplicate and are means ± SD.

Rescue of low $[K^+]_o$-treated neurons by high $[K^+]_o$, forskolin, IGF-1, and actinomycin D: commitment point

The drop of intracellular $[Ca^{2+}]_i$ is a very early event in the apoptotic cascade triggered by low $[K^+]_o$. The apoptotic process, on the other hand, is strictly dependent on transcriptional event(s) as we have previously shown by means of actinomycin D (D'Mello et al., 1993, and Fig. 1*e*). In order to define the timing of these transcriptional steps in cerebellar granule cells undergoing apoptosis, we determined the point of commitment to death (TD50), defined as the time at which 50% of K5 neurons can be rescued by the addition of a given survival agent. Neurons were shifted to low $[K^+]_o$ medium and, at different time (from 0 to 36 hr), high $[K^+]_o$, forskolin, IGF-1, or actinomycin D were added to the culture medium. Each recovery treatment was allowed to continue up to 48 hr from the initial KCl deprivation. At that time neuronal survival was evaluated. Time 0 refers to when neurons were treated with high $[K^+]_o$, forskolin, IGF-1, or actinomycin D from the beginning of the experiment and the relative viability was 100%, 82%, 78%, and 100% for these neurons, respectively. As can be seen in Figure 6, 50% of the neurons can be rescued by readdition of K25, forskolin or IGF-1, 6 hr after the shift in low $[K^+]_o$. Strikingly, the TD50 for actinomycin D is relatively early because in order to rescue 50% of neurons, this RNA transcription inhibitor has to be added within 1 hr in low KCl medium, implying that, in this lag of time, half of the neuronal population has transcribed enough message for a putative "suicide protein."

Rescue of low $[K^+]_o$-treated neurons by high $[K^+]_o$, forskolin, and IGF-1 is transcription independent

High $[K^+]_o$, forskolin, and IGF-1 may "neutralize" hypothetically newly synthesized "suicide proteins" by posttranslational mechanisms or, alternatively, may induce neosynthesis of other proteins which modulate the activity of the former so called "suicide proteins." In order to assess these two options, we have examined the ability of high $[K^+]_o$, forskolin, and IGF-1 to rescue neurons in presence of the transcription inhibitor actinomycin D. As described above (Fig. 6) the rescue capacity of actinomycin D dramatically drops from 100%, at the time of KCl deprivation (time 0), to 32% when the rescue treatment is initiated only 2 hr later. Hence, neurons were incubated in low $[K^+]_o$ for 2 hr and then each one of the survival agents was added either in presence or in absence of actinomycin D. Evaluation of survival was performed 48 hr after the initial KCl deprivation. No significant differences were observed between viability of neurons treated with high KCl, forskolin, or IGF-1 in the presence or in the absence of actinomycin D, as shown in Figure 7. The blockade of transcription was therefore unable to affect the survival promoting activity of each agent, clearly demonstrating that new RNA synthesis is not necessary for high KCl, forskolin, and IGF-1 to rescue neurons from apoptosis.

Discussion

Apoptosis is a process which has been observed and characterized in various cell types, including neurons (Oppenheim, 1991). The main features of apoptosis have been identified and found to vary considerably depending on the cellular model examined (Johnson and Deckwerth, 1993). We have recently found that the degeneration of cerebellar granule cells, deprived of high potassium, is an apoptotic process (D'Mello et al., 1993) and the present study has been conceived to characterize and dissect the signal transduction mechanism involved in this model. In particular we have examined several agents, which block apoptosis, and their relation and dependence on intracellular calcium and neosynthesis of RNA.

Cerebellar granule cells undergoing apoptosis as a consequence of lowering $[K^+]_o$ from 25 mM to 5 mM, exhibit the expected decrease in $[Ca^{2+}]_i$ due to the closure of voltage operated Ca^{2+} channels. The $[Ca^{2+}]_i$ remained low throughout the apoptotic process. The role of $[Ca^{2+}]_i$ in the survival activity of depolarizing KCl was confirmed as (1) 40% of the neurons died when incubated in high $[K^+]_o$ in presence of nifedipine, (2) nifedipine induced DNA laddering in high $[K^+]_o$-treated neurons, and (3) the calcium agonist Bay K8644 reduced the $[K^+]_o$ requirement for neuronal survival. Since depolarized neurons are heterogeneous with respect to their $[Ca^{2+}]_i$, these results taken together support the notion that neurons die by apoptosis when their $[Ca^{2+}]_i$ decreases below a certain threshold in the absence of the known survival agents (Franklin and Johnson, 1992). Thus, nifedipine may induce death in cells having a lower $[Ca^{2+}]_i$. Nifedipine-resistant cells are probably those neurons which maintain their intracellular $[Ca^{2+}]_i$ over this threshold in spite of the block of Ca^{2+} influx through L-type channels. Nifedipine-insensitive, voltage-operated Ca^{2+} channels may contribute to the maintenance of $[Ca^{2+}]_i$ above the survival threshold (Randall et al., 1993; Zhang et al., 1993).

Forskolin is able to prevent apoptosis triggered by low $[K^+]_o$ and proved very efficient, at early times, in maintaining a level of $[Ca^{2+}]_i$ higher than that observed in low $[K^+]_o$-treated neurons. The latter effect was inhibited by the PKA inhibitor KT5720 and it may be accounted for by the ability of PKA to phosphorylate Ca^{2+} channels, rendering them more permeable to Ca^{2+} ions, even in nondepolarizing conditions. It is known, in fact,

that PKA-mediated phosphorylation positively regulates the activity of voltage-dependent Ca^{2+} channels (Chad and Eckert, 1986; Armstrong and Eckert, 1987; Artalejo et al., 1992); thus, it may be possible that, in our model, forskolin prevents or reduces the repolarization-mediated closure of Ca^{2+} channels when cells are shifted from K25 to K5. Our results at early times demonstrate that forskolin can substitute depolarizing KCl in maintaining high $[Ca^{2+}]_i$ in cerebellar granule cells. However, since this effect of forskolin was lost at later times, it can not entirely explain long term survival promoted by forskolin.

If the initial effect of forskolin on $[Ca^{2+}]_i$ is important for its survival promoting activity, the channels involved seem to be neither nifedipine- nor ω-conotoxin-sensitive, since both these Ca^{2+} channels antagonist are ineffective in blocking the survival effect of forskolin. Alternatively, the initial effect of forskolin on intracellular $[Ca^{2+}]_i$ is not a prerequisite for its survival activity, but it is merely associated to the signal transduction cascade leading to the recovery of cells from apoptosis.

In vitro development of cerebellar granule cells needs serum, which can not be replaced by any of the known growth factors. However IGF-1 has the ability to restore the glutamate response of serum-deprived cerebellar granule cells (Calissano et al., 1993) and to save mature neurons from apoptosis (D'Mello et al., 1993). It was therefore interesting to examine the effect of IGF-1 on $[Ca^{2+}]_i$ to ascertain if its survival promoting activity was mediated by changes in this second messenger. Neither short nor long term effect of IGF-1 was observed on $[Ca^{2+}]_i$ of neurons shifted from high to low $[K^+]_o$, therefore other second messengers must be responsible for the IGF-1 ability to prevent apoptosis.

The fact that cerebellar granule cells can survive regardless of their low intracellular calcium, is in apparent disagreement with the calcium set hypothesis (Franklin and Johnson, 1992). However, IGF-1 and forskolin, modulating phosphorylations through receptor-mediated tyrosine kinase (Lamphere and Lienhard, 1992) and PKA, respectively, can probably bypass the increase of $[Ca^{2+}]_i$ which is a necessary step in the survival promoted by membrane depolarization.

The decrease of $[Ca^{2+}]_i$ triggered by low KCl may induce, through yet unknown mechanisms, the neosynthesis of killer protein(s), that will be of great importance to identify and characterize in further studies. Because of their abundance and homogeneity, cerebellar granule cells are a very convenient neuronal model for further biochemical and molecular studies on apoptosis. Thus, we characterized another aspect of apoptosis in this neuronal model, namely the role of RNA transcription in apoptosis as well as in the survival promoting activities of high KCl, IGF-1, and forskolin. We determined the TD50 for the high KCl, IGF-1, forskolin and actinomycin D. The TD50 found for high KCl, forskolin and IGF-1 was 6 hr. Moreover, 50% of neurons lose, after 1 hr, the ability to be rescued from apoptosis by actinomycin D. This finding suggests that a very early synthesis of "suicide proteins" occurs in cerebellar granule cells deprived of high KCl compared to NGF-deprived sympathetic neurons, in which Edward et al. (1991) have found a TD50 of 12 hr for actinomycin D or cycloheximide. As the entire process take approximately the same time in the two neuronal models, a possible explanation for the discrepancy of TD50 is that the earlier synthesis of mRNA for suicide proteins in cerebellar granule cells is followed by slower posttranscriptional events leading to apoptosis. Alternatively, the suicide proteins (or their mRNA) have different turnovers in the two models. Nevertheless, the hypothesis that agents which rescue neurons from apoptosis exert their action by affecting transcription of "suicide proteins" seems unlikely as the TD50s of high KCl, forskolin and IGF-1 are considerably larger than that of actinomycin D. Hence survival agents probably inactivate newly synthesized "suicide proteins" or interfere with the downstream cascade triggered by those newly made proteins (Johnson and Deckwerth, 1993). Finally, we investigated the requirement for RNA synthesis in the survival promoted by high $[K^+]_o$, forskolin, or IGF-1. The results shown here clearly demonstrate that survival induced by these agents is unaffected by the blockade of RNA synthesis by actinomycin D. This finding is in agreement with similar studies on NGF-deprived sympathetic neurons (Edwards et al., 1991). We therefore conclude that high KCl, forskolin and IGF-1 can counteract the effect of "suicide proteins" by a mechanism not requiring transcription of new mRNA.

In several neuronal models phosphorylation cascades affect neuronal survival (Graham and Burgoyne, 1993; Koike and Tanaka, 1993). In developing cerebellar granule cells in particular, it has been demonstrated that the trophic effect of high $[K^+]_o$ is mediated by Ca^{2+}/calmodulin-dependent protein kinase (Hack et al., 1993). Whether this is also true in mature neurons remains to be established. Indeed the maturation state of neurons greatly influences the response to physiological and pharmacological agents. NMDA exerts a trophic action on immature granule neurons (Balazs et al., 1988) while it induces excitotoxicity in mature neurons (Schramm et al., 1990; Mercanti et al., 1992); Li^{2+} provokes or prevents apoptosis in cerebellar granule neurons, according to their developmental state (D'Mello et al., 1994). Similarly, our results on forskolin-promoted survival suggest that activation of PKA can inhibit the apoptotic process in mature neurons while a previous report excluded a role for this cyclic nucleotide–dependent protein kinase in the survival of developing neurons (Balazs et al., 1992).

Further studies are necessary to dissect the signalling events placed downstream the increase of $[Ca^{2+}]_i$ in the pathway triggered by membrane depolarization or the "calcium independent" cascade induced by IGF-1 and forskolin in order to identify the possible common targets of these messages which both lead to prevention of apoptosis.

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