

Intracellular acidification by inhibition of the Na^+/H^+ -exchanger leads to caspase-independent death of cerebellar granule neurons resembling paraptosis

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Abstract

Potassium withdrawal is commonly used to induce caspase-mediated apoptosis in cerebellar granule neurons *in vitro*. However, the underlying and cell death-initiating mechanisms are unknown. We firstly investigated potassium efflux through the outward delayed rectifier K^+ current (I_k) as a potential mediator. However, tetraethylammoniumchloride, an inhibitor of I_k , was ineffective to block apoptosis after potassium withdrawal. Since potassium withdrawal reduced intracellular pH (pH_i) from 7.4 to 7.2, we secondly investigated the effects of intracellular acidosis. To study intracellular acidosis in cerebellar granule neurons, we inhibited the Na^+/H^+ exchanger (NHE) with 4-isopropyl-3-methylsulfonylbenzoylguanidine methanesulfonate (HOE 642) and 5-(*N*-ethyl-*N*-isopropyl)-amiloride. Both inhibitors concentration-dependently induced cell death and potentiated cell death after potassium withdrawal. Although inhibition of the NHE induced cell death with morphological criteria of apoptosis in light and electron microscopy including chromatin condensation, positive TUNEL staining and cell shrinkage, no internucleosomal DNA cleavage or activation of caspases was detected. In contrast to potassium withdrawal-induced apoptosis, cell death induced by intracellular acidification was not prevented by insulin-like growth factor-1, cyclo-adenosine-monophosphate, caspase inhibitors and transfection with an adenovirus expressing Bcl- X_L . However, cycloheximide protected cerebellar granule neurons from death induced by potassium withdrawal as well as from death after treatment with HOE 642. Therefore, the molecular mechanisms leading to cell death after acidification appear to be different from the mechanisms

after potassium withdrawal and resemble the biochemical but not the morphological characteristics of paraptosis.

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Abbreviations: I_k , outward delayed rectifier K^+ current; TEA, tetraethylammoniumchloride; pH_i , intracellular pH; NHE, Na^+/H^+ exchanger; HOE 642, 4-isopropyl-3-methylsulfonylbenzoylguanidine methanesulfonate; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; IGF-1, insulin-like growth factor; cAMP, cyclo-adenosine-monophosphate; Bcl- X_L , B cell leukemia gene; CHX, cycloheximide; DIV, day *in vitro*; NMDA, *N*-methyl-D-aspartate; HK, culture cell medium containing 25 mM potassium; LK, culture medium containing 5 mM potassium

Introduction

Depolarizing concentrations (25 mM) of potassium are a prerequisite for the survival of cerebellar granule neurons *in vitro*. These high potassium concentrations are likely to imitate the afferent input of cerebellar granule neurons, leading to their depolarization. This hypothesis is supported by the ability of cyclo-adenosine-monophosphate (cAMP) to block the death of cerebellar granule neurons in the absence of depolarizing potassium concentrations. Potassium withdrawal results in apoptosis imitating the loss of trophic support and synaptic afferences, and – like many other models of developmental cell death – is mediated by caspases. Therefore, caspase-mediated apoptosis of cerebellar granule neurons following potassium withdrawal appears to be a prototype model of developmental apoptosis.

In many instances, accidental cellular death of mature neurons is caused or at least accompanied by intracellular acidification. Examples of neurological disorders, in which acidosis is rapidly occurring, are ischemia/hypoxia,¹ trauma² or subarachnoid hemorrhage.³ Acidosis has been reported to induce necrosis and apoptosis of cultured primary neurons.⁴ Furthermore, in some (non-neuronal) paradigms, intracellular acidification appears to trigger apoptosis by the activation of caspases^{5,6} or enhances cytochrome *c*-mediated caspase activation.⁷

Members of the Na^+/H^+ exchanger (NHE) family constitute an extremely efficient system for protecting cells against internal acidification.⁸ A spontaneous mouse mutant identified to be deficient for NHE1 shows a neurological syndrome including ataxia, an epilepsy phenotype consisting of 3/s

absences and tonic-clonic seizures, and selective neuronal death in the cerebellum and brainstem, but otherwise is healthy.⁹ Surprisingly, inhibition of the NHE has been reported to protect rats from ischemia-induced brain damage.^{10–13} However, these effects may not be due to the block of the NHE-mediated control of intracellular pH, but due to the block of free fatty acid efflux¹⁴ and block of neutrophil accumulation.¹⁰

To study the role of potassium and acidification in neuronal apoptosis, we investigated the role of the outward delayed rectifier potassium current, changes of pH_i and the NHE in apoptosis of cerebellar granule neurons.

Results

Potassium withdrawal-induced apoptosis of cerebellar granule neurons is not mediated by an outward delayed rectifier potassium current

Lowering the extracellular potassium concentration from 25 to 5 mM at DIV8 induces apoptosis of differentiated

cerebellar granule neurons.^{15–18} When measured after 24 h, switch to 5 mM potassium decreases the viability of cerebellar granule neurons by >50%. Blocking the outward delayed rectifier potassium current by tetraethylammoniumchloride (TEA) or depolarizing potassium concentrations (25 mM) prevents apoptosis of murine cortical neurons induced by C_2 -ceramide, sphingomyelinase, β -amyloid, and NMDA.^{19–22} To test whether the potassium withdrawal-induced apoptosis of cerebellar granule neurons is also mediated by the outward delayed rectifier potassium current, cerebellar granule neurons were switched to serum-free medium either containing 25 or 5 mM potassium at DIV8 and treated with TEA (1 μ M up to 50 mM) at the same time (Figure 1a, b). Assessing the viability of neurons at 24 h showed that up to 5 mM TEA did not protect cerebellar granule neurons from potassium withdrawal-induced apoptosis. Treatment with 10 to 50 mM TEA was toxic and induced death independent of the potassium concentration.

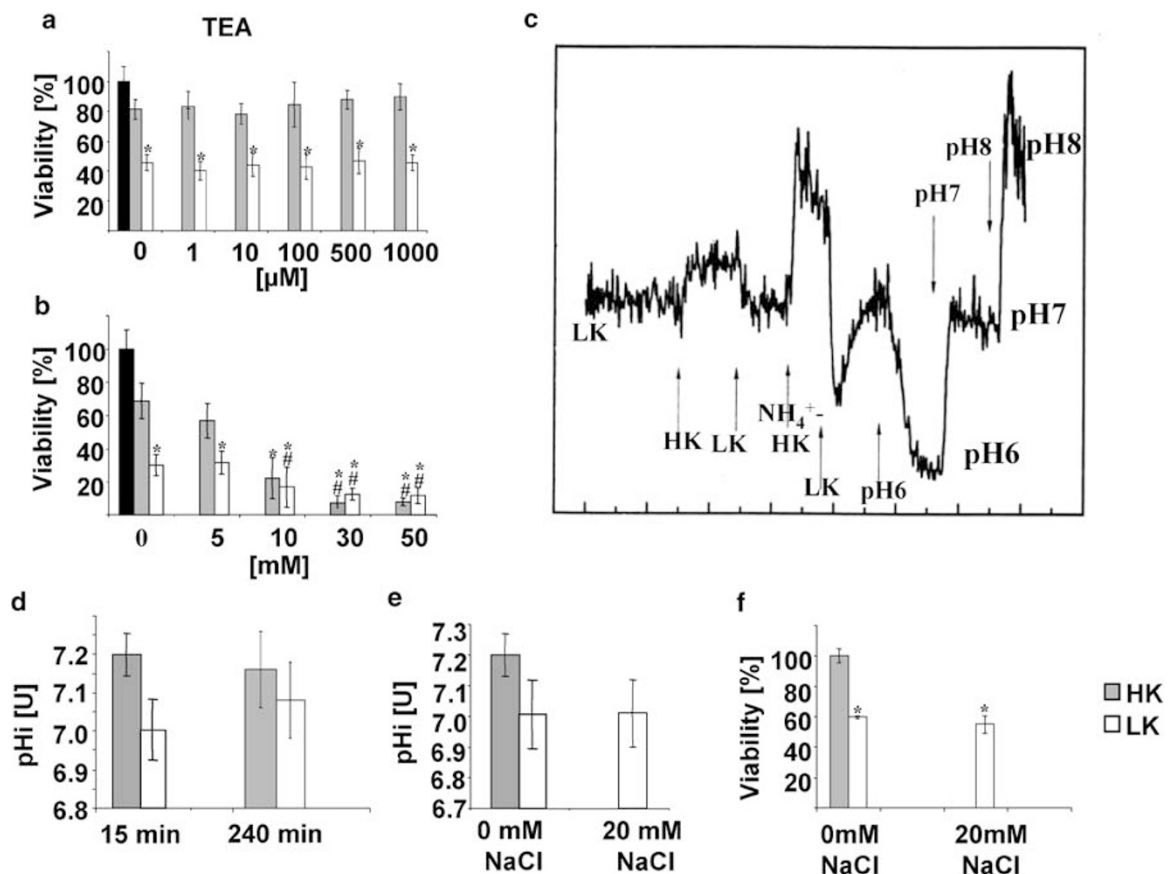


Figure 1 Role of the outward delayed rectifier potassium current and pH in potassium withdrawal-induced apoptosis of cerebellar granule neurons. (a, b) The voltage-dependent outward delayed potassium current was blocked by TEA (1 μ M–50 mM) in cerebellar granule neurons cultured in 25 mM potassium (HK, gray bars) or 5 mM potassium (LK, white bars). Viability was assessed at 24 h by using FDA staining and compared with neurons neither switched to serum-free medium nor treated with TEA (no switch, black bar; * $P < 0.001$ compared with HK not treated with TEA, # $P < 0.001$ compared with LK not treated with TEA). (c, d) The intracellular pH was assessed at 15 and 240 min after potassium withdrawal by using the pH-sensitive fluorescent dye BCECF-AM. The dye was used in its effective pH range, as determined by its pK_a (7.0). Calibration was accomplished by using the ionophore nigericin (HK, gray bars; LK, white bars). (e, f) To investigate the influence of changes in osmolarity of the culture medium after potassium withdrawal on intracellular acidification and cell death, the osmolarity of the culture medium was kept constant by adding 20 mM NaCl. The pH_i was assessed within 15 min after potassium deprivation by using BCECF-AM, viability was assessed 24 h after potassium withdrawal by using FDA staining (HK, gray bars; LK, white bars; * $P < 0.0001$ compared with HK)

Potassium withdrawal-dependent changes of osmolarity do not cause acidosis and cell death

As potassium is involved in many physiological processes of the cell, for example, the regulation of the pH_i ,²³ there possibly exists a calcium-independent mechanism in cerebellar granule neurons associated with the redistribution of potassium after its withdrawal. Therefore, we investigated the changes in pH_i occurring between 15 and 240 min after potassium deprivation (Figure 1c, d).

Lowering the extracellular potassium concentration from 25 to 5 mM reduced pH_i by 0.2 units (Figure 1c). When again increasing the potassium concentration to 25 mM, this reduction in pH_i was reversible. This effect was intensified by treating cerebellar granule neurons with NH_4^+ before switching them back to 25 mM potassium, leading to an increase in pH_i by 1 unit.

As switching from 25 to 5 mM potassium also causes changes in the osmolarity of the medium, we investigated whether keeping the osmolarity of the medium constant would prevent intracellular acidification. Adding 20 mM NaCl to the medium containing 5 mM potassium kept its osmolarity constant. Potassium deprivation without balancing the osmolarity as well as potassium withdrawal with balancing the osmolarity by adding 20 mM NaCl caused a decrease of pH_i by 0.1–0.2 units (Figure 1e). In addition, balancing the osmolarity did not block neuronal death induced by potassium deprivation (Figure 1f). Thus, intracellular acidification and induction of cell death after potassium withdrawal were not caused by changes of serum osmolarity.

Induction of intracellular acidosis by blocking the NHE induces death of cerebellar granule neurons

An important physiological mechanism regulating pH_i after intracellular acidification is the NHE which is expressed in cerebellar granular cells.²⁴ Meanwhile, five isoforms of the NHE have been described, NHE-1–5. Neurons mainly express NHE-1 and NHE-5. To investigate the role of intracellular acidification in neuronal apoptosis, we blocked the NHE by either 4-isopropyl-3-methylsulfonylbenzoyl-guanidine methanesulfonate (HOE 642) or 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA). Cerebellar granule neurons were switched at DIV8 to serum-free medium either containing 25 or 5 mM potassium, and in addition were treated with either HOE 642 or EIPA. Viability was assessed at 24 h (Figure 2a, b). Both HOE 642 and EIPA induced the death of cerebellar granule neurons cultured in the presence of 25 mM potassium and enhanced the death of neurons switched to 5 mM potassium. To investigate the changes in pH_i of cerebellar granule neurons treated with HOE 642 or EIPA, pH_i was measured over 3 h after treatment (Figure 2c). Treatment with either HOE 642 or EIPA decreased pH_i between 0.2 and 0.3 units within 1 h. This reduction of pH_i remained stable at all the following time points measured until morphological and biochemical features of apoptosis occurred (Figure 2c). Combined potassium withdrawal and treatment with HOE 642 caused an intracellular acidification by 0.35 units within 15 min, followed by an alkalization by 0.1–0.2 units when HOE 642 was removed (data not shown).

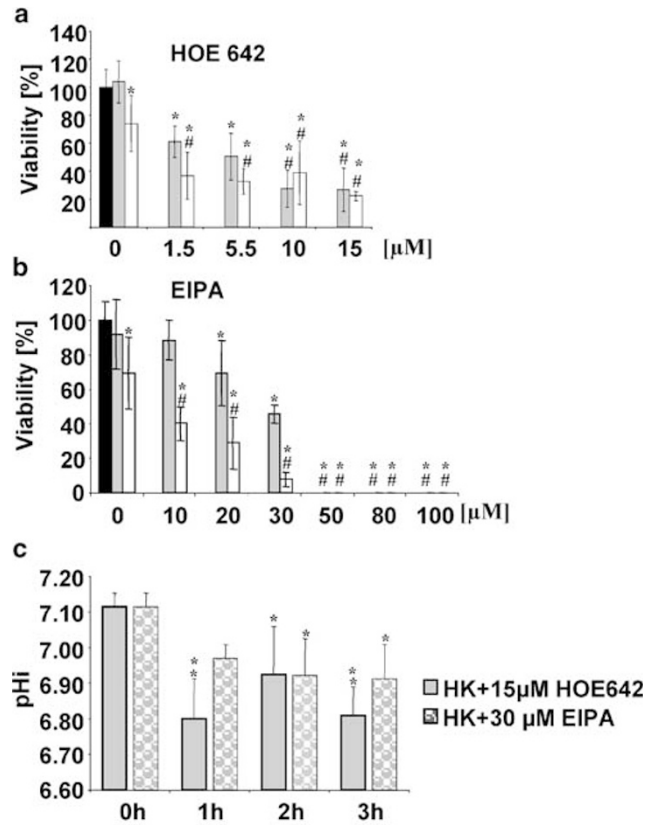


Figure 2 Effects of NHE inhibition. (a, b) NHE was blocked by increasing concentrations of either HOE 642 or EIPA. Viability was assessed 24 h after potassium withdrawal by FDA staining and compared with neurons neither switched to serum-free medium nor treated with HOE 642 or EIPA (no switch, black bar; HK, gray bars; LK, white bars; * $P < 0.01$ compared with HK; # $P < 0.001$ compared with LK^+). (c) pH_i was assessed at the time points indicated after switch to serum-free medium containing 25 mM potassium and treatment with either 15 μ M HOE 642 or 30 μ M EIPA (* $P < 0.05$, ** $P < 0.01$ compared with controls)

Intracellular alkalization prevents death of cerebellar granule neurons induced by treatment with HOE 642 or EIPA, but not by potassium withdrawal

Intracellular and extracellular pHs of the cell are changing in the same way. Increasing or decreasing extracellular pH by 1 unit is followed by an increase or decrease of intracellular pH by 0.5 units in murine cortical neurons.^{25,26} Likewise, increasing the extracellular pH of cerebellar granule neurons by 0.3 units caused an increase in pH_i by about 0.15 units (Figure 3a). We now investigated if counteracting intracellular acidification by alkalizing the culture medium would prevent death induced by potassium withdrawal or blocking the NHE. At DIV8, neurons were switched to serum-free medium with a pH of either 7.4 (usual pH of the medium), 7.6 or 7.9 containing either 25 or 5 mM potassium and were treated with HOE 642. Viability was assessed at 24 h (Figure 3b, c). Alkalizing the culture medium by titration with 1 M NaOH did not protect cerebellar granule neurons from potassium deprivation-induced apoptosis, but prevented death induced by blocking the NHE. Thus, intracellular acidification is

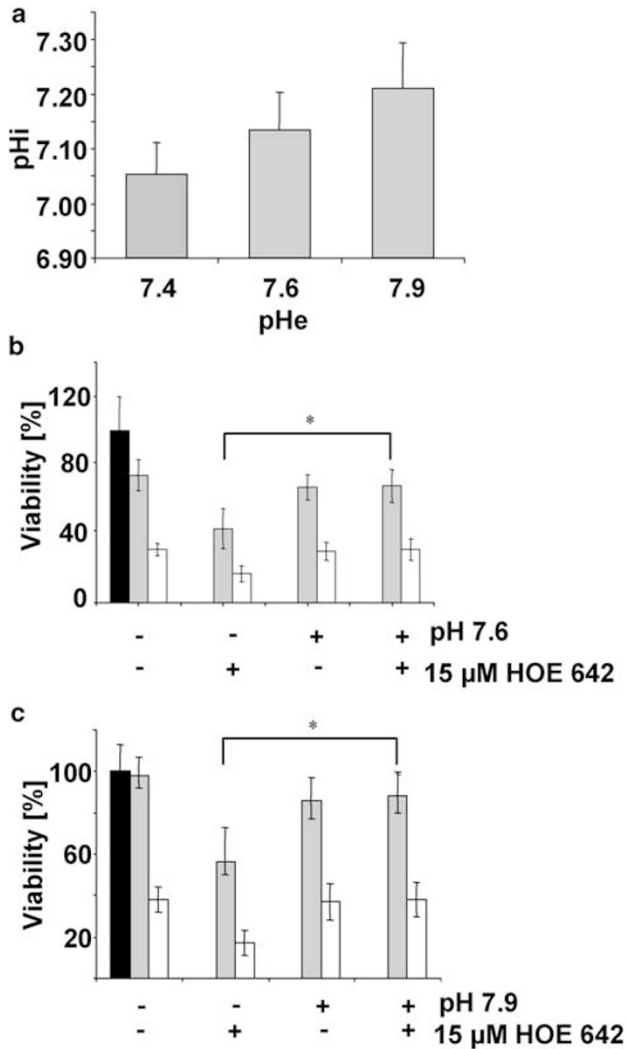


Figure 3 Effects of pH on survival of cerebellar granule neurons. (a) Culture medium (25 mM K⁺) was alkalinized as indicated (pH_e), changes in pHi dependent on pH_e were then assessed. (b, c) To investigate the effects of alkalinized culture medium on cell death induced by potassium withdrawal or blocking the NHE, neurons were switched to serum-free medium with a pH of either 7.4, 7.6 or 7.9, containing either depolarizing or physiological potassium concentrations and treated with HOE 642. Viability was assessed at 24 h after switch compared with neurons neither switched to serum-free medium nor treated with HOE 642 (no switch, black bar; HK⁺, gray bar; LK⁺, white bar; *P < 0.01 compared with the corresponding condition without alkalinization)

needed for cell death induced by blocking the NHE, but is not sufficient for death induced by potassium withdrawal.

Death of cerebellar granule neurons induced by HOE 642 or EIPA shows morphological features of apoptosis

To ensure that both potassium withdrawal and blockage of NHE induce apoptotic cell death, we investigated the morphologic features of apoptosis after treatment with HOE 642 or EIPA.

At DIV8, cerebellar granule neurons were switched to serum-free medium either containing depolarizing or

physiological potassium concentrations and treated with either 15 μM HOE 642 or 50 μM EIPA. Chromatin condensation of the nucleus was assessed by Hoechst staining at 4 and 24 h (Figure 4a). In the presence of 25 mM potassium, neurons either treated with HOE 642 or EIPA showed a considerable number of nuclei with chromatin condensation already at 4 h. In contrast, only a few neurons not treated with NHE inhibitors showed chromatin condensation at 4 h after switch to 25 or 5 mM potassium. Treatment with HOE 642 or EIPA for 24 h killed almost all neurons in the presence of 25 mM potassium. Only a few of the neurons cultured in the presence of 25 mM potassium for 24 h without blocking the NHE showed chromatin condensation, whereas neurons cultured in the presence of 5 mM potassium without blocking the NHE were diminished by 50% and the chromatin of almost all of the remaining cells was condensed.

In addition, cell shrinkage was assessed by measuring the cell volume at 24 h after switch of medium (Figure 4d). Treatment of cerebellar granule neurons cultured in the presence of 25 mM potassium with either HOE 642 or EIPA decreased the cell volume by about 33–50% (Figure 4d). The same result was observed when cell death was induced by either potassium withdrawal alone (Figure 4d) or combined potassium withdrawal and treatment with NHE inhibitors (data not shown).

DNA double-strand breaks were assessed by TUNEL staining at 6 h after switch of medium (Figure 4b). Many neurons cultured in the presence of 25 mM potassium treated either with HOE 642 or EIPA were TUNEL-positive compared to neurons cultured without blocking the NHE. In contrast, internucleosomal DNA cleavage, another feature of apoptosis, was only induced by potassium withdrawal, independent of whether the NHE was pharmacological blocked or not. Treatment of cerebellar granule neurons with HOE 642 or EIPA in the presence of 25 mM potassium did not cause typical DNA laddering (Figure 4e).

In addition, features of apoptotic cell death were assessed 24 h after potassium withdrawal by electron microscopy (Figure 4c). At 24 h, potassium withdrawal led to a severe compromise of the cell structure, condensation of the nucleoplasm, and a swelling of mitochondria. At this stage, the disruption of membranes points to a switch from apoptosis to secondary necrosis. The treatment with HOE 642 for 24 h led to an advanced stage of apoptosis with extreme condensation of the nucleoplasm and secondary disruption of the cytoplasmic integrity. Similarly, treatment with EIPA for 24 h or the combination of potassium withdrawal with Hoe 642 or EIPA showed apoptotic features, for example, nuclear condensation, and also signs of secondary necrosis, including extended fragmentation of cellular membranes (Figure 4c).

Death of cerebellar granule neurons induced by HOE 642 or EIPA does not require caspase-3-like activity

Apoptotic cell death induced by potassium deprivation is executed by effector caspases including caspase-3.^{27,28} Caspase-3 activity shows its maximum between 5 and 6 h after potassium withdrawal. We first investigated the activity of

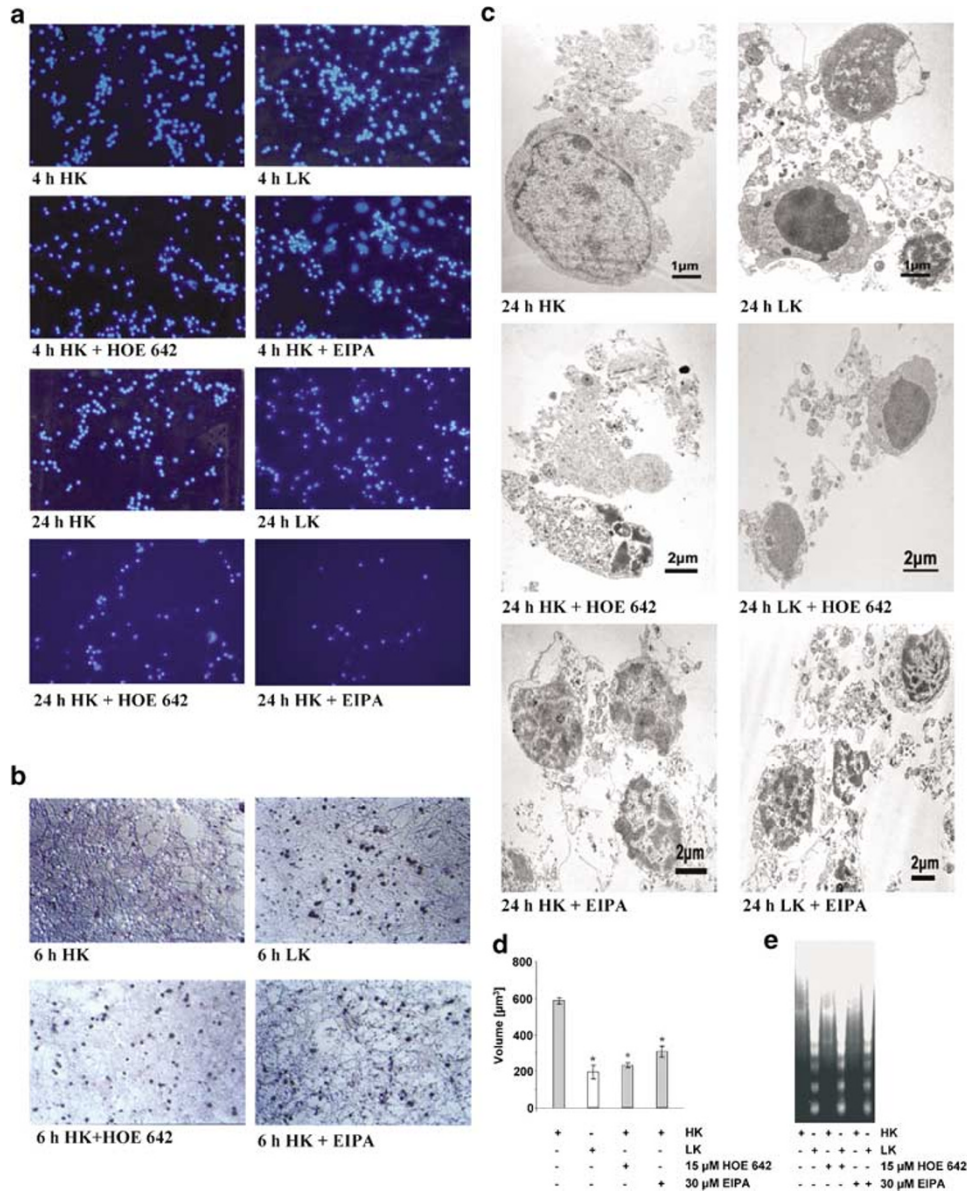


Figure 4 Morphology of cell death induced by NHE inhibition. **(a)** Hoechst 33258 nuclear staining was used to detect typical chromatin changes. Apoptotic cells were characterized by scoring condensed nuclei 4 h as well as 24 h after potassium withdrawal. **(b)** Profile of DNA strand breaks was studied by using terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) 6 h after potassium withdrawal. **(c)** Electron microscopy at 24 h after switch from high (HK) to low potassium (LK) concentrations or treatment with HOE 642 or EIPA. **(d)** Cell shrinkage and **(e)** DNA laddering were assessed 24 h after switch to serum-free medium containing either 5 or 25 mM potassium and treatment with HOE 642 or EIPA (HK⁺, gray bars; LK⁺, white bars; **P* < 0.001 compared with otherwise untreated HK⁺; *n* = 80–120 cells per group)

caspase-3-like caspases in neuronal apoptosis induced by blocking the NHE. Since the morphological characteristics of apoptosis following incubation with NHE blockers are detectable earlier than after potassium withdrawal, caspase activity should be detectable at the latest at 6 h. In cerebellar granule neurons withdrawn from serum but not from potassium and treated with Hoe 642 or EIPA, no increase in caspase-3-like activity occurred (Figure 5a).

To further confirm our results, we investigated the cleavage of caspase-3 at 3, 6 and 9 h after potassium withdrawal and after inducing death by blocking the NHE at DIV8 in cerebellar

granule neurons. The p17 cleavage product of caspase-3 was only identified after potassium withdrawal but not after treatment with HOE 642 or EIPA (Figure 5b). After potassium withdrawal, caspase-9 is activated, subsequently leading to the activation of caspase-3 and -8.²⁸ Here, we detected the p35/p37 cleavage product of caspase-9 and the cleaved p18-subunit of caspase-8 after potassium withdrawal, but not after treatment with HOE 642 and EIPA (data not shown). In addition, we investigated the effects of specific and pan-specific caspase inhibitors (caspase-3 inhibitor, DEVD-fmk; caspase-8 inhibitor, IETD-fmk; caspase-9 inhibitor,

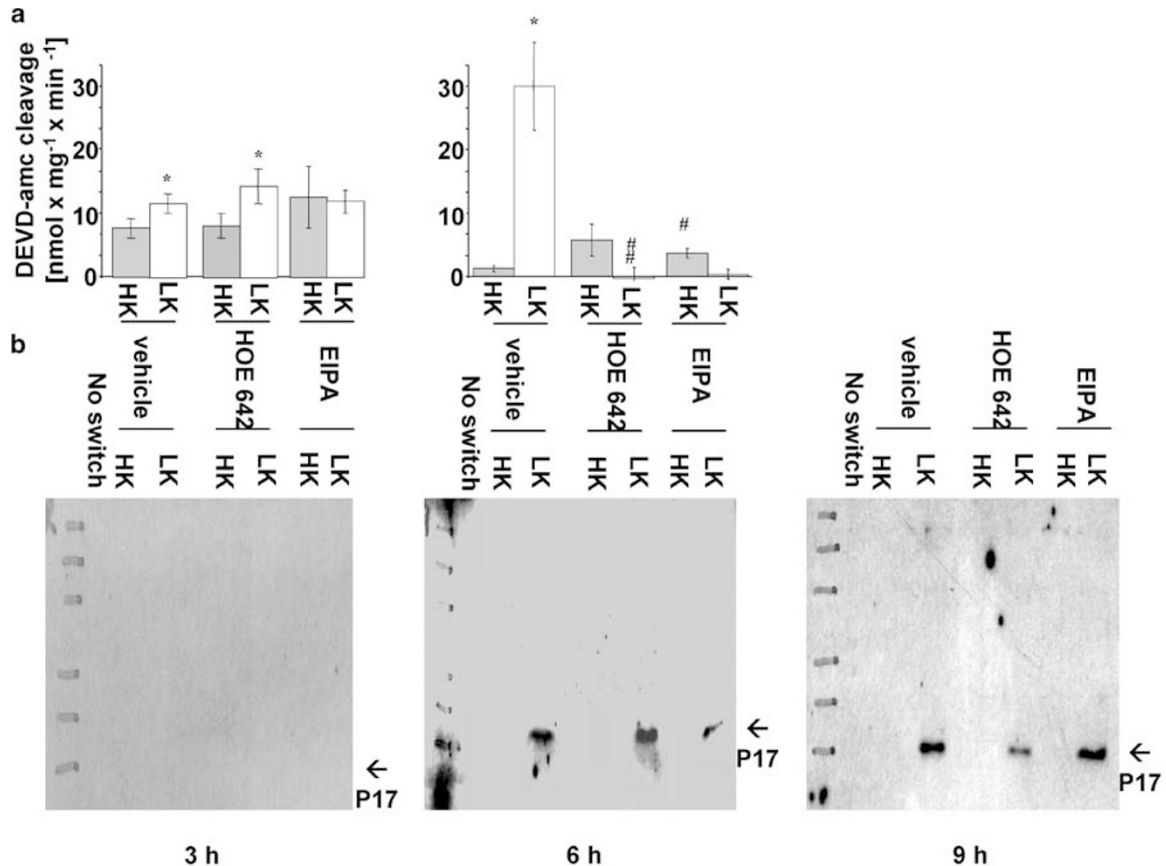


Figure 5 Activation of caspases in death of cerebellar granule neurons. (a) Activity of caspase-3-like caspases was accomplished by assessing the cleavage of the substrate of caspase-3-like caspases, DEVD-amc. The emission of the appearing fluorescence was measured 3 and 6 h after switch (HK⁺, gray bars; LK⁺, white bars; **P* < 0.01 compared with untreated HK⁺). (b) Cleavage of caspase-3 was assessed 3, 6 and 9 h after potassium withdrawal and/or treatment with HOE 642 or EIPA by Western blotting

LEHD-fmk; panspecific caspase inhibitor, zVAD-fmk) on cell death induced by Hoe 642 and EIPA. Treatment with DEVD-fmk, LEHD-fmk, and zVAD-fmk protected cerebellar granule neurons from apoptosis induced by potassium deprivation, whereas neurons treated with HOE 642 or EIPA died in spite of caspase inhibition (Figure 6a, b, d). Treatment with the inhibitor of caspase-8 did not show any effect (Figure 6c).

These results suggest that neither caspase-3 nor caspase-8 or -9 are needed for cell death induced by blocking the NHEs.

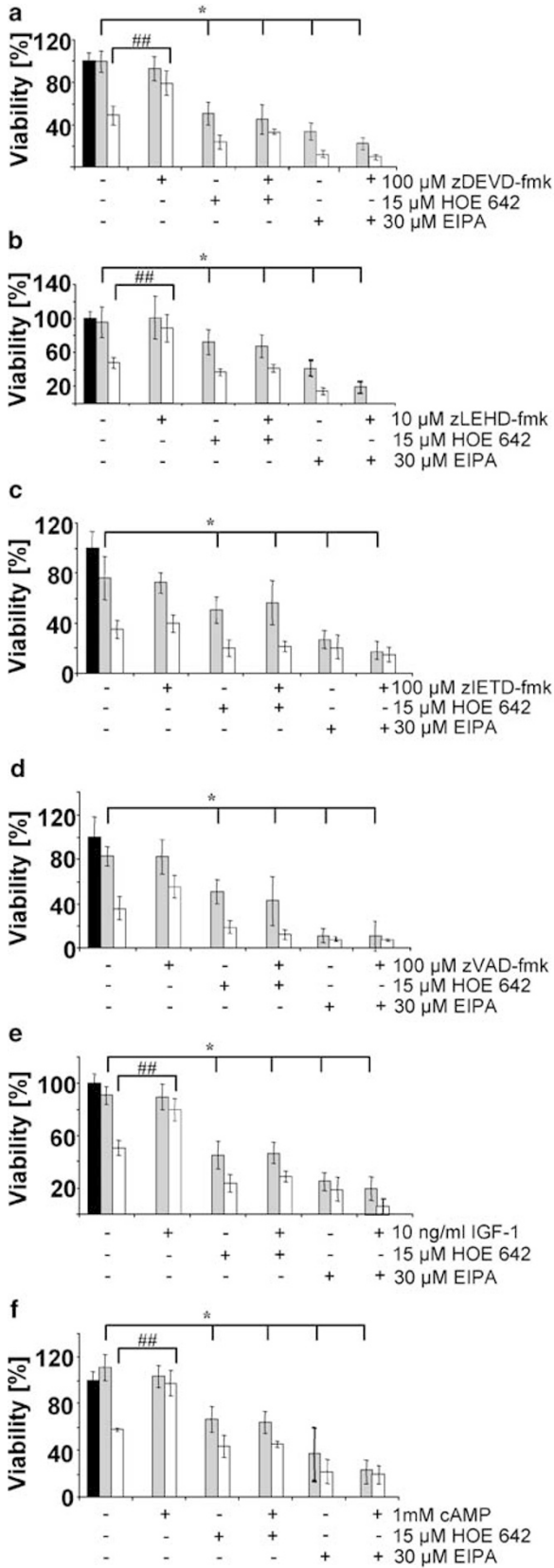
Cycloheximide, but not IGF-1, cAMP, and forced expression of Bcl-X_L protect cerebellar granule neurons from death induced by NHE inhibitors

cAMP and insulin-like growth factor-1 protect cerebellar granule neurons from apoptosis induced by potassium deprivation.^{17,18,29} We investigated whether treatment with either IGF-1 or cAMP also protected cerebellar granule neurons from death induced by HOE 642 or EIPA. Both IGF-1 and cAMP protected cerebellar granule cells from apoptosis induced by potassium deprivation, but did not show any effect when neurons were treated with either HOE 642 or EIPA independent of the potassium concentration

(Figure 6e, f). These results suggest that neuronal death induced by either potassium deprivation or blocking the NHE is mediated by different cell death pathways. Sperandio *et al.* (2000)³⁰ recently introduced the concept of 'paraptosis', an alternative, nonapoptotic form of programmed cell death, that did not show a response to caspase inhibitors and Bcl-X_L, but to cycloheximide. Treatment with cycloheximide blocked the death of cerebellar granule neurons after potassium withdrawal and after treatment with HOE 642 (Figure 7a). Incubation of cerebellar granule neurons with an adenovirus at DIV1 leads to a transduction efficacy of about 80%.²⁸ We have recently shown that transfection of cerebellar granule neurons with AdV-Bcl-X_L leads to the expression of Bcl-X_L at DIV7 and provides protection against potassium withdrawal-induced apoptosis.³¹ We here confirm these results (Figure 7b). However, although transfection with Bcl-X_L provided protection against potassium withdrawal-induced death, it did not protect against cell induced by HOE 642 (Figure 7b).

Discussion

Our results suggest that apoptosis of cerebellar granule neurons induced by potassium withdrawal is neither mediated



by an outward delayed rectifier potassium current nor by the intracellular acidosis occurring after potassium deprivation. However, neuronal death induced by the treatment with either HOE 642 or EIPA seems to be mediated by intracellular acidification, shows morphological features of apoptosis, but is not mediated by caspases and is not prevented by IGF-1 or cAMP. Therefore, apoptosis of cerebellar granule neurons

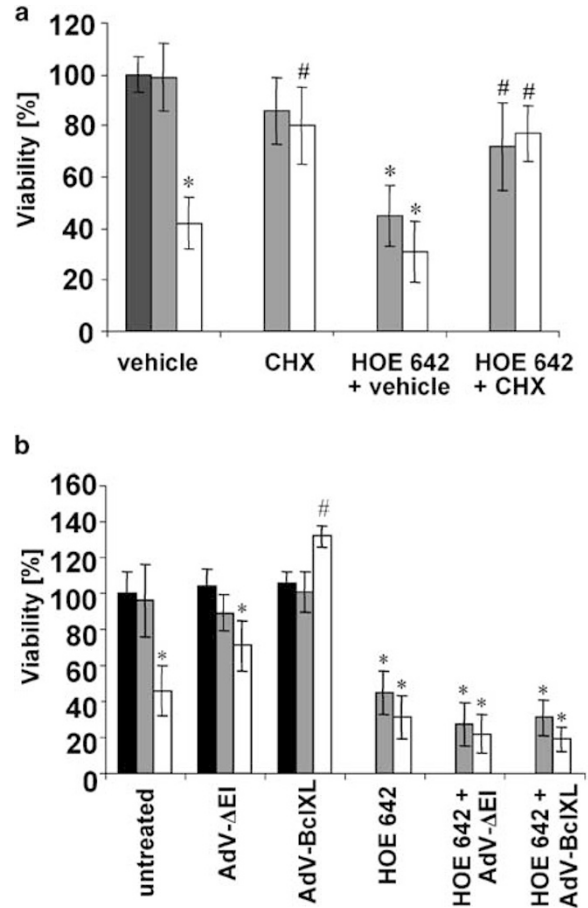


Figure 7 Effects of cycloheximide and Bcl-X_L on death in cerebellar granule neurons. (a,b) Cell viability was assessed by FDA staining at 24 h after potassium withdrawal or treatment with 15 μ M HOE 642. Cerebellar granule neurons were treated with 10 μ g/ml cycloheximide or vehicle at the time of potassium withdrawal or HOE 642 treatment (a) or were transfected with AdV-Bcl-X_L or the control vector AdV- Δ E1 at DIV1 (b) (no switch, black bar; HK⁺, gray bars; LK⁺, white bars; *P < 0.0001 compared with HK⁺; #P < 0.001, P < 0.0001 compared with LK⁺)

Figure 6 Effects of caspase inhibition, IGF-1, and cAMP on the death of cerebellar granule neurons. (a-d) The effects of specific and panspecific caspase inhibitors (caspase-3 inhibitor, DEVD-fmk; caspase-8 inhibitor, IETD-fmk; caspase-9 inhibitor, LEHD-fmk; panspecific caspase inhibitor, zVAD-fmk) on cell death induced by HOE 642 or EIPA were investigated by assessing the viability by FDA staining 24 h after switch compared with neurons neither switched to serum-free medium nor treated with HOE 642 or EIPA (no switch, black bar; HK⁺, gray bars; LK⁺, white bars; *P < 0.001 compared with otherwise untreated HK⁺; ##P < 0.001 compared with otherwise untreated LK⁺). (e, f) The effect of IGF-1 and cAMP on cell death induced by blocking the NHE with either HOE 642 or EIPA were investigated by assessing the viability by FDA staining 24 h after switch compared with neurons neither switched to serum-free medium nor treated with either HOE 642 or EIPA (no switch, black bar; HK⁺, gray bars; LK⁺, white bars; *P < 0.001 compared with otherwise untreated HK⁺; ##P < 0.001 compared with otherwise untreated LK⁺)

induced by acidification is fundamentally different from apoptosis occurring after potassium withdrawal.

Apoptosis is thought to play an important role in neuronal death in many neurological disorders, including stroke, Alzheimer's disease, and Huntington's disease. Potassium deprivation-induced apoptosis is a well-characterized *in vitro* model that is widely used to study the cellular mechanisms underlying neuronal apoptosis. Cerebellar granule neurons cultured in the presence of serum and depolarizing potassium concentrations undergo caspase-dependent apoptosis when switched to serum-free medium containing physiological potassium concentrations, but remain viable after serum deprivation alone.^{18,16,15} The initial mechanisms leading to the activation of caspases still have to be elucidated. Depolarizing potassium concentrations of the culture medium induce increased calcium influx into the neurons or increased calcium turnover of the cells.¹⁷ Thus, it has to be assumed that potassium deprivation induces decreased calcium influx caused by decreased activity of the L-type calcium channels or decreased calcium turnover. Possibly, the expression of different caspases is regulated by the concentration of intracellular calcium.³²

However, it has never been examined in which way intracellular potassium is redistributed after potassium withdrawal and which processes regulated by potassium are involved in the induction of neuronal apoptosis. It has been suggested that in cortical cultures ceramide-, NMDA-, and β -amyloid-induced apoptosis is mediated by the enhancement of the potassium outward current.^{19–22} Inhibition of this current by TEA provided protection in cortical cultures in these cell death paradigms. To investigate whether the potassium outward current also plays an important role in potassium withdrawal-induced apoptosis of cerebellar granule neurons, we treated cerebellar granule neurons with TEA. In nontoxic concentrations, we did not find any protective effect of TEA using a wide range of concentrations (Figure 1). These results show that the redistribution of potassium through the potassium outward current is not the mediator of apoptosis in cerebellar granule neurons.

However, potassium also plays an important role in the regulation of intracellular pH (pH_i) and the cellular volume.²³ We report here for the first time that potassium withdrawal leads to acidification in cerebellar granule neurons that is not due to changes in osmolarity (Figure 1d). However, alkalization of the medium did not block potassium withdrawal-induced apoptosis (Figure 3). In summary, after potassium withdrawal, neither acidification nor redistribution of potassium through the potassium outward current are essential mediators of cell death. In contrast and in agreement with published literature, treatment with IGF-1 and cAMP provided protection (Figure 6). Therefore, continuous depolarization and the IGF-1, PI3 kinase/Akt pathway appear to be the most important mediators of survival after potassium withdrawal.

To investigate whether disturbed regulation of pH_i nevertheless is an important mediator of cell death in cerebellar granule neurons, we inhibited the NHE with Hoe 642 and EIPA. Both inhibitors concentration-dependently induced death in cerebellar granule neurons (Figure 2). Although the death fulfilled the morphological criteria of apoptosis, in contrast to apoptosis induced by potassium withdrawal, we

did not find any evidence for caspase-dependent apoptosis. At first sight, this appears surprising because in some non-neuronal paradigms acidification triggers the activation of caspases^{5,6} and enhances cytochrome *c*-mediated caspase activation.⁷ Especially interesting is the report by Roy and colleagues showing that acidification of the NT2 cell cytosol removes a 'safety catch' of pro-caspase-3 and leads to its autoactivation. However, this only occurs in a pH range of 5.25–6.05, which we did not achieve by the inhibition of the NHE. In contrast, in cytosolic extracts, a decrease of the pH from 7.4–7.1 leads to a 1.7-fold increase of caspase activity.⁷ In line with these findings, we observed a potentiation of potassium withdrawal-induced caspase activation in Hoe 642 cotreated cerebellar granule neurons at 3 h after potassium withdrawal, although Hoe 642 by its own did not induce caspase activity (Figure 5a).

By morphology, death induced by NHE inhibition fulfills most of the criteria that are characteristic for apoptosis, including chromatin condensation, DNA strand breaks, cell shrinkage, and blebbing. However, we also observed membrane fragmentation and mitochondrial swelling (Figure 4), which are not features of apoptosis. Surprisingly, block of the NHE induced caspase-independent apoptosis. Although initially disputed, several forms of caspase-independent apoptosis^{33–35} or forms that are similar to apoptosis ('paraptosis') but also do not involve caspases have been reported.^{30,36} Sperandio and colleagues (2000) characterized 'paraptosis' in 293T cells and mouse embryonic fibroblasts as a form of cell death without nuclear fragmentation and apoptotic bodies, but with cytoplasmic vacuolation and late mitochondrial swelling. In addition, death was independent of caspase activity, Bcl-X_L, and was executed without DNA fragmentation. However, inhibition of new mRNA and protein synthesis by actinomycin D and cycloheximide protected against this form of cell death. From the biochemical aspects, death induced by NHE inhibition is very similar, because it is not sensitive to caspase inhibition or forced Bcl-X_L expression, but blocked by cycloheximide. In addition, cell death induced by the block of NHE is not prevented by treatment with cAMP or IGF-1.

In summary, we provide evidence that NHE is important for the regulation of pH_i in neurons. Its block leads to cell death that is molecularly different from potassium withdrawal-induced death and fulfills the biochemical and pharmacological criteria of paraptosis, but not of apoptosis (Table 1). However, most of the morphological criteria that we observed after NHE inhibition resemble apoptosis and not paraptosis.

Materials and Methods

Materials

DEVD-fmk, IEDT-fmk, and LEHD-fmk were purchased from Calbiochem (Bad Soden, Germany). zVAD-fmk was purchased from Bachem Biochemika GmbH (Heidelberg, Germany). The antibody against cleaved caspase-3 (Asp175, catalog #9664) was purchased from Cell Signaling, New England Biolabs GmbH (Frankfurt a.M., Germany). Antibodies against caspase-8 (SK-441) and caspase-9 (Bur 4) were kindly provided by K Kikly (SmithKline Beecham Pharmaceuticals, Research Triangle Park, NC, USA) and S Krajewski (Burnham Institute, La Jolla, CA, USA).

Table 1 Characteristics of apoptosis, paraptosis, and cell death induced by acidification

	Apoptosis	Paraptosis	NHE inhibition (acidification)
<i>Morphology</i>			
Nuclear fragmentation	+	–	+
Chromatin condensation	+	–/+	+
Apoptotic bodies	+	–	+
Cytoplasmic vacuolation	–	+	–
Mitochondrial swelling	Sometimes	Late	Late
Cell shrinkage	+	?	+
<i>Genomic effects</i>			
TUNEL	+	–	+
Internucleosomal DNA fragmentation	+	–	–
<i>Caspase activity</i>			
DEVD-cleaving activity	+	–	–
Caspase-3 processing	+	–	–
<i>Inhibition by</i>			
zVAD-fmk	+	–	–
Bcl-XL	+	–	–
Cycloheximide	+	+	+
IGF-1	+	Not done	–
cAMP	+	Not done	–

+, present; –, not present; ?, not reported; ±, ambiguous.

HOE 642 (cariporide-mesilat) was made available by the Hoechst AG (Frankfurt a.M., Germany). TEA and EIPA were purchased from Fluka Biochemika (Neu-Ulm, Germany) and Sigma Aldrich (Steinheim, Germany). Unless otherwise stated, all other materials were obtained from Sigma (Deisenhofen, Germany).

Cell culture and survival assays

Cerebellar granule neurons were prepared from 7-day-old Sprague–Dawley rats (Charles River, Sulzfeld, Germany) as previously described.¹⁶ Briefly, freshly dissected cerebella were dissociated by mechanical disruption and by incubation at 37°C for 15 min in 0.3 mg/ml trypsin. Cells were plated in poly-L-lysine-precoated 35, 60 or 100 mm culture plates or 24- or 96-well plates and seeded at a density of 2×10^5 cells/cm² in BME medium (H₂O, 2.2 g/l NaHCO₃, Earle's salts; Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum, 2 mM glutamine, 20 µg/ml gentamycin and 25 mM KCl. Cytosine arabinoside was added at a concentration of 10 µM after 24 h to prevent the growth of non-neuronal cells. Contamination with glial cells was typically <5%.¹⁶ In all experiments, neurons were cultured for 7 days in 25 mM potassium and 10% fetal calf serum before use. For potassium deprivation, the culture medium was replaced by serum-free BME medium containing 5 mM potassium (low K⁺, LK⁺) or 25 mM potassium (high K⁺, HK⁺) and supplemented with glutamine and gentamycin, as indicated above.

Cell culture medium was alkalinized using 1 M NaOH. During the titration, the pH was constantly assessed.

TUNEL staining

The profile of DNA strand breaks was studied by using terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL).

This method is much less sensitive for single- than for double-strand breaks. Cells were fixed for 10 min in PBS containing 4% paraformaldehyde and 1.5% glutaraldehyde, then two times permeabilized with PBS containing 0.5% Triton, each time for 5 min. Permeabilized neurons were incubated with TdT buffer (1 M potassium cacodylate, 125 mM Tris–HCl, 1.25 mg/ml BSA) containing 37.5 µl/ml cobalt chloride for 5 min. Then cells were incubated with 10 µM biotin/UTP, 50 U/ml terminal transferase, 0.1 M TdT buffer, and 25 mM cobalt chloride (which was not used for negative control) for 20 min at 37°C. The reaction was stopped by incubation with SSC buffer (15 mM NaCl, 150 mM Na-citrate) for 10 min, followed by washing cells twice with PBS. Neurons then were blocked with BSA/PBS (200 mg/10 ml) for 10 min at room temperature and then washed with PBS again. Incorporation of dUTP was assessed by streptavidin-alkalized phosphatase (diluted 1:500 in PBS, 10 µl/5 ml); then the cells were washed again for two times. Staining of the double-strand breaks was ensued by NBT buffer (10 ml; 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris–HCl) containing 0.41 mM NBT and 0.38 mM BCIP for 20 min. Staining was stopped by washing the cells with water. Stained double-strand breaks were assessed by phase contrast (Mikroskop DM IRBE, Leica, Wetzlar, Germany) and photographed at 400-fold magnification (camera: Ricoh XR-X-3000, Ricoh Europe B.V., Eschborn, Germany).

Hoechst nuclear staining

Hoechst 33258 nuclear staining was used to detect typical chromatin changes. Apoptotic cells were characterized by scoring condensed nuclei. Cells were fixed with PBS containing 4% paraformaldehyde for 20 min, then washed two times with PBS. After incubation with Hoechst 33258 (diluted 1:5000 in PBS containing 0.3% Triton), neurons were washed again two times with PBS. Then IMMU-MOUNT-oil and a coverslip were put on each well and fluorescent cells, magnified 400-fold, were photographed using UV by a microscope (Mikroskop DM IRBE, Leica, Wetzlar, Germany) (camera: Ricoh XR-X-3000, Ricoh Europe B.V., Eschborn, Germany).

Internucleosomal DNA cleavage

DNA was isolated from cerebellar granule neurons using the 'QIAquick Gel extraction kit' (Qiagen, Hilden, Germany). Cells were detached, mechanically disrupted, and DNA was precipitated with 100% isopropanol. DNA was then purified according to the manufacturer's instructions and analyzed on a 0.8% agarose gel.

Cell shrinkage

Cells were cultured on coverslips as described above. At DIV 8, cells were switched to serum-free medium either containing 5 or 25 mM potassium and treated with either HOE 642 or EIPA. After 24 h, neurons on coverslips were fixed in paraformaldehyde (4%) covered with mounting medium and put on slides. Cell volume was then assessed at ×400 magnification (Axioplan 2, Carl Zeiss, Jena, Germany) using the Stereoinvestigator 4.34 (MicroBrightField, Inc., Colchester, USA).

DEVD-AMC cleavage studies

Cleavage of the caspase substrate DEVD-AMC was determined as described.²⁸ Briefly, neurons cultured in 96-well plates were lysed in 50 µl of buffer A (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM MgCl₂, 1 mM PMSF, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 5 µg/ml aprotinin, 0.5% CHAPS). Subsequently, cells

were incubated for 30 min in buffer B (25 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 3 mM DTT, pH 7.5) containing 10 μ M DEVD-AMC. Fluorescence was measured at excitation 360 nm, emission 460 nm, using a Cytofluor fluorescent plate reader (Cyto Fluor II, PE Biosystems, Weiterstadt, Germany).

Immunoblot analysis

Blotting was performed essentially as described previously.¹⁶ Cerebellar granule neurons were seeded on 60 mm dishes. The growth medium was removed from cultures and cerebellar granule neurons were once washed in cold PBS before being lysed for 15 min on ice in lysis buffer (1% Triton X-100, 0.1% SDS with 10 μ g/ μ l leupeptin and aprotinin). Cell debris was removed by high-speed centrifugation at 4°C. Samples (containing 20 μ g of protein) were boiled in 1% SDS and 1% β -mercaptoethanol for 5 min, separated by 10–15% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose. Immunodetection involved blocking for 1 h in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% TWEEN, 5% skim milk, and 2% BSA; incubation with primary antibody to caspase-3 overnight at 4°C; incubation with horseradish peroxidase-linked secondary antibody (anti-rabbit IgG-peroxidase conjugate (Sigma, Dreisenhofen, Germany) or anti-mouse IgG-horseradish peroxidase (Amersham Life Sciences, Freiburg, Germany)) for 1 h room temperature. The bound antibody was visualized using enhanced chemoluminescence (ECL, Amersham, Braunschweig, Germany).

Intracellular pH measurements

Cerebellar granule neurons were cultured on coverslips overnight. Coverslips with cells were transferred to a perfusion chamber and incubated in cell culture medium containing the pH-sensitive dye BCECF-AM (2',7'-bis-[2-carboxyethyl]-5-[and-6] carboxyfluorescein-acetoxy-methylester; 10 μ mol/l) (Molecular Probes, Eugene, OR, USA). This dye is an esterified component that readily penetrates membranes. In the cell, the dye ester is hydrolyzed by cytosolic esterases to membrane-impermeable ions. The dye was used in its effective pH range, as determined by its pK_a (7.0). The cells were dye-loaded by incubation with 1 μ M BCECF-AM at 37°C for 30 min, followed by incubation in serum-free medium at 37°C for another 30 min. Then the perfusion chamber was transferred to an inverted microscope (Axiovert 135, Carl Zeiss, Oberkochen, Germany) and attached to a free-flow perfusion system that allowed rapid exchange of the superfusate. Solutions were maintained at 37°C during the course of the experiments. Once focused on the stage of the microscope under $\times 40$ magnification, cells were washed with culture medium to remove de-esterified dye. After washing, areas of interest were identified with a custom-made high-speed ratiometric program and these areas along with the images were recorded to a hard disk. Excitation resulted in alternating at 440 and 485 nm, emission was analyzed at 515 nm (Longpass, Schott, Mainz, Germany) and 521 nm (Ramon Longpass, Omega) after having passed a 535-nm beam-splitter (Bandpass, Omega) by an inverted microscope (Axiovert 135, Carl Zeiss, Oberkochen, Germany) with a video-imaging system (IMG8, Lindemann und Meiser, Homburg, Germany). The intensity of emission was pH-dependent with an increase in intensity when pH was rising. Calibration was accomplished by using the ionophore nigericin.

Electron microscopy

Cultured cells were immersion-fixed in 2% glutaraldehyde, buffered in 0.1 M cacodylate buffer for 1 h and then washed in the same buffer without

the fixative. For ultrathin sectioning, cells were postfixed in 1% buffered OsO₄, washed several times in pure buffer and dehydrated in a graded ethanol series. The 70% ethanol was saturated with uranyl acetate for contrast enhancement. The dehydrated cells were embedded in Araldite (Serva, FRG), polymerized at 60°C for 12 h and then stored at 90°C for another 24 h. From the Araldite blocks, ultrathin sections (thickness 50 nm) were cut by a diamond knife (Diatome, Biel, Switzerland) on an ultramicrotome (Ultracut E, Reichert Jung), drawn up on Formvar (Merck, Darmstadt, Germany)-coated copper grids (Veco GmbH, Solingen, Germany), contrasted by lead citrate for 5 min, immediately washed with purest water and then dried. Electron microscopy was accomplished by a transmission electron microscope (EM 10, Carl Zeiss, Oberkochen, Germany) at 80 kV. Agfa Scientia negative material (Agfa, Leverkusen, Germany) was used for photographic documentation. Development of the negative material was accomplished by Agfa Metinol developer. Positives were made on Rapitone Fotopapier P-3 magnified by 1.8-fold.

Viral vectors

Replication-deficient recombinant adenoviruses that had the E1 region replaced by the transgene expression cassette (Bcl-X_L) were constructed by cotransfection of the shuttle vector pMH4 containing the transgene cDNA and genomic adenoviral DNA using the previously described procedures.²⁸ The expression of the transgene cDNA is controlled by the human cytomegalovirus (CMV) immediate early promoter and enhancer. The viral construct called Ad-dE1 had a deletion of the E1 region and was used as a control vector for viral infection studies. Following cotransfection of the adenoviral genomic DNA and shuttle vectors into the adenovirus-transformed human embryonic kidney cell line 293 (ATCC#CRL 1573) by calcium phosphate-mediated gene transfer, plaques were screened for the presence of transgene expression by RT-PCR or Northern blot analysis. Viral plaques containing the transgene sequences and devoid of adenoviral E1 sequence were grown in 293 cells and purified by cesium chloride gradient.

Infection of neurons and detection of transgene expression

For optimal infection of cerebellar granule neurons, cultures at DIV1 were used. The volume of conditioned medium was reduced to one-third and recombinant adenovirus was added at 100 multiplicity of infection (MOI). After allowing the virus to adsorb for 1 h at 37°C, the conditioned medium was added back to its original volume. Neurons were further cultured until DIV8 *in vitro*, and transgene expression was quantified by immunocytochemistry as previously described.²⁸

Statistical analysis

Data are expressed as mean \pm S.D. Statistical analysis was assessed by one-way ANOVA followed by Fisher's PLSD *post hoc test*. If not stated otherwise, all experiments reported represent at least three independent replications performed in triplicate.

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