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Inhibition of protein phosphatases induces IGF-1-blocked neurotrophin-insensitive neuronal apoptosis

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Abstract We have previously described the marine toxin okadaic acid (OKA) to be a potent neurotoxin for cultured rat cerebellar neurons. Here we show that OKA-induced neurodegeneration involves the DNA fragmentation characteristic of apoptosis and is protein synthesis-dependent. DNA fragmentation and neurotoxicity correlated with inhibition of protein phosphatase (PP) 2A rather than PP1 activity. Neurotrophins NT-3 and BDNF failed to protect from OKA-induced apoptotic neurotoxicity that was, however, totally prevented by insulin-like growth factor-1. Neuronal death by OKA was significantly reduced by protein kinase C inhibitors and by the L-type calcium channel agonist Bay K8644, while it was potentiated by the reduction of free extracellular calcium concentrations.

Key words: Okadaic acid; Neurotoxicity; Cerebellar neuron; Calcium channel; Protein kinase C; Phosphorylation

1. Introduction

Programmed neuronal cell death appears to result from a cascade of molecular and cellular events that occur at a specific stage of differentiation and may allow for the removal of excess neurons and the establishment of correct synaptic connections [1,2]. Despite the rapid progress that has taken place in the last year or two in the apoptosis field, the biochemical and molecular mechanisms underlying neuronal apoptosis are not yet very well defined. Several agents have been demonstrated to interfere with the apoptotic process in different neuronal cultures. Thus, cAMP and high KCl block apoptosis in NGF-deprived sympathetic neurons [3], while brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) all prevented apoptotic death in cultured cortical neurons following serum deprivation or exposure to the calcium channel antagonist nimodipine [4]. Cerebellar granule neurons in primary culture have recently been identified as a very convenient model to study central nervous system neuronal apoptosis in vitro [5]. Most of these neurons undergo apoptosis in culture unless they are grown in the presence of depolarizing conditions [5]. Naturally occurring DNA fragmentation has also been found in the granular layer of newborn rat cerebellum [6], suggesting the existence of an in vivo correlate of the apoptosis observed for neurons in culture. In cultured cerebellar granule neurons, apoptotic death triggered by lowering extracellular KCl concentration is associated with an immediate and permanent decrease in the levels of free intracellular calcium, $[Ca^{2+}]_i$, and could be inhibited by insulin-like growth factor 1 (IGF-1), by the cAMP-elevating agent forskolin and by cAMP analogues [5,7]. Interestingly, the signalling pathways by which these agents rescue neurons from apoptosis does not involve long-term modifications of $[Ca^{2+}]_i$, suggesting that the ability of IGF-1 and forskolin to prevent low K^+-induced apoptosis may come from a common phosphorylation pathway that does not involve $[Ca^{2+}]_i$ increase.

Although a possible relationship between apoptosis and protein phosphorylation has been suggested in some tumour cell lines [8–10], there is no direct evidence for a role of protein kinases and/or protein phosphatases in neuronal apoptosis. We have previously reported that exposure of cultured cerebellar granule neurons to very low concentrations (0.5–5 nM) of the protein phosphatase (PP) 1 and 2A inhibitor okadaic acid (OKA) [11,12], results in widespread neurotoxicity [13,14]. Here we have investigated the role of protein phosphorylation in apoptotic neurodegeneration of cultured cerebellar granule neurons, and found that apoptosis can be triggered by inhibiting protein phosphatases, and in particular PP2A. Our results also suggest the involvement of protein kinase C (PKC) in this apoptotic process. The ability of intracellular calcium, IGF-1 and neurotrophins to prevent OKA-induced apoptosis has also been investigated.

2. Materials and methods

2.1. Cell culture

Primary cultures of rat cerebellar granule neurons were prepared as described previously [15]. Briefly, cerebella from 8-day-old pups were dissected, cells were dissociated and suspended in basal Eagle's medium (BME, Gibco) supplemented with 25 mM KCl, 2 mM glutamine, 100 μg/ml gentamycin and 10% fetal calf serum. Cells were seeded in poly(L-lysine)-coated (5 μg/ml) dishes (NUNC) at 2.5×10^5 cells/cm^2 and incubated at 37°C in a 5% CO_2, 95% humidity, atmosphere. Cytosine arabinoside (10 μM) was added after 20–24 h of culture to prevent proliferation of non-neuronal cells. After 8 days in vitro, morphologically identifiable granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons and astrocytes did not exceed 3% of the overall number of cells in culture [16]. Cerebellar granule neurons were kept alive for more than 40 days in culture, by replenishing the growth medium with glucose every 4 days and compensating for lost amounts of water, due to evaporation.

2.2. Neuronal treatment and survival

Neurons were used between 10 and 25 days in culture. Drugs were added in the growth medium for the indicated times. Then, growth medium was removed and cultures were incubated for 5 min with 1 ml incubation buffer containing 154 mM NaCl, 5.6 mM glucose, 8.6 mM

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HEPES, 1 mM $MgCl_2$, 2.3 mM $CaCl_2$, pH 7.4, to which the vital stain fluorescein diacetate (5 μg/ml) was added. The staining mixture was then aspirated, replaced with incubation buffer, and cultures were examined for neurotoxicity. Under fluorescent light, live neurons showed a bright green colour in the cell body and neurites, while dead neurons did not retain any fluorescein diacetate, and their nuclei could be stained red by 1 min exposure to 50 μg/ml ethidium bromide. Photographs of three randomly selected culture fields were taken, and live and dead neurons were counted. Total number of neurons per dish was calculated considering the ratio between the area of the dish and the area of the picture (~2000).

2.3. Data presentation and analysis

The mean ± S.D. of the data is reported. For statistical analysis one-way or two-way analysis of variance (ANOVA) was used to identify overall treatment effects. The unpaired two-tailed Student's *t*-test was used for selective comparison of individual data groups. Only significances relevant for the discussion of the data are indicated in each figure.

2.4. DNA fragmentation analysis

Soluble DNA was extracted as described [17] and subjected to electrophoresis in a 1.5% agarose gel. After treatment with RNAse A (20 μg/ml for 3 h at 37°C), the gel was visualized by ethidium bromide staining. DNA molecular size (b) markers were included for comparison.

2.5. Materials

Recombinant human IGF-1 was from Boehringer Mannheim. Calyculin A was from Moana Bioproducts Inc. (Honolulu, Hawai). Tautomycin was a generous gift from Dr. K. Isono (Riken, Wako, Saitama, Japan). Okadaic acid and okadaic acid methyl ester tetramethyl ether were kindly provided by Dr. H. Fujiki (Saitama Cancer Center, Inc., Saitama, Japan). MK801 and nifedipine were a generous gift from Dr. G.J. Kaczarowski (Merck Sharp and Dohme Laboratories, NJ, USA). NT-3 and BDNF were from Alomone Labs (Jerusalem, Israel). (±)Bay K8644 was from Research Biochemicals International (Natick, MA, USA. GF 109203X was from Boehringer Mannheim. All other chemicals were from Sigma.

3. Results

3.1. Okadaic acid induces neuronal gene expression-dependent apoptosis

Consistent with previous data [13,14], exposure of cultured cerebellar granule neurons to 5 nM OKA resulted in widespread neurotoxicity over 24 h, decreasing the number of live neurons from $850 \pm 100 \times 10^3$ ($n = 6$) to $200 \pm 60 \times 10^3$ ($n = 5$) (Fig. 1C,F). Under fluorescent light, neurons treated with 5 nM OKA for 24 h and then stained with fluorescein diacetate (see Section 2) showed considerable disintegration of neurites and swelling of cell bodies as compared to OKA-untreated cultures (Fig. 1A,C). The presence of vacuoles and cellular fragmentation was also evident. Neurotoxicity by OKA was a long-term process since neither cell death nor morphological signs of toxicity were observed in neurons which had been exposed to 5 nM OKA for 18 h (Fig. 1B,E) ($860 \pm 87 \times 10^3$ neurons ($n = 5$)).

The morphological changes accompanying OKA-induced neurotoxicity resembled those reported for cells undergoing apoptosis [1,18]. To verify that death induced by OKA in cerebellar neurons was due to apoptosis, we examined the DNA of these cells. As shown in Fig. 1E, agarose gel electrophoresis of soluble DNA extracted from neurons treated with 5 nM OKA for 18 h revealed considerable DNA fragmentation characteristic of apoptotic cells, resulting from cleavage of nuclear DNA in internucleosomal regions [19]. Fragmentation of DNA was a very early event in the death process for it was already evident in DNA extracted from neurons which had been exposed to 5 nM OKA for only 9 h, while at least 24 h were necessary to observe clear morphological signs of toxicity and a significant decrease in neuronal survival (Fig. 1C,E). Based on the intensity of ethidium bromide staining, DNA from 9-h-treated neurons showed quantitatively lower fragmentation than DNA from 18-h-treated neurons, whereas no soluble fragmented DNA was obtained from control neurons (Fig. 1F).

We examined whether OKA-induced apoptotic pathways required RNA synthesis by using the transcriptional inhibitor actinomycin D. We found that the presence of actinomycin D (1 μg/ml) totally abrogated the neurotoxic effects of OKA (Fig. 1D,E) ($770 \pm 93 \times 10^3$ neurons ($n = 4$)), suggesting that newly synthesized protein(s) are necessary for OKA-mediated neurotoxicity.

3.2. Role of PP1 and PP2A inhibition on apoptotic neurotoxicity by OKA.

The low concentration of OKA (5 nM) we used here as well as in previous work [13,14] suggested that PP2A rather than PP1 inhibition was responsible for the apoptotic degeneration induced by OKA. To determine further the specific role of PP1 and PP2A in inducing neuronal apoptotic death we used calyculin A and tautomycin, two potent PPs inhibitors with higher affinity for PP1 as compared to OKA. Thus, calyculin A inhibits both enzymes with similar IC_{50} [20], whereas tautomycin inhibits PP1 with approx. 10-fold greater affinity than PP2A [21]. As shown in Fig. 2A, concentrations above 1 nM OKA and 5 nM calyculin A caused a concentration-dependent reduction in neuronal survival after 24 h. In contrast, as much as 1 μM tautomycin was necessary to induce significant neurotoxicity. The concentrations producing half-maximal reduction in neuronal survival were 3.5 and 10 nM for OKA and calyculin A, respectively. When OKA was replaced by okadaic acid methyl ester tetramethyl ether (OKA-TME), a derivative lacking PP inhibitory activity [22], no reduction in neuronal survival was observed up to 1 μM OKA-TME (Fig. 2A).

In agreement with these observations, exposure of neurons to calyculin A (25 nM, 19 h) but not to tautomycin or OKA-TME (both at 100 nM for 48 h), induced fragmentation of soluble DNA characteristic of apoptotic cells (Fig. 2B), further suggesting a role for protein phosphorylation due to PP2A inhibition in the apoptotic death induced by OKA and calyculin A.

3.3. OKA-induced apoptotic neurotoxicity is prevented by IGF-1 but not by neurotrophins

Neurotrophins have been documented to attenuate neuronal apoptotic death induced by different stimuli including deprivation of serum or lowering of $[Ca^{2+}]_i$ [4,23]. We checked the ability of cerebellar granule neurons to respond to NT-3 and BDNF by testing whether these neurotrophins could prevent toxicity induced by exposure of neurons to dihydropyridine calcium channel antagonists. This treatment has been demonstrated to induce neurodegeneration in cultured cerebellar granule neurons [24] possibly via apoptosis [7] as it has been reported for other neuronal cultures [4]. Addition of 1 μM nifedipine (NIF) to the culture medium resulted in neuronal degeneration evolving slowly over 72 h, and reduced the number of surviving cells from $800 \pm 62 \times 10^3$ to $251 \pm 71 \times 10^3$ neurons ($n = 3$). Pretreatment of neurons for 24 h with NT-3

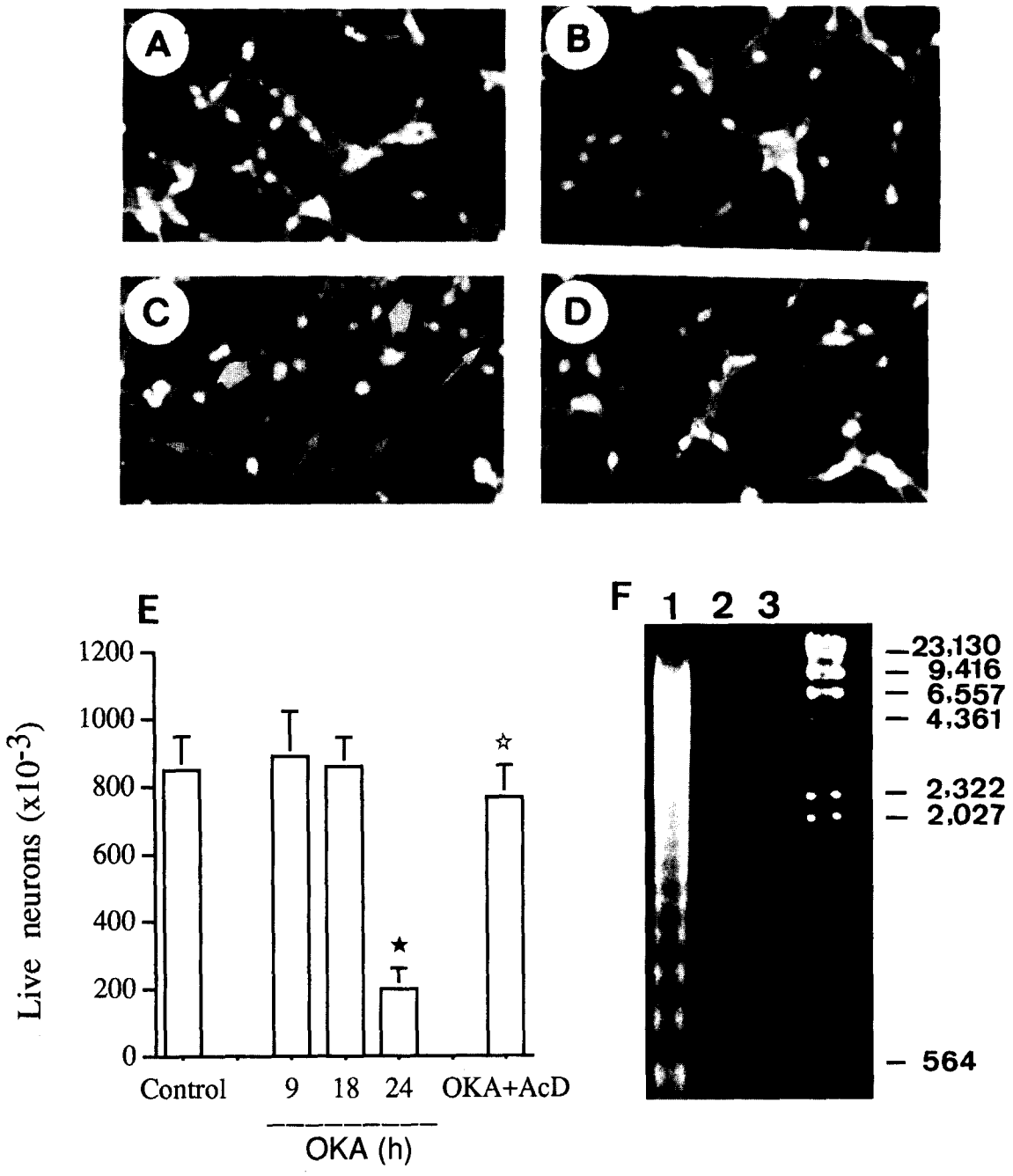

Fig. 1. Okadaic acid induces neuronal gene expression-dependent apoptosis. Fluorescence photomicrographs of neuronal cerebellar cultures before (A) and after 18 h (B) or 24 h (C) exposure to 5 nM okadaic acid (OKA). Live neurons showed a bright green color in the cell body and the neurites (large arrows), while nuclei of dead neurons appeared stained in red (small arrows). Addition of the transcriptional inhibitor actinomycin D (1 μg/ml) prevented neuronal degeneration and death (D). (E) Number of live neurons per dish (mean ± S.D., $n = 4$–6), before (control) or after exposure to OKA for the indicated times. Neuronal survival after 24 h treatment with OKA (5 nM) in the presence of actinomycin D (1 μg/ml) is also represented (OKA+AcD). $^{\star}P < 0.01$ vs control, and $^{\star}P < 0.01$ vs OKA (24 h). (F) Agarose gel electrophoresis of cerebellar neurons soluble DNA reveals a ladder pattern after 18 h exposure to 5 nM OKA (lane 1). Slight DNA fragmentation is also evident in neurons treated with 5 nM OKA for 9 h (lane 2), but not in control neurons (lane 3).

or BDNF (both at 20 ng/ml) reduced the incidence of this cell death by 50% (NIF + NT-3 = $540 \pm 65 \times 10^3$ neurons; NIF + BDNF = $514 \pm 37 \times 10^3$ neurons ($n = 3$); $P < 0.01$ vs NIF alone).

We therefore investigated the effect of NT-3 and BDNF on OKA-induced apoptotic neurotoxicity. We also examined IGF-1, which has been previously reported to prevent apoptosis by low K^+ in cultured granule neurons [7]. As shown in Fig. 3A, pretreatment of neurons with 2.5 nM IGF-1 for 24 h prior to OKA addition, significantly increased the number of surviving neurons from $145 \pm 110 \times 10^3$ ($n = 6$) to $750 \pm 150 \times 10^3$ ($n = 6$). In contrast, neither NT-3 nor BDNF (both at 20 ng/ml, up to 72 h) showed any protective effect, and typical neurite disintegration (not shown) and cell death (Fig. 3A) were observed after 24 h exposure to OKA in neurons pretreated with each neurotrophin [NT-3 = $200 \pm 50 \times 10^3$ neurons ($n = 6$); BDNF = $60 \pm 22 \times 10^3$ neurons ($n = 6$)].

Consistent with these observations, preincubation of neu-

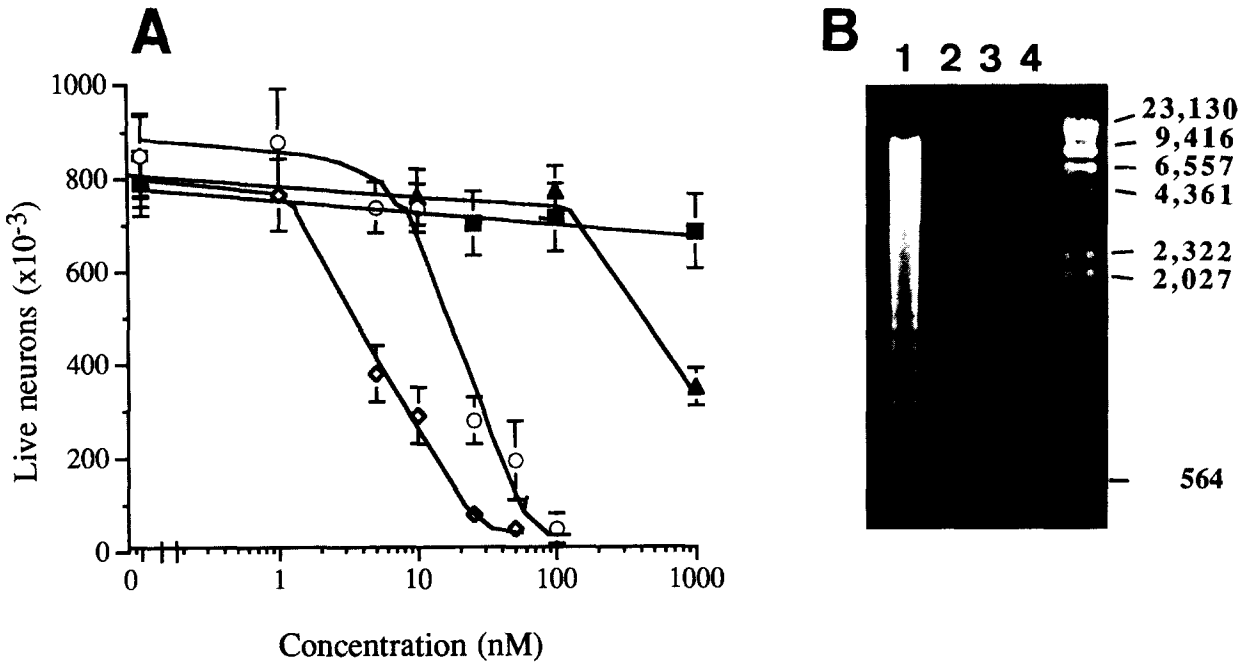

Fig. 2. Induction of apoptotic neurotoxicity by protein phosphatase inhibitors with high affinity for PP2A. (A) Number of live neurons per dish (mean ± S.D., $n = 4$) after treatment of cultures for 24 h with OKA (◇) or calyculin A (○), or for 48 h with tautomycin (▲) or okadaic acid methyl ester tetramethyl ether (OKA-TME) (■), at the indicated concentrations. (B) Soluble DNA agarose gel electrophoresis reveals considerable DNA fragmentation in neurons exposed for 18 h to 25 nM calyculin A (lane 1) but not in neurons treated with 100 nM tautomycin (lane 2), 100 nM OKA-TME (lane 3), or in control neurons (lane 4).

rons with 2.5 nM IGF-1 for 24 h totally prevented DNA fragmentation induced by OKA, while similar treatments with 20 ng/ml NT-3 and BDNF had no effect (Fig. 3B).

3.4. *Neurotoxicity by OKA is attenuated by calcium channel agonists*

To investigate the involvement of Ca^{2+} in the apoptosis induced by OKA, we examined the dependence of OKA neurotoxicity upon $[Ca^{2+}]_i$. We found that by adding the L-type Ca^{2+} channel agonist Bay K8644 (1 μM) to the culture medium 24 h prior to OKA, neuronal survival following 24 h exposure to 5 nM OKA significantly increased from $300 \pm 47 \times 10^3$ to $743 \pm 56 \times 10^3$ neurons ($n = 10$) (Fig. 4). The non-competitive NMDA-receptor antagonist MK801 (1 μM)

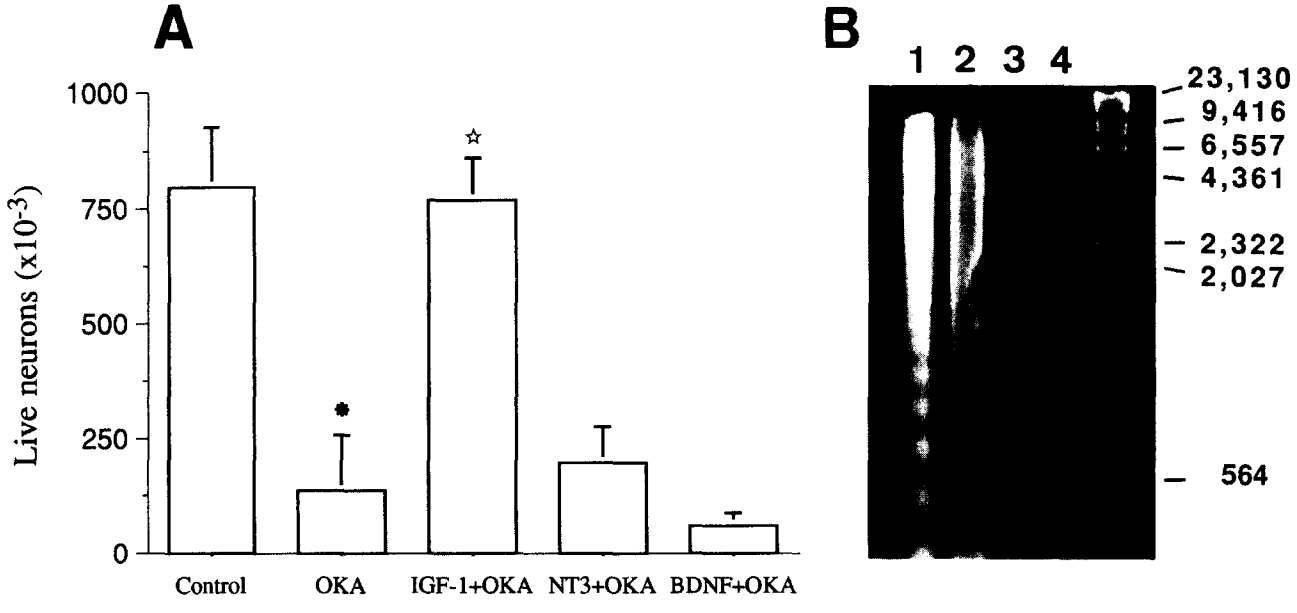

Fig. 3. Inhibition of OKA-induced apoptotic neurotoxicity by IGF-1 but not by neurotrophins. (A) Number of surviving neurons (mean ± S.D., $n = 6$) before or after exposure of cerebellar neurons to 5 nM OKA without or with 24 h pretreatment with IGF-1 (2.5 nM), NT3 or BDNF (both at 20 ng/ml), respectively. $^*P < 0.01$ vs control, and $^☆P < 0.01$ vs. OKA. (B) Agarose gel electrophoresis of soluble DNA shows extensive DNA fragmentation in neurons pretreated for 24 h with 20 ng/ml NT3 (lane 1) or BDNF (lane 2) and then 5 nM OKA for 18 h. No DNA fragmentation is observed in neurons pretreated for 24 h with 2.5 nM IGF-1 prior to 18 h exposure to 5 nM OKA (lane 3) or in control neurons (lane 4).

was also added in these experiments in order to prevent toxicity by excitatory amino acids endogenously released into the culture medium from Bay K8644-treated neurons [25]. Ca^{2+}-mediated protection was not permanent and no significant differences in neuronal survival were found between Bay K8644-treated and untreated neurons after 48 h exposure to 5 nM OKA (data not shown). The role of extracellular Ca^{2+} influx in attenuating the neurotoxic effects of OKA was further confirmed by using the membrane-impermeable Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA). Reduction of free extracellular Ca^{2+} concentrations due to preincubation of neurons with 1 mM BAPTA for 24 h reduced the number of surviving neurons after 24 h exposure to OKA by 50% (Fig. 4), and the time of exposure to OKA required to induce neuronal death, which was at least 6 h shorter in BAPTA-pretreated neurons compared to OKA alone. No significant differences in neuronal survival were observed in cultures treated with Bay K8644+MK801 ($950 \pm 75 \times 10^3$ neurons ($n = 10$)) or BAPTA ($780 \pm 85 \times 10^3$ neurons ($n = 10$)) in the absence of OKA, compared to untreated cultures ($860 \pm 60 \times 10^3$ neurons ($n = 10$)).

3.5. Role of protein kinase A and protein kinase C activity in OKA-induced neurotoxicity

To investigate whether OKA affected the function of proteins phosphorylated by protein kinase A or protein kinase C (PKC), we first examined the effects of forskolin and phorbol esters on neuronal survival. Neither addition of 10 μM forskolin, capable of increasing intracellular cAMP concentration from 26 ± 2 to 2760 ± 275 pmol/mg protein, nor activation of PKC by PMA (10–100 nM), resulted in significant reduction of neuronal survival after 24 h, compared to untreated neurons (Table 1). No differences in the final number of surviving neurons or in the time of exposure to OKA needed to induce toxicity were observed when forskolin was added to the neurons simultaneously with OKA. However, when 10–100 nM PMA was co-added with OKA, neurotoxicity was totally prevented (Table 1). Given the prolonged treatments (24 h) needed in these experiments, we considered the possibility that down-regulation rather than activation of PKC activity could be responsible for the protection observed. For this purpose we used 5-isoquinolinylsulphonyl-2-methylpiperazine (H7), an inhibitor of PKC and several other protein kinases, and bisindolylmaleimide (GF 109203X), a potent and selective inhibitor of PKC activity [26]. As shown in Table 1, preincubation of neurons for 1 h with 50 μM H7 or 9 μM GF 109203X prevented neurotoxicity by 24 h exposure to 5 nM OKA, suggesting the involvement of PKC in the phosphorylation of substrates affected by OKA and leading to apoptotic death.

Table 1
Role of protein kinase A and protein kinase C activity in OKA-induced neurotoxicity

Treatment	Live neurons ($\times 10^{-3}$)	
	None	OKA
None	950 ± 100	279 ± 65[a]
Forskolin	870 ± 120	350 ± 39
PMA	800 ± 200	850 ± 70[b]
H7	980 ± 90	880 ± 150[b]
GF108203X	900 ± 90	894 ± 60[b]

The number of live cells was determined as described in Section 2. Results are the mean ± S.D. for triplicate cultures. Neurons were exposed to 5 nM okadaic acid (OKA) for 24 h. Forskolin (10 μM) was added 10 min before OKA. H7 (50–100 μM) and GF108203X were added 1 h prior to OKA. PMA (10–100 nM) was added simultaneously with OKA.
[a] $P < 0.01$ vs None in the same line.
[b] $P < 0.01$ vs OKA in the same column.

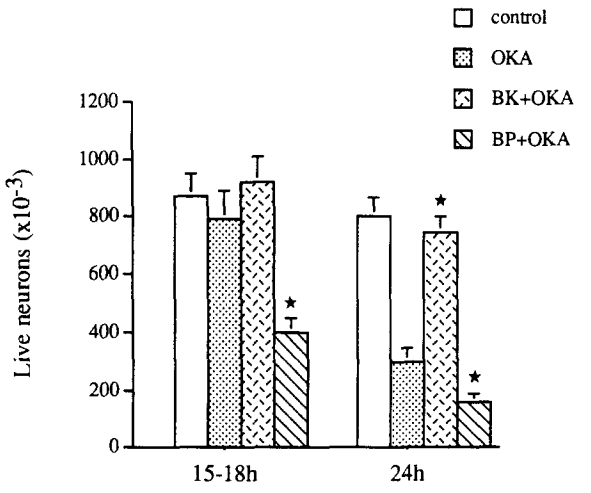

Fig. 4. Involvement of calcium in OKA-induced neurotoxicity. Neurons were exposed to 5 nM OKA for 15–18 or 24 h as indicated, in the absence or presence of Bay K8644 (1 μM) or BAPTA (1 mM). Control represents untreated cultures. Results represent the number of surviving neurons (mean ± S.D., $n = 10$). $^{\star}P < 0.01$ vs OKA in the same group.

4. Discussion

Cultures exposed for at least 24 h to low concentrations of the PP1 and PP2A inhibitor OKA presented swollen cell bodies and extensive disintegration of the neurites, as well as a large number of vacuoles as a result of cellular fragmentation (Fig. 1C). Most of these features have been previously reported in neurons undergoing apoptosis [5]. Indeed, when DNA extracted from neurons exposed to OKA for at least 9 h was run on an agarose gel, it appeared as a ladder-like series of bands (Fig. 1F), a hallmark of apoptosis [18]. It is worth noting that DNA fragmentation was detectable long before neuronal degeneration and death could be observed, supporting the idea that it is an early event in the death process. Our observation that OKA-induced apoptosis can be inhibited by inhibitors of RNA and protein synthesis indicates that apoptosis by OKA is a genetically controlled process. Interestingly, the later two characteristics have also been described for low K^+-induced apoptosis in cerebellar granule neurons [5], suggesting possible common targets in the pathway triggered by membrane hyperpolarization or by OKA.

The fact that both OKA and calyculin A were effective in inducing neuronal apoptosis strengthens the evidence that it is a cellular process controlled by phosphorylation. Moreover, a closely related structural analog of OKA that is inactive toward phosphatases failed to induce apoptosis (Fig. 2). Although the specificities of the three PP inhibitors may be different in vivo, our results very much suggest that inhibition of PP2A rather than PP1 activity has a major contribution in the induction of apoptosis by OKA and calyculin A. Thus, at the concentration we used (5 nM), OKA is likely to affect

mainly PP2A and not PP1, since OKA at concentrations as low as 1 nM has been shown to inhibit totally PP2A activity in vitro, whereas complete inhibition of PP1 activity needs at least 500 nM OKA [12,20]. Moreover, no DNA fragmentation or cell death (Fig. 2) was observed in cells treated with 100 nM tautomycin, conditions under which the activity of native PP1 but not of PP2A should be completely inhibited [21]. Concentrations of inhibitors producing neurotoxicity were usually 5–10-times higher than those reported for the inhibition of catalytic activity in in vitro assays. In this respect, it should be considered that the catalytic subunits of PP1 and PP2A are complexed to regulatory and/or targetting subunits in vivo [27], and therefore inhibition of native PPs may require higher concentrations of inhibitors than the free catalytic subunits [21,28]. Although a relative cell impermeability to any of the PP inhibitors we used cannot be ruled out completely, their lipophilic structures renders this possibility unlikely.

A general protective effect of neurotrophins against neuronal apoptosis has been previously suggested by Koh and coworkers who found NT-3, BDNF and NT-4/5 to be effective in protecting murine cortical neurons against apoptotic degeneration induced by Ca^{2+} channel antagonists and serum deprivation [4]. In cerebellar granule neurons, however, only a small but statistically significant effect has been found for NT-3 in promoting survival of neurons maintained in low K^+, while the protecting role of BDNF remained controversial [5,23]. The inability exhibited by NT-3 and BDNF to protect neurons against apoptosis induced by OKA suggests that the protective effect of neurotrophins may be restricted to apoptosis elicited by stimuli inducing a decrease in $[Ca^{2+}]_i$. In fact, no differences in $[Ca^{2+}]_i$ were observed using confocal laser microscopy in neurons exposed to OKA for 24 h (data not shown). Alternatively, neurotrophins may be interfering upstream with the signalling events triggered by OKA.

IGF-1 prevention of granule cell death by OKA further supports a role for IGF-1 as a crucial physiological neurotrophic factor for cerebellar granule neuron survival and development. Several lines of evidence have contributed to this concept. Thus, IGF-1 induces the expression of functionally active glutamate receptors in these neurons, and neutralization of serum IGF-1 by a specific antibody reduces neuronal survival by 30–40% [29]. Although cerebellar granule neurons do not produce IGF-1 at any stage of development, they do express the IGF-1 receptor both in vivo [30] and in vitro (data not shown). Moreover, IGF-1 inhibits low K^+-induced granule neuron apoptosis [3]. The role of IGF-1 in supporting granule cell survival and development could be particularly significant in vivo. Apoptosis has been shown to be a mechanism of physiological cell death of cerebellar granule neurons during the first 2 weeks of postnatal development [6], when granule cells migrate from the external to the internal granule layer, crossing a monolayer of postmitotic Purkinje neurons with which the granule neurons make synapses. Since IGF-1 is synthesized and secreted by cerebellar Purkinje cells, especially during early postnatal life [31,32], taking up the IGF-1 secreted by Purkinje cells may be a crucial step for selecting cerebellar granule neurons that will successfully differentiate from those that will undergo apoptotic degeneration. It is tempting to speculate that this effect of IGF-1 in vivo may involve modulation of protein phosphatases, and in particular of PP2A activity or substrates.

The observation that neuronal pretreatment with the calcium agonist Bay K8644 attenuates apoptosis by OKA was not surprising in that $[Ca^{2+}]_i$ has previously been associated with neuronal survival in several neuronal models including cerebellar granule cells [24,33–35]. Moreover, convincing data have been reported in the last few years suggesting that neurons may die by apoptosis when their $[Ca^{2+}]_i$ levels decrease below a certain threshold in the absence of survival factors [7,36]. Ca^{2+}-mediated protection from neuronal apoptosis probably results from a combination of modulatory effects of this ion on several intracellular targets, including protein kinases and phosphatases. PP1 as well as PP2A and most PP2A-type activities are independent of Ca^{2+}. However, elevated $[Ca^{2+}]_i$ levels are known to stimulate the activity of the Ca^{2+}-dependent calmodulin-stimulated PP2B calcineurin [37], which in turn activates PP1 via the dephosphorylation of cAMP- and cGMP-regulated PP1 inhibitors (see [38] for a review). Mammalian PP1 and PP2A show 43% overall sequence identity and have overlapping specificities in vitro [39]. It may therefore be possible that a larger fraction of PP1 molecules in the active form in Bay K8644-pretreated neurons could partially compensate the imbalance of the kinase/phosphatase system induced by OKA. The observation that Bay K8644 delayed rather than prevented neuronal death would be consistent with this possibility. Alternatively, calcineurin may dephosphorylate substrates such as the microtubule-associated proteins MAP-2 and tau [38], of which the phosphorylation level may be particularly relevant for neuronal survival and function [40]. Indeed, severe disturbances in the phosphorylation of microtubule associated proteins are known to accompany the neuronal cytoskeletal pathology characteristic of Alzheimer's disease brains [41–43]. Although more studies are needed to establish the nature of protein kinases responsible for the phosphorylation of substrates affected by OKA and leading to apoptotic death, the protecting effect observed for H7 and GF 109203X indicates that PKC may be involved. Interestingly, PKC activity has been reported to be altered in Alzheimer's disease brain [44,45] and both OKA and PKC activity-stimulating agents led to an abnormal processing of Alzheimer β/A4 amyloid precursor protein [46], further suggesting a key role for PP2A and PKC activities in maintaining the physiological phosphorylation/dephosphorylation balance.

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