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Prevention of Dopaminergic Neuronal Death by Cyclic AMP in Mixed Neuronal/Glial Mesencephalic Cultures Requires the Repression of Presumptive Astrocytes

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ABSTRACT

Cyclic AMP-elevating agents are highly effective in preventing the loss of dopaminergic neurons that occurs spontaneously in neuronal-glial mesencephalic cultures. We demonstrate here that cAMP causes a concomitant decline in the number of dividing non-neuronal cells, suggesting that inhibition of proliferation contributes to neuroprotection. Consistent with this hypothesis, a transient treatment with the antimitotic cytosine arabinoside, at concentrations that induce long-term repression of glial cell proliferation, mimicked the neuroprotective action of cAMP and also obviated the need for the cyclic nucleotide. Treatment with cAMP-elevating agents reduced the population of OX-42–positive microglial cells and the number of immature astrocytes expressing vimentin and low levels of the astrocytic marker glial fibrillary acidic protein. The effect on the immature astrocytes, however, seemed essential for neuroprotection. Ciliary neurotrophic factor and leukemia inhibitory factor, which stimulate astrocyte differentiation without reducing cell proliferation, failed to reproduce the protective effects of the cyclic nucleotide. Cyclic AMP did not operate by counteracting the action of the astrocyte mitogen epidermal growth factor or by reducing activation of the mitogen-activated protein kinase signaling pathway. The neuroprotective and antiproliferative actions of cAMP, however, were closely mimicked by olomoucine and roscovitine, potent inhibitors of the cyclin-dependent kinase CDK1 that are structurally related to cAMP. Measurement of CDK1 activity confirmed that neuroprotection was closely correlated with inhibition of this kinase by cAMP. In summary, neuroprotection of mesencephalic dopaminergic neurons by cAMP most probably requires the repression of presumptive astrocytes through inhibition of CDK1.

Cyclic AMP is an archetypal second messenger that functions as an intracellular mediator for neurotransmitters and hormones (Sutherland, 1972). Several studies indicate that the cyclic nucleotide can also act as a potent neurotrophic signal for a variety of nerve cells (Goldberg and Barres, 2000). In particular, the elevation of cAMP levels was reported to promote the survival of sympathetic ganglion cells in the peripheral nervous system, retinal ganglionic and cerebellar granule cells, spinal motoneurons, basal forebrain cholinergic neurons, and brainstem noradrenergic neurons in the central nervous system (reviewed in Goldberg and Barres, 2000).

Several mechanisms have been proposed to explain the survival promotion by cAMP in neurons. Cyclic AMP-elevating agents were found to enhance the responsiveness of retinal ganglionic cells and spinal motoneurons to brain-derived neurotrophic factor by recruiting trkB receptors to the membrane from intracellular stores (Meyer-Franke et al., 1998). Direct inhibition of proapoptotic signaling pathways may also occur in other experimental paradigms. More specifically, it has been suggested that cAMP could operate by phosphorylation and inhibition of the glycogen synthase kinase 3β (Li et al., 2000) or of Bad, a pro-apoptotic member of the Bcl-2 protein family (Virdee et al., 2000), by interaction with the ceramide-dependent signaling pathway (Brugg et al., 1996), by preventing the activation of proapoptotic caspases (Vaudry et al., 2000), by activating Bcl-2 (Riccio et al., 1999), or by stimulating the mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinases 1,2 ($ERK_{1/2}$) signaling pathway (Villalba et al., 1997; Troadec et al., 2002).

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; ara-C, cytosine arabinoside; CDK, cyclin-dependent kinase; MK-801, dizocilpine maleate; ; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; EGF, epidermal growth factor; PD98059, 2′-amino-3′-methoxyflavone; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; dbcAMP, dibutyryl cAMP; FK, forskolin; ddFK, dideoxyforskolin; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; DIV, days in vitro; tyrphostin AG-1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline.

We and others have shown that cyclic AMP-elevating agents can also prevent the death of dopaminergic neurons that occurs spontaneously in mesencephalic cultures (Hartikka et al., 1992; Mena et al., 1995; Michel and Agid, 1996). The mechanisms that underlie the protection of dopaminergic neurons by cAMP have only been addressed, however, using neuron-enriched cultures in which the density of glial cells is very low (Engele and Franke, 1996; Troadec et al., 2002). In these conditions, cAMP was found essentially to cooperate with other factors such as glial-derived neurotrophic factor (Engele and Franke, 1996), noradrenaline, other catecholamines, and some antioxidants (Troadec et al., 2002). The cyclic nucleotide was also neuroprotective in mixed glial-neuronal cultures, although another mechanism was probably involved. Indeed, when glial cells were present, the effects of cAMP in mesencephalic cultures were mimicked by low concentrations of several antimitotics such as cytosine arabinoside (ara-C), fluorodeoxyuridine, and aphidicolin that are inhibitors of the DNA polymerase (Michel et al., 1997). We established previously that the neuroprotective effect of ara-C and its congeners was indirect and resulted from elimination of dividing glial cells from the cultures (Michel et al., 1997). The aims of the present study were 1) to determine whether the neuroprotective action of cAMP for dopaminergic neurons in mixed mesencephalic cultures was also mediated by an effect on glial cells, 2) to identify the glial cell population that is the putative target of the cyclic nucleotide, and 3) to characterize the underlying mechanisms.

Our results show that cAMP was neuroprotective via the repression of a subpopulation of immature glial cells that express vimentin and low levels of GFAP. The effect of the cyclic nucleotide required inhibition of the cyclin-dependent kinase CDK1 ($p34^{cdc2}$), a key component of the cell cycle. Our model might be relevant to such neurodegenerative conditions as Parkinson's disease and closely related disorders in which the selective loss of dopaminergic neurons might be dependent, at least in part, on a glia-mediated mechanism (Hirsch et al., 2003; Teismann et al., 2003).

Materials and Methods

Mesencephalic Cell Cultures. Animals were treated in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health). The embryos were removed at embryonic day 15.5 from pregnant Wistar rats (Elevage Janvier, Le Genest St. Isle, France) that had been anesthetized then decapitated. The ventral mesencephalon was dissected as described previously (Douhou et al., 2001). Cell suspensions prepared by mechanical trituration without proteolytic enzymes were plated in culture wells precoated overnight with 1 mg/ml polyethylenimine (Sigma, Saint Quentin Fallavier, France) in borate buffer, pH 8.3. The cells were then maintained for maturation and differentiation in N5 culture medium (Kawamoto and Barrett, 1986) supplemented with 5 mM glucose, 5% horse serum, and 0.5% fetal calf serum, except for the first 3 days in vitro, when the concentration of fetal calf serum was 2.5%. Cultures were fed daily by replacing 300 of the 500 μl of medium in the culture wells. Ara-C–treated cultures, which contain a very low number of astrocytes and are therefore highly vulnerable to excitotoxic stress caused by culture medium changes, were maintained in the presence of 1 μM MK-801. Excitotoxic stress was not observed in the other test experimental conditions.

Peptides and Pharmacological Agents. Peptides such as leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and epidermal growth factor (EGF), and most of the pharmacological agents were purchased from Sigma/RBI-Aldrich (Saint Quentin Fallavier, France). PD98059, a cell-permeable inhibitor of the mitogen-activated protein kinase (MAPK) kinases (MEK) was obtained from Calbiochem (Darmstadt, Germany). Stock solutions of dibutyryl cyclic AMP (dbcAMP) were made in sterile distilled water. Forskolin (FK), dideoxyforskolin (ddFK) and PD98059 were prepared in dimethyl sulfoxide. The highest final concentrations of dimethyl sulfoxide (0.5%) added to the cultures had no impact on neuronal survival. All these compounds were stored at −20°C. [*methyl*-^{3}H]thymidine and [γ-^{32}P]ATP were purchased from Amersham Biosciences (Orsay, France).

Identification of Dopaminergic Neurons and Glial Cells. Tyrosine hydroxylase (TH) immunocytochemistry was used to quantify the survival of dopaminergic neurons, as described previously (Douhou et al., 2001). Briefly, after fixation with 4% formaldehyde in PBS for 15 min, the cells were incubated overnight at 4°C with a mouse anti-TH monoclonal antibody (Chemicon, Temecula, CA) diluted 1/250 in phosphate-buffered saline containing 0.2% Triton X-100. Subsequent incubations were performed at room temperature with a biotinylated secondary anti-mouse IgG (1/500 in phosphate-buffered saline; Jackson ImmunoResearch, West Grove, PA) followed by amplification with a preformed avidin-biotin horseradish peroxidase complex (Vectastain; Vector, Burlingame, CA). The peroxidase was revealed by incubation with a solution of diaminobenzidine (1 mg/ml) containing 0.006% H_2O_2. Astrocytes were identified with a rabbit anti-glial fibrillary acidic protein (GFAP) antibody diluted 1/100 (DAKO Corporation, Carpinteria, CA) followed by a fluorescein isothiocyanate-conjugated anti-rabbit IgG (DAKO). Vimentin-positive cells were labeled with a monoclonal antibody (clone V9; DAKO) diluted 1/100, followed by immunofluorescence detection with tetramethylrhodamine isothiocyanate-conjugated anti-mouse immunoglobulins (DAKO). Macrophages and microglial cells were identified with a mouse anti-CD11b antibody (clone MRC OX-42; Serotec, Oxford, England) diluted 1/50. No Triton X-100 was used with this antibody.

Incorporation of [*methyl*-^{3}H]Thymidine. [*methyl*-^{3}H]thymidine was used as a marker of DNA synthesis to label proliferating cells as described previously (Douhou et al., 2001). Briefly, mesencephalic cultures, maintained for 6 to 10 days in vitro in the presence of the various test treatments, were washed twice, and then exposed to [*methyl*-^{3}H]thymidine (1 μCi/16-mm culture well; 40 Ci/mmol; Amersham Biosciences) for 2 h at 37°C in serum-free N5 medium supplemented with 5 mM glucose. After two rapid washes, the cells were allowed to recover for 2 h further in the same culture medium. This step permitted the elimination of radioactivity that accumulated in the cells but was not irreversibly incorporated into DNA. The cultures were then fixed with 4% formaldehyde for 15 min and processed for immunodetection of TH, GFAP, vimentin, or OX-42. Finally, thymidine-positive nuclei were visualized with the Hypercoat LM-1 emulsion (Amersham Biosciences) after incubation for 4 days at 4°C.

Western Blot Analysis. MAPK activation was investigated by Western blot with an anti-phospho-$ERK_{1/2}$ (p-$ERK_{1/2}$) antibody. Mesencephalic cultures were exposed to the test treatments, then recovered in 50 μl of lysis buffer containing 20 mM Tris/HCl, 20 mM NaCl, 2 mM EDTA, pH 8, 1% Triton, 10% glycerol, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml pepstatin A. Proteins (30 μg/lane) were separated on a 10% polyacrylamide gel and blotted onto nitrocellulose membranes. After incubation for 1 h with 5% low-fat milk in phosphate-buffered saline at room temperature, to inhibit nonspecific binding, the blot was incubated for 48 h at 4°C with a phospho-$ERK_{1/2}$ antibody (New England Biolabs, Beverly, MA) diluted 1/500. The membranes were washed, then incubated for 2 h with an anti-mouse peroxidase-conjugated secondary antibody (Amersham Biosciences) diluted 1/5000 before detection using the Super Signal

enhanced chemiluminescence kit (Pierce, Rockford, IL). Equal protein loading was verified by immunolabeling with a total $ERK_{1/2}$ antibody (New England Biolabs) visualized with an anti-rabbit peroxidase conjugated secondary antibody (Amersham Biosciences).

Protein Kinase Assay for CDK1. The activity of the cyclin-dependent kinase CDK1 was assessed using a biotinylated peptide substrate (PKTPKKAKKL) derived from histone H1 (SignaTECT kit; Promega; Charbonnières, France). Briefly, mesencephalic cultures that had been synchronized by serum starvation for 18 h were harvested with an extraction buffer containing 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, pH 8, 0.1% Triton X-100, 50 mM sodium fluoride, 10 μM leupeptin, 100 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride. The cell lysates were clarified by centrifugation (10 min at 4°C, 13,000*g*), and 10 μg of protein was incubated at 30°C in the presence of the reaction mixture (total volume, 25 μl) containing the biotinylated peptide substrate, the test compounds and [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Biosciences). After 10 min, the reaction was terminated with 7.5 M guanidine hydrochloride, and the radiolabeled, phosphorylated substrate was spotted onto a streptavidin matrix (SAM^2 biotin capture membrane). After washing and drying, the radioactivity on the membrane was counted by liquid scintillation counting.

Statistical Analysis. Multiple comparisons against a single reference group were made by one-way analysis of variance followed by Dunnett's test. When all pair-wise comparisons were carried out, the Student-Newman-Keuls test was used. The results are expressed as the mean ± S.E.M. of three independent experiments.

Results

Agents That Elevate Intracellular cAMP Levels Promote the Survival of Mesencephalic Dopaminergic Neurons

Mesencephalic dopaminergic neurons degenerate spontaneously and progressively when maintained in a culture medium supplemented with serum proteins, whereas other types of neurons survive (Michel and Agid, 1996; Douhou et al., 2001). In the present study, we found that less than 20% of initially plated TH^+ neurons remained detectable after 10 days in vitro (Fig. 1A). Consistent with our initial observation (Michel and Agid, 1996), the cell permeant analog of cyclic AMP, dbcAMP (1 mM), prevented the death of TH^+ neurons (Figs. 1A and 2B). Combining the adenylate cyclase activator FK (25 μM) with the type IV phosphodiesterase inhibitor rolipram (100 μM) was as effective as dbcAMP alone (Figs. 1A and 2C). Rolipram (100 μM) alone, ddFK (25 μM; an inactive analog of FK), and native cAMP, which does not cross plasma membranes, had no effect on TH^+ cell survival (Fig. 1A). A chronic treatment with the antimitotic ara-C, used at a concentration of 1 μM, reproduced the effect of cAMP-elevating agents on the survival of dopaminergic neurons (Figs. 1A and 2D).

Neuroprotection by Agents That Elevate cAMP Is Correlated with Their Antiproliferative Action on Dividing Cells

Dividing glial cells are thought to be responsible for the demise of TH^+ cells in our culture system (Michel et al., 1997, 1999). We speculated, therefore, that the cyclic nucleotide analog dbcAMP and other cyclic AMP-elevating agents might exert their neuroprotective effects by preventing glial cell proliferation. To test this hypothesis, we quantified the number of cells that had incorporated [^{3}H]thymidine into their DNA at a culture time (DIV6) when the cell death process was ongoing but not yet complete (Michel and Agid, 1996; Douhou et al., 2001). Dibutyryl cAMP produced a marked reduction in the number of dividing cells that accumulate the radioactive label in their nuclei (Figs. 1B and 2F). Again, FK combined to rolipram mimicked the effects of dbcAMP (Figs. 1B and 2G), whereas cell-impermeable cAMP and ddFK, which did not prevent the loss of the dopaminergic neurons, did not significantly reduce cell proliferation (Fig. 1B). At a concentration of 1 μM, ara-C eliminated >98% thymidine-positive cells from the cultures (Figs. 1B and 2H).

Cyclic AMP Becomes Inactive in Cultures in Which Dividing Cells Are Eliminated by Treatment with the Antimitotic ara-C

To explore the possibility that cAMP could also act directly on the TH^+ neurons, in addition to arresting glial proliferation, we analyzed the effect of dbcAMP in cultures from which dividing cells had been permanently eliminated by a transient treatment with 1 μM ara-C during the first 2 days in vitro (Fig. 3, A and B). In these cultures, subsequent application of the cyclic nucleotide from days 3 to 10 had no significant impact on the number of TH^+ cells present at DIV10 (Fig. 3). FK combined with rolipram was also inactive (not shown). These data further support the notion that cAMP analogs promoted survival of the TH^+ neurons indirectly via their antimitotic action on glial cells.

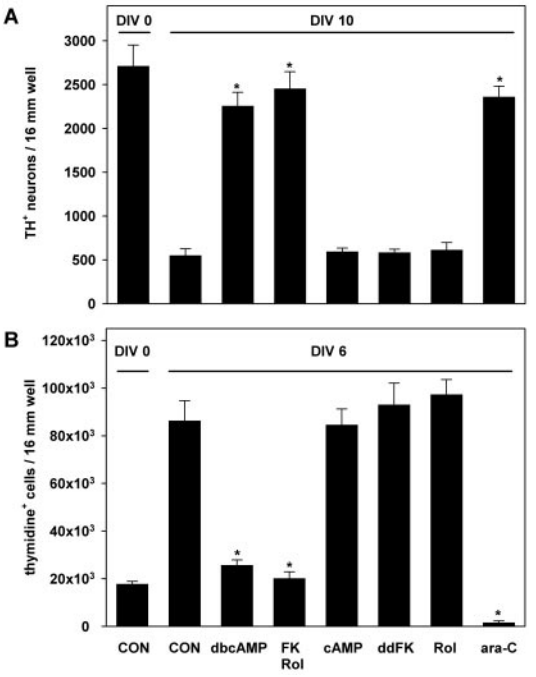

Fig. 1. The increase in the number of TH^+ neurons caused by cAMP-elevating agents is correlated with the repression of cell proliferation. A, treatments with dbcAMP (1 mM), a permeant analog of cAMP, or with the adenylate cyclase activator FK (25 μM) in the presence of the phosphodiesterase inhibitor rolipram (Rol; 100 μM) produced a robust increase in the number of TH^+ cells. Native cAMP (1 mM), ddFK (25 μM; an inactive analog of FK), or rolipram alone were ineffective. The antimitotic ara-C (1 μM) produced an increase in TH^+ cell survival comparable with that obtained with dbcAMP. B, survival promotion by dbcAMP or FK combined with rolipram was correlated with a proportional reduction in the number of non-neuronal cells that incorporated [^{3}H]thymidine into their DNA. Treatments with native cAMP, ddFK, or rolipram, which did not reduce cell proliferation, had no effect on TH^+ cell survival. The number of TH^+ cells was quantified immediately after plating at DIV0 and later at DIV10. Thymidine-positive nuclei were counted at DIV0 and DIV6. *, $p < 0.05$, versus untreated cultures.

What Population of Dividing Cells Is Targeted by cAMP-Elevating Agents?

Next, we wished to determine whether the neuroprotective action of cAMP resulted from an effect on a specific subpopulation of dividing glial cells.

Prevention of Dopaminergic Neuronal Death by cAMP Is Not Correlated with the Repression of OX-42$^+$ Microglial Cells. Some microglial cells detected by OX-42 immunofluorescence incorporated [^{3}H]thymidine into their nuclei in control conditions (Fig. 4A). Cyclic AMP-elevating agents prevented the replication of these cells. Long-term treatment with dbcAMP for 10 days eradicated virtually all OX-42$^+$ cells from the cultures (Fig. 4C), an effect that was mimicked by a cotreatment with FK and rolipram (data not shown). Microglial cells, however, were probably not essential in the death process of dopaminergic neurons for two reasons: 1) OX-42$^+$ cells represented only a small fraction of the cells that were dividing in the cultures (Fig. 4C) and 2) a transient treatment with dbcAMP (DIV0–2) that was sufficient to irreversibly eliminate all OX-42$^+$ cells from the cultures reduced only marginally the death of the dopaminergic neurons quantified at DIV10 (Fig. 4, B and C). Interestingly, the results obtained with OX-42 were confirmed using ED-1, an antibody that recognizes another protein specific to microglial cells and macrophages (results not shown).

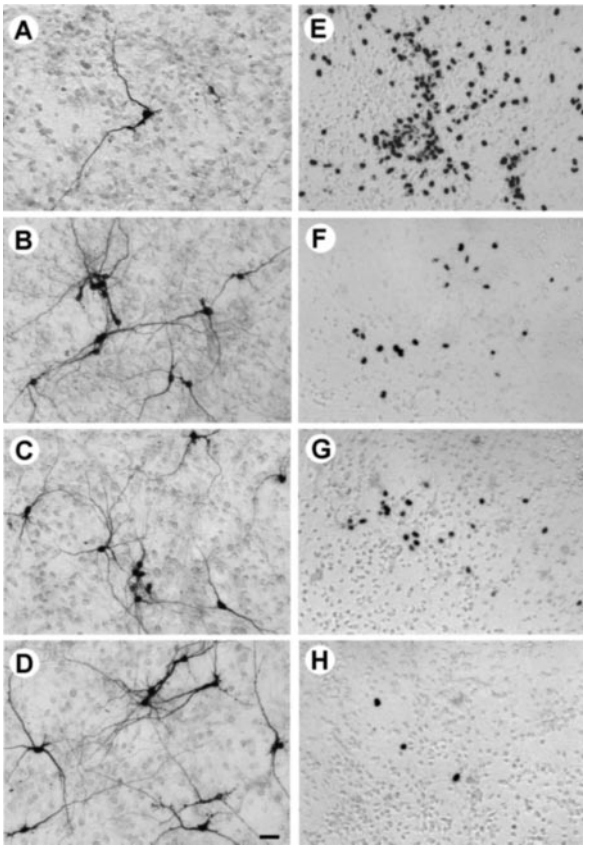

Fig. 2. Illustration of the effects of cAMP on TH$^+$ neurons and proliferating cells. Dopaminergic neurons were visualized using TH immunostaining at DIV10 (A–D) and dividing cells by microautoradiographic detection of [^{3}H]thymidine-positive nuclei at DIV6 (E–H). A and E, control cultures. Cultures exposed to 1 mM dbcAMP (B and F), 25 μM FK combined with 100 μM rolipram (C and G), or 1 μM ara-C (D and H). Scale bar, 50 μm.

Prevention of Dopaminergic Neuronal Death by cAMP Results from the Repression of a Population of Immature Astrocytes Expressing Vimentin and Low Levels of GFAP. The vast majority of cells with thymidine-positive nuclei (>90%) were astrocytes or their precursor cells that express vimentin as well as variable levels of GFAP (Figs. 5 and 6). Approximately 60% of these cells were weakly immunopositive for GFAP (Fig. 5). dbcAMP and other cAMP-elevating agents effectively prevented the replication of this cell population. After 6 days of treatment with the cyclic nucleotide, the cells expressing low levels of GFAP represented <1% of the vimentin-positive cells. This treatment had only a limited impact on the number of cells expressing high levels of GFAP (Fig. 5). Vimentin-positive cells expressing low levels of GFAP were generally found in clusters and seemed to divide more rapidly than the more mature astrocytes containing higher levels of GFAP (Fig. 5). Unlike OX-42$^+$ cells, the survival rate of TH$^+$ neurons promoted by cAMP was closely correlated with the repression of vimentin-positive cells expressing low levels of GFAP (Fig. 6). In particular, a transient treatment with dbcAMP from DIV0 to DIV3, which increased TH$^+$ cell survival moderately, as measured at DIV10, reduced proportionally the number of vimentin/thymidine-positive cells expressing low levels or no GFAP (Fig. 6, A and B). A treatment limited to the first 6 days of culture afforded a degree of protection at DIV10 that was only slightly smaller than that obtained with long-term treatment (Fig. 6A). Interestingly, when the treatment was interrupted after only 6 days, the reduction in the number of

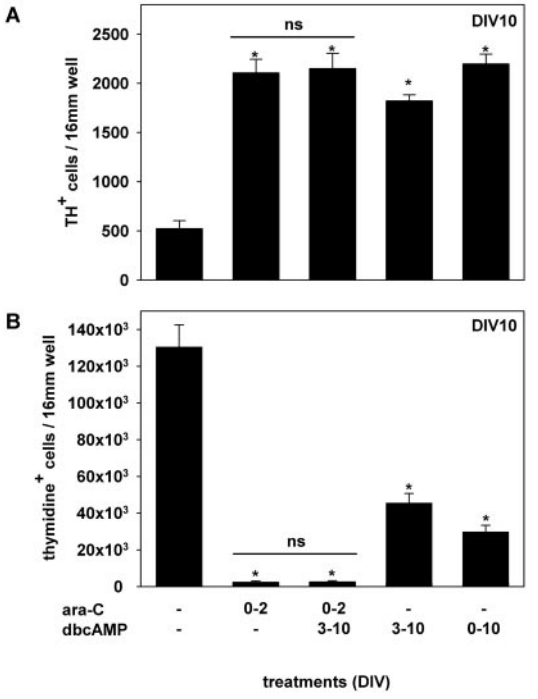

Fig. 3. dbcAMP does not further increase TH$^+$ cell survival in cultures where dividing cells were eliminated by the antimitotic ara-C. A, determination of TH$^+$ cells in cultures treated with ara-C (1 μM) between DIV0 and DIV2, then with or without dbcAMP (1 mM) between DIV3 and DIV10. Sister cultures were exposed to dbcAMP alone between DIV0–10 and DIV3–10. Note that dbcAMP did not further increase the number of TH$^+$ cells in cultures that had previously been exposed to ara-C for the first 48 h after plating. B, counts of thymidine-positive nuclei in cultures exposed to the same treatments as above. *, $p < 0.05$, versus untreated cultures; ns, not significantly different from each other.

proliferating vimentin cells expressing low levels of GFAP was only slightly smaller than when the cultures underwent long-term exposure to the cyclic nucleotide (Fig. 6B).

Inducing GFAP Expression with LIF or CNTF Does Not Mimic the Neuroprotective Effect of cAMP on Dopaminergic Neurons

To determine whether we could mimic the trophic action of cAMP on dopaminergic neurons by inducing a high level of GFAP expression in the immature vimentin-positive astrocytes, we exposed our cultures to LIF and CNTF, cytokines known to stimulate the expression of this protein (Galli et al., 2000). CNTF (Fig. 7A) and LIF (data not shown), both at 25 ng/ml, strongly stimulated GFAP expression in dividing cells that initially expressed low levels of the protein (>90% of the dividing vimentin-positive cells expressed high levels of GFAP after these treatments), but did not rescue dopaminergic neurons from death (Fig. 7B). Unlike cAMP, neither LIF nor CNTF was able to reduce cell proliferation (Fig. 7C), further demonstrating that the antiproliferative activity of cyclic AMP-elevating agents, not their ability to induce and/or maintain GFAP expression, was required for neuroprotection.

Cyclic AMP Is Not Protective by Counteracting EGF-Dependent Proliferation

We hypothesized that cAMP-elevating agents might protect TH^+ neurons by counteracting an effect of EGF, a mitogen known to stimulate the division of astrocytes (Leutz and Schachner, 1981). At its optimal concentration (25 ng/ml), EGF increased the number of proliferating cells incorporating [^{3}H]thymidine by more than 2.5-fold, compared with cultures that were supplemented with serum only, and amplified the spontaneous death of dopaminergic neurons by about 50%, effects that were both prevented by cAMP (Fig. 8, A and B). This supported the notion that EGF in serum may have contributed to the death process of TH^+ neurons in control conditions. This was probably not the case, however, because tyrphostin AG-1478, at a concentration (3 μM) that specifically inhibits the EGF receptor tyrosine kinase (Santiskulvong et al., 2001), abolished both the mitogenic and toxic effects of exogenous EGF (Fig. 8) but provided no protection against serum, suggesting that dbcAMP prevented the effects of mitogens other than EGF in control culture conditions.

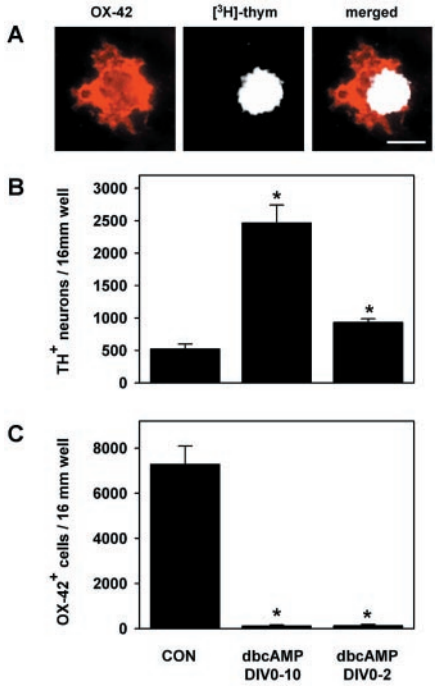

Fig. 4. Neuroprotection of dopaminergic neurons by cAMP is not correlated with the repression of microglial cell proliferation. A, microautoradiographic detection in a control culture of a thymidine-positive nucleus in a cell expressing the microglial marker OX-42. Quantification of TH^+ (B) and OX-42^+ (C) cells in DIV10 cultures undergoing extended treatment with dbcAMP for 10 days or transiently between DIV0 and DIV2. *, $p < 0.05$, versus untreated-cultures.

Cyclic AMP Does Not Prevent Neuronal Death by Inhibiting Activation of the MAPK/$ERK_{1/2}$ Signaling Pathway

It has long been recognized that cAMP can inhibit cell proliferation by blocking growth factor activation of the Ras/MAPK signaling pathway (reviewed in Stork and Schmitt, 2002). To determine whether the effects of cAMP in our cultures were also mediated by MAPK inhibition, we examined the state of activation (phosphorylation) of $ERK_{1/2}$. Serum mitogens strongly induced ERK_1 and, to a lesser extent, ERK_2 phosphorylation (Fig. 9A). However, rather than inhibiting ERK phosphorylation, treatment with dbcAMP increased it even further (Fig. 9A), indicating that the antimitotic effect of cAMP was probably not mediated by inhibition of MAPK-mediated signal transduction. Furthermore, treatment with PD98059 (20 μM), that prevents the activation of $ERK_{1/2}$ by its upstream kinase MEK, reduced the phosphorylation of $ERK_{1/2}$ to levels comparable with those observed in the absence of serum supplementation, but it neither inhibited cell proliferation nor prevented dopaminergic cell death (Fig. 9, B and C). As expected, however, PD98059 totally prevented the mitogenic and toxic effects of EGF (data not shown). Taken together, these results suggest that cAMP is not neuroprotective by interfering with serum factors that promote proliferation through an ERK-dependent signaling cascade.

Inhibition of CDK1 Activity Reproduces the Survival Promoting Effects of cAMP

Several purine derivatives structurally related to cAMP, in particular olomoucine and roscovitine, are antimitotic agents that directly inhibit CDK1, a cyclin-dependent kinase that triggers and coordinates the transition between the G_2/M phases of the cell division cycle (Vesely et al., 1994). Interestingly, both olomoucine and roscovitine at optimal concentrations of 100 and 25 μM, respectively, prevented the death of TH^+ neurons that occurs spontaneously in mesencephalic cultures (Fig. 10A). These compounds also mimicked the antiproliferative action of cAMP-elevating agents (Fig. 10B), suggesting that cAMP might act by reducing CDK1 activity in dividing cells. To explore this possibility, we measured the activity of the kinase in cell culture homogenates. As expected, concentrations of olomoucine or roscovitine that promoted TH^+ cell survival caused a profound reduction in CDK1 activity (Fig. 10C). The same was true of cAMP (Fig. 10C), whereas the anticancer drug ara-C (1 μM), which is

known to target the DNA polymerase (Harrington and Perrino, 1995), had no significant effect on CDK1 activity.

Discussion

We showed that cAMP promotes survival of mesencephalic dopaminergic neurons under conditions in which their death is caused by dividing glia. Cyclic AMP acted indirectly by arresting the proliferation of a population of immature astrocytes that are deleterious to dopaminergic neurons. Cyclic AMP probably stopped cell cycle progression through direct inhibition of CDK1.

Cyclic AMP Acts as a True Neuroprotective Agent for TH$^+$ Neurons. The cAMP-dependent increase in the number of TH$^+$ neurons in mixed glial-neuronal mesencephalic cultures could logically be explained by a neuroprotective effect of cAMP, but there are other possibilities. Cyclic AMP could operate as a mitogen (Stork and Schmitt, 2002) for TH$^+$ neuroblasts or their precursors. This is unlikely, however. TH$^+$ cells are already postmitotic at embryonic day 15.5, when the cultures are prepared (Rothman et al., 1980). Furthermore, cAMP reduced rather than increased the number of dividing mesencephalic cells in cultures. The cAMP-induced acquisition of a dopaminergic phenotype by neurons not initially committed to this lineage is also possible (Du and Iacovitti, 1997). However, there were always fewer TH$^+$ neurons in dbcAMP-treated cultures than TH$^+$ neuroblasts detectable immediately after plating. Furthermore, [^{3}H]thymidine was never incorporated into TH$^+$ neurons exposed to dbcAMP (not shown). Finally, it is also unlikely that cAMP simply maintained or restored the expression of TH (Lim et al., 2000) in cells in which the protein had been reduced to undetectable levels by suffering. When treatment with dbcAMP was delayed, the number of TH$^+$ neurons could not be restored to the number of neurons present immediately after plating (Michel and Agid, 1996). It may therefore be concluded that cAMP-elevating agents acted as true neuroprotective agents.

The Neuroprotective Effect of cAMP Results from Its Antiproliferative Effect on Dividing Glial Cells. We previously showed that the antimitotic ara-C was able to prevent the death of dopaminergic neurons in mesencephalic cultures. This effect was indirect and resulted from inhibition of the proliferation of glial cells (Michel et al., 1997). We therefore hypothesized that cAMP might act similarly. Our results support this view: 1) The number of proliferating mesencephalic cells was dramatically reduced by treatment with cAMP-elevating agents. 2) The trophic activity of cAMP was abolished when dividing cells were permanently eliminated from the cultures by transient treatment with ara-C. 3) When cAMP was withdrawn after prolonged treatment, dopaminergic neurons did not begin to die immediately, as one would expect in a classic paradigm of trophic factor deprivation (Deshmukh and Johnson, 1997), but after a time lag of several days, during which proliferation of non-neuronal cells progressively increased.

These results do not exclude, however, a direct effect of

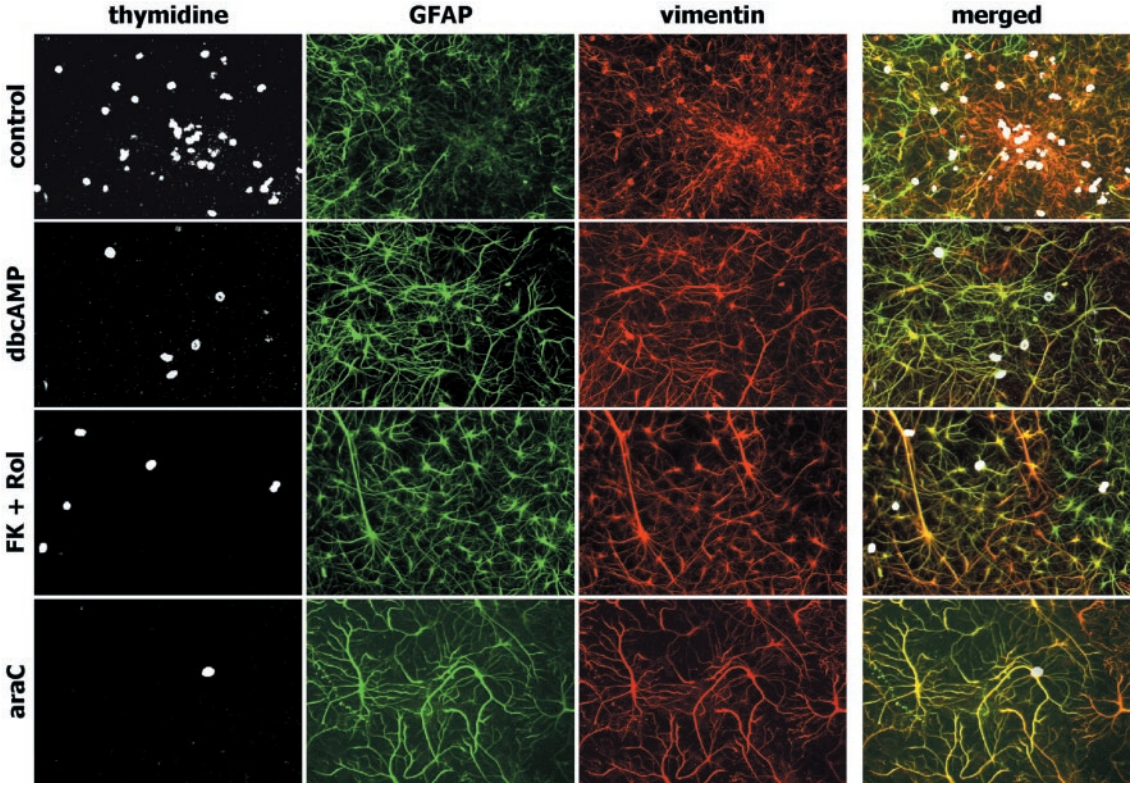

Fig. 5. Illustration of the effects of cAMP on proliferating astrocytes. Microautoradiographic detection of nuclei that incorporated [^{3}H]thymidine in control cultures or in cultures treated chronically for 6 days with dbcAMP (1 mM), FK (25 μM) combined with rolipram (100 μM), or ara-C (1 μM). Phenotypic identification of the thymidine-positive nuclei was performed by immunofluorescent detection of GFAP and vimentin, revealed by fluorescein isothiocyanate (green) and tetramethylrhodamine isothiocyanate (red), respectively. The pulse of tritiated thymidine was given at DIV6 for 2 h in the absence of any other treatment and in a serum-free culture medium. In control cultures, vimentin-positive cells with low GFAP staining seem orange in the merged image and are distributed as clusters.

cAMP on TH$^+$ neurons. In mixed mesencephalic cultures, the death of dopaminergic neurons can be prevented without reducing cell proliferation by increasing the levels of extracellular K^+ to depolarizing concentrations (Douhou et al., 2001). Furthermore, in astrocyte-poor mesencephalic cultures, dopaminergic neurons can be rescued by a cAMP-dependent mechanism that obviously does not necessitate the repression of cell division (Engele and Franke, 1996; Troadec et al., 2002). However, survival promotion by a direct effect of cAMP on dopaminergic neurons in mixed neuronal-glial mesencephalic cultures remains unlikely for several reasons. 1) In neuron-enriched cultures, cAMP is inactive by itself and requires the presence of cofactors such as catecholamines, antioxidants, and neurotrophic peptides (Engele and Franke, 1996; Troadec et al., 2002). 2) In these conditions, survival promotion by cAMP is observed only if treatment is initiated within the first 2 days in vitro then maintained (Troadec et al., 2002), whereas in the presence of glial cells, the cyclic nucleotide was neuroprotective up to the last stages of the culture (Michel and Agid, 1996). It is likely, therefore, that cAMP can prevent the death of dopaminergic neurons through two distinct modes of action, depending on the mechanisms by which dopaminergic neurons degenerate: oxidative damage in the absence of glial cells (Stull et al., 2002; Troadec et al., 2002), or activation of a cell death pathway by a proliferating glial cell in mixed mesencephalic cultures.

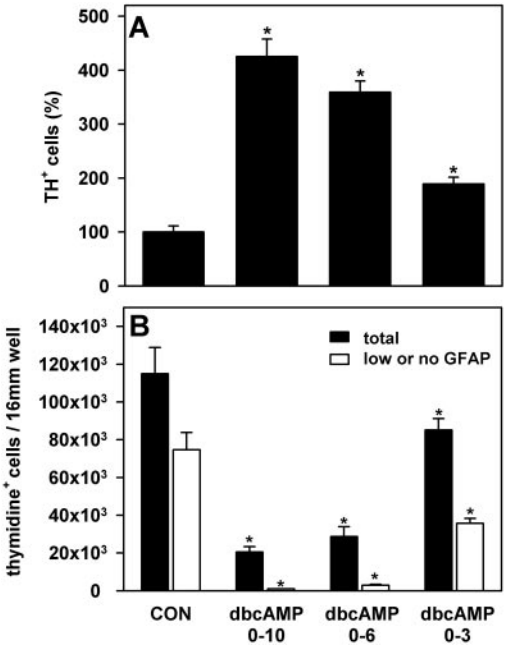

Fig. 6. Neuroprotection of dopaminergic neurons by cAMP is correlated with the repression of proliferating vimentin-positive cells expressing low levels of GFAP. A, dbcAMP (1 mM) was applied to mesencephalic cultures either persistently or transiently between DIV0–6 and DIV0–3. TH$^+$ neurons were counted, in all conditions, at DIV10. Note that TH$^+$ cell death did not resume immediately when the treatment was interrupted but after a time lag of several days. B, after dbcAMP withdrawal, the number of thymidine/vimentin-positive cells with no or low GFAP staining increased progressively up to DIV10. *, $p < 0.05$, versus corresponding control cultures.

Cyclic AMP-Elevating Agents Act Specifically on a Subpopulation of Immature Astrocytes. Because cAMP acted on proliferating glial cells, it was necessary to determine whether these cells were astrocytes or microglia. Microglial cells are potentially harmful to neurons (Streit et al., 1999; Hirsch et al., 2003), and their proliferation can be prevented by cAMP (Dalmau et al., 1996; Fujita et al., 1998). In our conditions, cAMP abolished the replication of OX-42 microglial cells. However, these cells were almost certainly not involved in death of dopaminergic neurons for two reasons: 1) they represented only a small fraction of the dividing cells and 2) although a transient treatment with dbcAMP (DIV0–2) was sufficient to permanently eliminate virtually

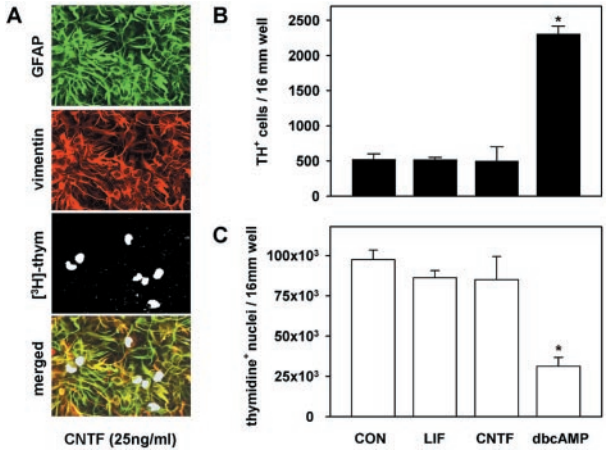

Fig. 7. Induction of GFAP expression with LIF or CNTF does not reproduce the neuroprotective action of cAMP on dopaminergic neurons. A, illustration of CNTF (25 ng/ml)-induced expression of GFAP in proliferating glial cells. The number of dopaminergic neurons (B) and thymidine-positive nuclei (C) was estimated at DIV10 and DIV6, respectively, in mesencephalic cultures treated with CNTF or LIF (both at 25 ng/ml) and dbcAMP (1 mM). Note that LIF and CNTF, which did not rescue TH$^+$ neurons from death, were also unable to reduce cell proliferation. *, $p < 0.05$, versus corresponding control cultures.

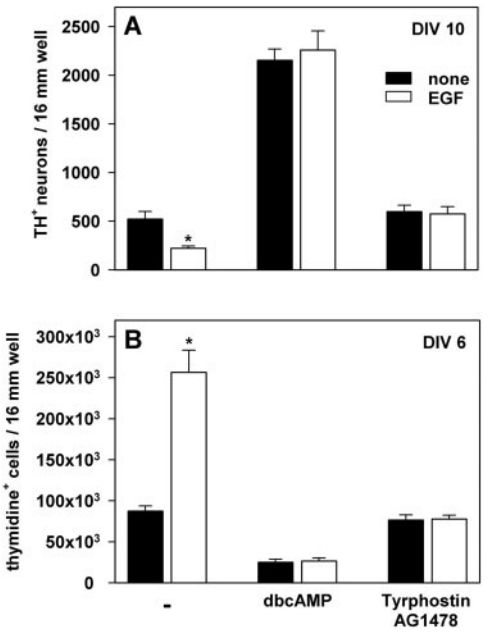

Fig. 8. Cyclic AMP does not protect TH$^+$ neurons by inhibiting EGF-induced proliferation. A, assessment of TH$^+$ cells in serum-supplemented mesencephalic cultures in the presence or absence of EGF (25 ng/ml). Some sets of cultures were also exposed concomitantly for 10 days to dbcAMP (1 mM) or tyrphostin AG1478 (3 μM). B, effects of the same treatments on the number of thymidine-positive nuclei at DIV6. *, $p < 0.05$, versus corresponding cultures without EGF.

all OX-42$^+$ cells, it led to only a marginal increase in the number of dopaminergic cells.

The vast majority of dividing cells (>95%) were astrocytes expressing vimentin and low to high levels of GFAP. Cyclic AMP was mostly effective in repressing the subpopulation that expressed low levels of GFAP. Interestingly, the time course of dopaminergic neuronal death was strictly correlated with the rate of proliferation of this cell population. This suggests that these cells express a phenotypic trait that is deleterious to dopaminergic neurons, or that cells expressing high levels of GFAP permit or enhance the survival of these neurons. The former possibility is more likely, because the induction of GFAP expression in still dividing cells by treatment with LIF or CNTF (Galli et al., 2000) did not result in a concomitant suppression of cell death. This undoubtedly occurred because neither CNTF nor LIF had significant effects on cell division, again supporting the notion that the antiproliferative activity of cAMP-elevating agents was instrumental to rescue dopaminergic neurons.

Cyclic AMP Is Not Protective by Neutralizing an EGF-Dependent Mechanism. EGF is a prototypical mitogen for astrocytes (Leutz and Schachner, 1981). Therefore, we hypothesized that cAMP-elevating agents might protect TH$^+$ neurons by counteracting the effect of this cytokine. Consistent with this hypothesis, EGF amplified the death of dopaminergic neurons and increased the number of proliferating cells in comparison with cultures that were supplemented with serum only. dbcAMP abolished neuronal death produced by a combined treatment with EGF and serum and prevented both EGF- and serum-induced cell proliferation, again supporting the notion that EGF was involved in the spontaneous death process caused by serum proteins. However, inhibition of the EGF receptor tyrosine kinase by tyrphostin AG-1478 (Santiskulvong et al., 2001) prevented both the mitogenic and toxic effects of EGF but provided no protection in the presence of serum alone, suggesting that dbcAMP prevented the effects of mitogens other than EGF in control culture conditions.

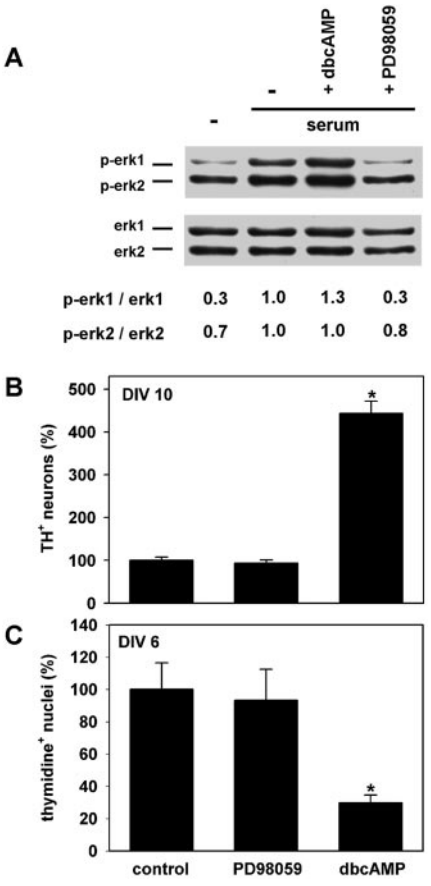

Fig. 9. Cyclic AMP does not prevent neuronal death by inhibiting the MAPK/ERK$_{1/2}$ signaling pathway. A, Western blotting analysis of pERK$_{1/2}$ expression in DIV6 mesencephalic cultures initially deprived of serum proteins for 18 h then re-exposed to serum in the presence of dbcAMP (1 mM) or PD 98059 (25 μM) for 60 min. Protein loading was controlled by additionally staining blots with a total ERK$_{1/2}$ antibody recognizing ERK regardless of its state of phosphorylation. Results are expressed as the ratios of pERK to ERK. Note that the serum-dependent increase in ERK$_{1/2}$ phosphorylation was inhibited by PD98059 but not by dbcAMP. Number of TH$^+$ neurons (B) and thymidine-positive nuclei (C) in cultures exposed persistently to 1 mM dbcAMP or 25 μM PD98059 for 10 days. Unlike dbcAMP, PD98059 failed to inhibit cell proliferation and did not prevent TH$^+$ cell death. *, $p < 0.05$, versus corresponding control cultures.

Inhibition of Cell Proliferation by cAMP Does Not Result from Inhibition of the MAPK/ERK$_{1/2}$ Signaling Pathway.

The MAPK/ERK$_{1/2}$ signaling pathway is thought to transduce the effects of a wide range of mitogens (Stork and Schmitt, 2002). Several studies suggest that inhibition of

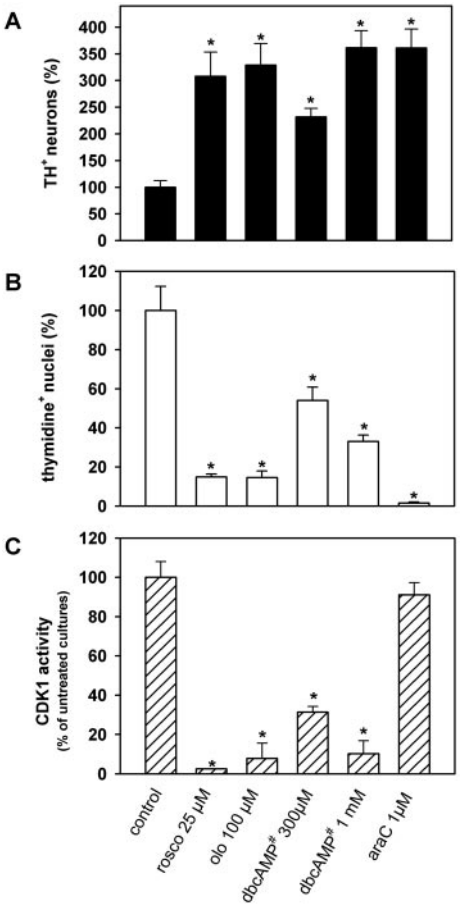

Fig. 10. Inhibition of CDK1 activity mimics the prosurvival effects of cAMP-elevating agents. The numbers of TH$^+$ cells (A) and thymidine nuclei (B) were determined in mesencephalic cultures exposed persistently for 10 days to roscovitine (rosco; 25 μM), olomoucine (olo; 100 μM), dbcAMP (300 μM or 1 mM) or ara-C (1 μM). C, activity of CDK1 in homogenates of mesencephalic cultures exposed for 10 min to the same treatments as above. #, for the enzymatic assay, native cAMP was used instead of dbcAMP. *, $p < 0.05$, versus corresponding control cultures.

ERKs by cAMP could account for the antimitotic activity of the cyclic nucleotide (Bayatti and Engele, 2001; Stork and Schmitt, 2002). We therefore examined the expression of the phosphorylated (active) forms of $ERK_{1/2}$ in mesencephalic cultures. As expected, serum mitogens produced a robust activation of $ERK_{1/2}$. cAMP, however, instead of inhibiting these kinases, activated them even more, indicating that the cyclic nucleotide did not act by inhibiting the MAPK cascade. In experimental conditions in which ERK activation was not required for the mitotic process (McKenzie and Pouyssegur, 1996), cAMP can halt proliferation without interfering with ERK-dependent signaling. This is probably the case in our cultures, because inhibition of MEK, the upstream kinase of $ERK_{1/2}$, by PD98059 failed to halt cell proliferation and consequently dopaminergic cell death.

Cyclic AMP Acts by Direct Inhibition of Cell Cycle Progression

Olomoucine and roscovitine, two purine analogs of cAMP (Vesely et al., 1994), prevented the proliferation of immature astrocytes and, as a result, mimicked the prosurvival effects of cAMP. Both compounds, at the concentrations used, are reported to specifically inhibit CDK1, the main mitotic kinase that drives the G_2/M transition in the cell cycle (Knockaert et al., 2002). In homogenates from mesencephalic cultures, the degree of inhibition of this kinase by cAMP was directly correlated with the capacity of the cyclic nucleotide to prevent the death of dopaminergic neurons. This means that CDK1 was the likely target of cAMP. However, an effect on related kinases, such as CDK2 and CDK5, the activity of which is also reduced by olomoucine and roscovitine (Malumbres and Barbacid, 2001), cannot be excluded. The inhibition of CDK5, however, is an unlikely explanation of the antimitotic effect of cAMP, because this is a non–cell-cycle-related cyclin-dependent kinase (Knockaert et al., 2002).

In summary, we have shown that cAMP-elevating agents were neuroprotective for dopaminergic neurons in culture by reducing the proliferation of a subpopulation of immature and potentially deleterious astrocytes. This model may be relevant to pathologies such as Parkinson's disease or striatonigral degeneration, in which astrocytes are suspected to play a role in the loss of dopaminergic neurons (Wenning et al., 2002; Hirsch et al., 2003).

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