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Activation of the Mitogen-Activated Protein Kinase ($ERK_{1/2}$) Signaling Pathway by Cyclic AMP Potentiates the Neuroprotective Effect of the Neurotransmitter Noradrenaline on Dopaminergic Neurons

JEAN-DENIS TROADEC, MARC MARIEN, SOPHIE MOURLEVAT, THOMAS DEBEIR, MERLE RUBERG, FRANCIS COLPAERT, and PATRICK P. MICHEL

Institut National de la Santé et de la Recherche Médicale U289, Experimental Neurology and Therapeutics, Hôpital de la Salpêtrière, Paris (J.-D.T., S.M., T.D., M.R., P.P.M.); Centre de Recherche Pierre Fabre, Castres, France (M.M., F.C., P.P.M.)

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ABSTRACT

We have shown previously that low concentrations of noradrenaline (NA) confer long-term but partial protection to tyrosine hydroxylase (TH^+) dopaminergic neurons by reducing spontaneously occurring oxidative stress. We demonstrate here that the effect of NA is strongly enhanced by cAMP-elevating agents, in particular forskolin (FK), through a mechanism that does not involve activation of adrenoceptors. FK also enhanced the neuroprotective action of antioxidants that mimic the trophic effects of NA, such as trolox and pyrocatechol, but was totally ineffective by itself, suggesting that inhibition of oxidative stress was a required step to reveal the cAMP-dependent mechanism. Neuroprotection afforded by FK was rapidly reversible, optimal when the treatment was initiated in the early phase of the culture and exquisitely specific to dopaminergic neurons. FK stimulated the phosphorylation of extracellular signal-activated kinases $(ERK)_{1/2}$ in a subpopulation of dopaminergic neurons, suggesting that the mitogen-activated protein kinase (MAPK) pathway was involved in the effects of cAMP-elevating agents. Accordingly, inhibition of the upstream kinases of $ERK_{1/2}$ by 2′-amino-3′-methoxyflavone (PD98059) not only suppressed MAPK activation caused by FK but also abolished the survival promoting activity that this compound exerts on TH^+ neurons. PD98059 did not reduce, however, the trophic effects provided by NA alone. Surprisingly, the archetypal cAMP-dependent protein kinase was apparently not responsible for $ERK_{1/2}$ activation. The data suggest that the MAPK signaling pathway plays a key role in the trophic effects that cAMP elevating agents and NA cooperatively exert on TH^+ neurons.

Noradrenaline (NA) is a neurotransmitter produced in the central nervous system by neurons localized essentially in the brainstem, particularly in the locus ceruleus (LC) (Foote et al., 1983). Through the activation of adrenoceptors and corresponding second messenger systems (Molinoff, 1984), NA participates in a variety of motor and mental functions, such as locomotor control, cognition, motivation, and attention (Foote et al., 1983). In addition to these classic transmitter signaling functions, NA is thought to play a key role in neuronal survival, differentiation and plasticity, and to participate in brain repair mechanisms. For instance, compounds that mimic or increase LC output, including NA itself, its precursor L-*threo*-3,4-dihydroxyphenylserine, and amphetamine, improve behavioral recovery (Feeney, 1998), stimulate neural sprouting and synaptogenesis (Lee et al., 1994; Stroemer et al., 1998) and limit neuronal death in experimental models of cerebral ischemia (Lee et al., 1994). Another class of molecules that elevates extracellular levels of NA (i.e., α_2 adrenoceptor antagonists) is effective in protecting brain cholinergic neurons against cortical devascularization (Debeir et al., 2001) and excitotoxin-induced lesioning (Martel et al., 1998). These compounds also reduce ischemia-induced neuronal damage (Gustafson et al., 1989) and improve functional recovery after brain injury and stroke (Goldstein, 2000; Puurunen et al., 2001). In line with these observations, lesions of the LC-NA system aggravate post-ischemic damage in the rat brain (Goldstein, 2000) and

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ABBREVIATIONS: NA, noradrenaline; LC, locus ceruleus; MAP, mitogen-activated protein; TH, tyrosine hydroxylase; MAP-2, microtubule associated protein-2; CREB, cyclic AMP response element binding protein; ERK, extracellular signal-regulated kinases; pCREB, phospho-cAMP binding protein; PBS, phosphate-buffered saline; DA, dopamine; 5-HT, 5-hydroxytryptamine; DIV, day(s) in vitro; TRITC, tetramethyl-rhodamine isothiocyanate; ROS, reactive oxygen species; PD98059, 2′-amino-3′-methoxyflavone; MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase.

worsen neurological deficits in animal models of Parkinson's disease (Marien et al., 1993; Gesi et al., 2000). Furthermore, drugs that decrease norepinephrine release or block its postsynaptic effects at α_1 adrenoceptors aggravate or even reinstate neurological deficits, and impede recovery after stroke in animals and man (Goldstein, 2000). Taken together, these data support the hypothesis that stimulation of the LC-NA system would be beneficial in the treatment of acute and chronic neurodegenerative diseases and their progression (Colpaert, 1994).

To better understand the molecular mechanisms underlying the neuroprotective effects of NA, we have developed a model of mesencephalic cells in culture in which low concentrations of NA confer long-term protection to dopaminergic neurons by a mechanism that does not seem to require adrenoceptor activation (Troadec et al., 2001). The neuroprotective action of NA that was substantial and long-lived, however, remained partial (Troadec et al., 2001). This led us to examine whether other trophic signals might improve the survival-promoting effects of NA on dopaminergic neurons.

Cyclic AMP is an archetypal second messenger that functions as an intracellular mediator of hormone and neurotransmitter actions. More recent studies have revealed that the cyclic nucleotide can also act as a trophic signal for various populations of neuronal cells in the PNS and central nervous system. In particular, cAMP was described as a prosurvival molecule for several populations of catecholaminergic neurons, such as sympathetic ganglionic cells and noradrenergic neurons in the brainstem (for review, see Goldberg and Barres, 2000). Other studies have revealed that elevated cyclic AMP levels can also promote the development, maturation and survival of midbrain dopaminergic neurons (Mena et al., 1995; Michel and Agid, 1996) and can protect them from toxic insults (Hulley et al., 1995). Even if in some experimental paradigms (Goldberg and Barres, 2000; Michel and Agid, 1996) the elevation of intracellular levels of cAMP is thought to be sufficient per se to promote the survival of catecholaminergic and other neurons, a number of studies have reported on the ability of the cyclic nucleotide to cooperate with additional trophic peptides (Meyer-Franke et al., 1995). In particular, activation of the cAMP signaling pathway increased the survival-promoting effects of glial cell line-derived neurotrophic factor, a prototypical trophic peptide for dopaminergic neurons (Engele and Franke, 1996).

The aim of the present study, therefore, was to determine whether cAMP-elevating agents can improve the neuroprotective effects of the neurotransmitter NA on dopaminergic neurons and to characterize the underlying molecular mechanisms. The results demonstrate that cyclic AMP elevation powerfully potentiated the neuroprotective action of NA. The effect of cAMP but not that of NA seemed to require the activation of the Ras-MAP-kinase signaling pathway.

Materials and Methods

Mesencephalic Cell Cultures. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 1996), the European Directive N886/609, and the guidelines of the local institutional committee for animal care and use. Cultures of postmitotic dopaminergic neurons were prepared from the ventral mesencephalon of Wistar rat embryos dissected at embryonic day 15.5, as described previously (Michel and Agid, 1996; Troadec et al., 2001). Mesencephalic cells in suspension were plated onto polyethylenimine (1 mg/ml; Sigma/RBI-Aldrich, Saint Quentin Fallavier, France) precoated culture plates (24 wells) and maintained in 500 μl of chemically defined serum-free medium consisting of equal volumes of minimal essential medium containing Earle's salts and glutamine and Ham's F12 nutrient mixture (Invitrogen, Cergy Pontoise, France). The culture medium was supplemented with 10 μg/ml insulin, 30 mM glucose, and 100 U/ml penicillin and streptomycin. To favor cell attachment, 10% fetal calf serum was also added for the first hour immediately after plating. Cells were fed daily by replacing 350 μl of the culture medium twice. Pharmacological reagents were generally obtained from Sigma-RBI-Aldrich (Saint Quentin Fallavier, France). Dihydrorhodamine 123, the cell permeant probe used for the detection of reactive oxygen species, was purchased from Molecular Probes (Interchim, Montluçon, France). All tritiated compounds were obtained from Amersham Biosciences (Orsay, France). Stock solutions of FK were prepared in dimethyl sulfoxide whereas trolox was dissolved in ethanol. The highest final concentrations of dimethyl sulfoxide or ethanol (0.5 and 0.0025%, respectively) added to the cultures had no impact on neuronal survival.

Quantification of Neuronal Survival. Tyrosine hydroxylase (TH^+) immunocytochemistry was used to quantify the survival of dopaminergic neurons as described previously (Troadec et al., 2001). Briefly, after fixation with a mixture of glutaraldehyde/formaldehyde (0.5%:4%) in PBS for 12 min, the cells were incubated overnight at 4°C with a mouse anti-TH monoclonal antibody (MAB-5280; Chemicon, Temecula, CA) diluted 1:250 in PBS containing 0.2% Triton X-100. Subsequent incubations were performed, at room temperature, with a secondary biotinylated anti-mouse IgG (1:500 in PBS; Jackson Immunoresearch, West Grove, PA) followed by amplification with an avidin-biotinylated horseradish peroxidase preformed complex (Vectastain; Vector, Burlingame, CA). The peroxidase was revealed by incubation with a solution of diaminobenzidine (1 mg/ml) containing 0.006% hydrogen peroxide (H_2O_2). Microtubule-associated protein-2 (MAP-2) immunocytochemistry was used to assess the survival of the entire population of mesencephalic neurons regardless of the neurotransmitter phenotype, as described previously (Douhou et al., 2001).

Immunodetection of Phospho-CREB and Phospho-ERK$_{1/2}$. We investigated a possible implication of phospho-cAMP response element binding protein (pCREB) and phospho-extracellular regulated kinases $_{1/2}$ (pERK$_{1/2}$) in the survival promoting action of FK using mesencephalic cultures at DIV-1 (i.e., at a time when neuronal death has started but is still limited). After termination of the test treatments by a single wash with cold PBS, the cultures were fixed in 4% formaldehyde for 15 min, permeabilized for 1 h with 0.2% Triton X-100, exposed for another hour to a blocking buffer containing 10% horse serum, and finally for 30 min to 0.6% hydrogen peroxide in PBS to inactivate the endogenous peroxidases. Afterward, the cells were incubated for two days, at 4°C, with a rabbit anti-pCREB or a mouse anti-pERK$_{1/2}$ monoclonal antibody (each at 1:300 in PBS; New England Biolabs, Beverly, MA) and then with corresponding biotinylated immunoglobulins (1:500 in PBS, Jackson Immunoresearch). The staining was revealed as described above using diaminobenzidine as the chromogen. The expression of pCREB or pERK$_{1/2}$ was assessed more specifically in dopaminergic neurons by combining the detection of the phosphoproteins to that of TH. Concurrent visualization of pERK$_{1/2}$ and TH required using a rabbit anti-TH antibody (AB#151; Chemicon, Temecula, CA), which was detected with fluorescein isothiocyanate-conjugated immunoglobulins (DAKO, Glostrup, Denmark).

Neurotransmitter Uptake Studies. The functional integrity of dopaminergic neurons was evaluated by their ability to take up dopamine (DA) by active transport (Douhou et al., 2001). After preincubation for 10 min in 500 μl of PBS containing 5 mM glucose and 100 μM ascorbic acid, the uptake was initiated by addition of 50 nM [^{3}H]DA (40 Ci/mmol) to the cultures and terminated after 15 min by removal of the incubation solution followed by two rapid washes with

cold PBS. Cells were scraped off the culture wells and counted by liquid scintillation spectrometry. The uptake of [^{3}H]DA was also visualized by microautoradiography. In that case, the incubation time with [^{3}H]DA was prolonged to 30 min and the concentration of the neurotransmitter raised to 100 nM to improve the sensitivity of detection. The accumulation was terminated by two extensive washes with cold PBS. The cultures were fixed with a mixture of glutaraldehyde/formaldehyde (0.5%:4%) and then dehydrated with ethanol. Incorporation of [^{3}H]DA was detected with the Hypercoat LM-1 emulsion (Amersham Biosciences) after an exposure of 7 to 10 days in the dark at 4°C. In both paradigms, blank values were obtained in the presence of 5 μM GBR-12,909 (Sigma-Aldrich, St. Louis, MO). GABA uptake was measured, as described previously (Michel and Agid, 1996), at 37°C with 50 nM [^{3}H]GABA (85 Ci/mmol) using an incubation time of 5 min. Blank values were obtained at 4°C. Serotonin (5-HT) uptake was estimated after treatment of the cultures with 20 nM [^{3}H]5-HT (120 Ci/mmol) for 15 min. Blank values were determined in the presence of 3 μM citalopram (Lundbeck and Co., Copenhagen, Denmark).

Uptake of [*methyl*-^{3}H]Thymidine. [*methyl*-^{3}H]thymidine, a marker of DNA synthesis was used to label proliferating cells. Mesencephalic cultures maintained in regular serum-free medium were exposed for the first 24 h in vitro to [*methyl*-^{3}H]thymidine (40 Ci/mmol; 0.5 μCi per 16-mm culture well) in the presence of the test compounds, then allowed to recover until DIV 5 in the same conditions of treatment but in the absence of the tritiated nucleoside. After two rapid washes with PBS, the cultures were fixed with a mixture of glutaraldehyde/formaldehyde (0.5%:4%) in PBS for 20 min. The staining of TH and MAP-2 positive cells was performed as described above. The characterization of vimentin-positive cells was carried out with a monoclonal antibody (clone V9) from DAKO Corporation (Carpinteria, CA) followed by immunofluorescence detection with tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated anti-mouse immunoglobulins (DAKO). Thymidine positive nuclei were then visualized using the Hypercoat LM-1 emulsion (Amersham Biosciences) after an incubation of 4 days at 4°C.

Quantification of Reactive Oxygen Species. The production of intracellular reactive oxygen species (ROS) was quantified in DIV 3 mesencephalic cultures (i.e., at a stage when the cell death process is substantial). ROS measurement was performed using dihydrorhodamine 123 (Molecular Probes, Eugene, OR; Troadec et al., 2001). Briefly, cultured neurons were exposed for 30 min to 50 μM DHR-123 and then washed twice to eliminate the unincorporated fluorescent label. ROS measurements were performed in serum-free medium supplemented with the test treatments. The fluorescent signal, visualized by epifluorescence microscopy (excitation, 488 nm; emission, 515 nm), was quantified by computer-assisted image analysis (Fluostar software; Imstar, Paris, France).

Western Immunoblotting of ERK $_{1/2}$. After exposure of the cultures with the test treatment for various incubation times, the cells were recovered in a lysis buffer containing 20 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, pH 8, 1% Triton, 10% glycerol, 2% complete miniprotease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany), 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 50 mM sodium fluoride. Samples were electrophoresed through a 10% acrylamide gel and blotted onto a nitrocellulose membrane. The membranes were incubated with a pERK$_{1/2}$ antibody (1:300; New England Biolabs) and developed with the enhanced chemiluminescence detection kit (Pierce; Rockford, IL). Membranes were stripped using a mixture containing 62.5 mM Tris-HCl, pH 7.4, 2% SDS, 100 mM β-mercaptoethanol, and 0.1% sodium azide, incubated with an anti-ERK$_{1/2}$ antibody (1:1000, New England Biolabs) and then developed as described above.

Statistical Analysis. Comparisons between two groups were performed with Student's unpaired *t* test. Multiple comparisons against a single reference group were made by one-way analysis of variance followed by Dunnett's test. S.E.M. values were derived from at least three independent experiments.

Results

Forskolin Enhances the Trophic Action of NA on Dopaminergic Neurons. Mesencephalic TH$^+$ neurons degenerate progressively when maintained in a serum-free culture medium minimally supplemented with insulin and glucose (Troadec et al., 2001). Consistent with our initial observation (Troadec et al., 2001), a concentration of 1 μM NA afforded long-lasting protective effects on TH$^+$ cells (Fig. 1A). These rescuing effects were partial, however, because approximately 60 and 40% of TH$^+$ neurons remained alive after 6 and 10 days, respectively (Fig. 1A). Interestingly, the effects of the neurotransmitter were improved substantially by concomitant exposure to the diterpene derivative FK, a potent activator of the adenylate cyclase (Figs. 1 and 2C). The additional protection provided by FK was concentration-dependent and optimal at 25 μM (Fig. 1B). Surprisingly, FK afforded protection when combined with concentrations of NA that promote neuronal survival (0.3–10 μM) but was totally inactive per se (Fig. 1C). Consequently, the EC$_{50}$ of

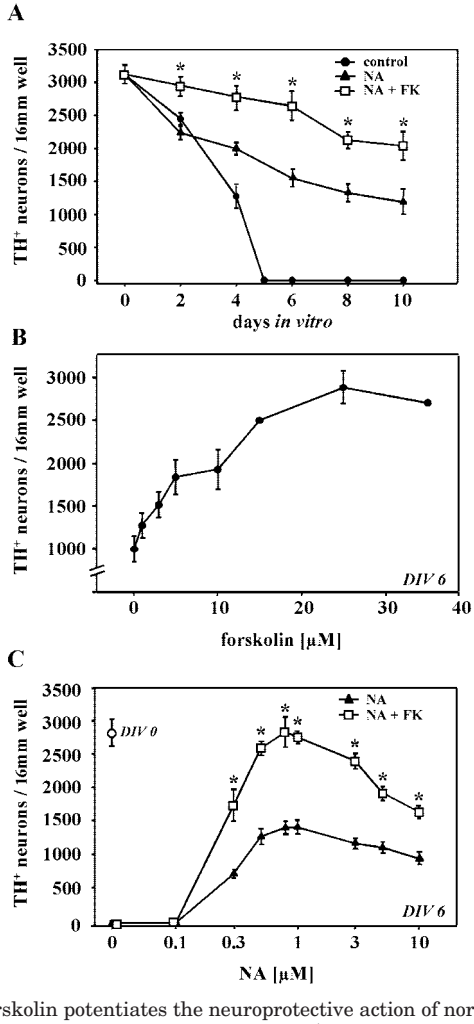

Fig. 1. Forskolin potentiates the neuroprotective action of noradrenaline on dopaminergic neurons. A, number of TH$^+$ neurons in mesencephalic cultures treated with 1 μM NA in the presence or absence of FK as a function of time (DIV 0 to DIV 10). Note that a cotreatment with NA and FK rescued more than 70% of TH$^+$ cells at DIV 10. B, neuroprotective effect of increasing concentrations of FK (1–35 μM) on TH$^+$ cells in DIV 6 cultures exposed to NA (1 μM). C, concentration-dependent effects of NA (0.1–10 μM) on TH$^+$ cell survival in the presence or the absence of FK (25 μM). $\star$, $p < 0.05$, versus corresponding NA-treated cultures.

NA in the presence of 25 μM forskolin was similar to that observed in the presence of NA alone (~0.4 μM) (Fig. 1C). At DIV 6, treatment with optimal concentrations of both FK and NA (25 μM and 1 μM, respectively) promoted the survival of virtually all the dopaminergic neurons initially plated in the cultures. The combined effect of NA and FK was still highly significant 10 days after plating because approximately 70% of the TH^+ neurons remained viable under these conditions of treatment (Fig. 1A). It is noteworthy that the trophic action of FK on NA-treated TH^+ neurons was optimal if the treatment was initiated early after plating. Delayed application of FK resulted in a progressive reduction of its trophic activity; treatment of the cultures with FK after DIV 3 failed to improve the survival of NA-treated TH^+ neurons (Fig. 3A). Conversely, the trophic effect obtained with optimal concentrations of NA and FK applied chronically to mesencephalic neurons was rapidly reversible when the adenylate cyclase activator was removed prematurely from the culture medium (Fig. 3B). For instance, FK produced no net increase in the survival of TH^+ cell if the cultures were cotreated with NA and FK up to DIV 3 and then with NA alone for the last three days of culture (Fig. 3B).

TH^+ Neurons Rescued by Cotreatment with NA and Forskolin Accumulate Dopamine Efficiently. We have shown previously that TH^+ neurons protected by treatment with NA alone were capable of accumulating exogenous DA, suggesting that those rescued neurons were functionally active and well differentiated (Troadec et al., 2001). In the presence of NA, FK increased the number of TH^+ neurons and the number of cells accumulating [^{3}H]DA approximately to the same extent (Figs. 2 and 4), indicating that the population of TH^+ neurons saved by treatment with the adenylate cyclase activator expressed the high-affinity transport system for DA as well. The treatment of NA-treated cultures with FK, however, did not modify the estimated rate of [^{3}H]DA uptake per neuron (Fig. 4).

Specificity of The Effects of Cotreatment with Forskolin and Noradrenaline. We have shown previously that NA was equally protective for dopaminergic and nondopaminergic neurons in mesencephalic cultures (Troadec et al., 2001). To determine whether FK also had an effect on nondopaminergic neurons, we labeled the entire population of mesencephalic neurons using MAP-2 immunostaining. The number of MAP-2^+ neuronal cells was roughly similar in NA and NA+FK-treated DIV 6 cultures (Fig. 5A), 91,200 ± 9,600 and 92,600 ± 10,700 per 16-mm culture well, respectively. Consistent with this result, FK failed to increase the uptakes of tritiated GABA or 5-HT, functional markers of GABAergic and serotoninergic neurons, respectively (Fig. 5B), suggesting that the action of FK was exquisitely specific for dopaminergic neurons in this model.

Does Forskolin Act as a Mitogen for TH^+ Neuroblasts or Their Precursor Cells? We next studied the possibility that the increase in TH^+ cell numbers could result, at least in part, from a mitogenic action of NA and/or FK on TH^+ neuroblasts or their precursor cells. Mesencephalic cultures were exposed to the test treatments in the presence of [*methyl*-^{3}H]thymidine, a marker of DNA synthesis used to label proliferating cells. Some mesencephalic cells (~5% of the total) were found to accumulate the tritiated label in their nucleus in the presence of NA alone. The number of positive nuclei, however, was not significantly increased by the presence of FK (Fig. 6A). Moreover, in cultures treated with NA or NA+FK, positive nuclei were never found in association with TH or MAP-2 immunopositive neurons (Fig. 6, B, C, and D). A significant proportion of the cells exhibiting radiolabeling expressed vimentin and had the morphological appearance of astrocytes (Fig. 6E). Together, these findings suggest that the FK-dependent increase in TH^+ cell numbers was not caused by a proliferation of TH^+ neuroblasts or their precursor cells.

The Potentiation of the Effect of NA by Forskolin Occurs by a cAMP-Dependent Mechanism That Does Not Require β Adrenoceptor Activation. Consistent with the idea that a cyclic AMP-dependent mechanism was involved in the trophic effect produced by FK, dibutyryl cAMP, a membrane permeant analog of the cyclic nucleotide, mimicked entirely the survival-promoting action of FK, when used at an optimal concentration of 1 mM (Fig. 7). Application of exogenous cyclic AMP, which is not membrane-permeant, was inactive. The survival-promoting effects elicited by a cotreatment with FK and NA could possibly involve β adrenoceptors, which are coupled positively to the adenylate cyclase and therefore to cAMP-dependent signaling (Molinoff, 1984). This was not the case, however, because the β adrenoceptor blocker propanolol (1 μM) failed to reduce TH^+ cell survival in cultures exposed concomitantly to NA and FK (Fig. 7). Blockade of α adrenoceptors by phentolamine (1 μM) was similarly ineffective (not shown).

Forskolin Does Not Potentiate the Antioxidant Activity of NA. We have shown previously that NA was neuroprotective in this model by reducing oxidative stress (Troa-

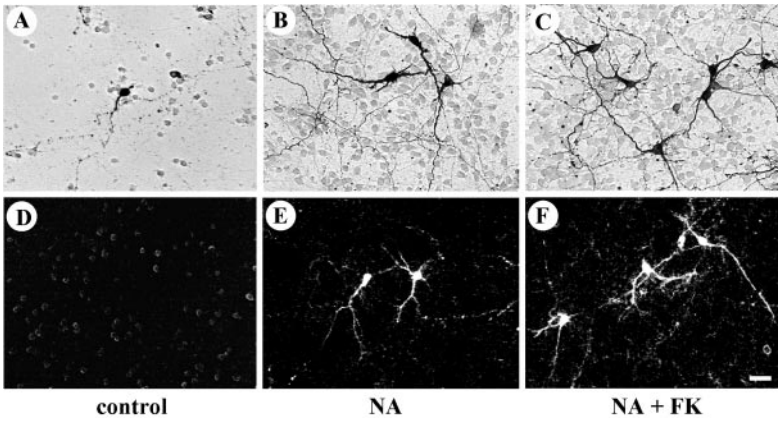

Fig. 2. Synergistic effects provided by NA and forskolin. Dopaminergic neurons visualized by immunostaining of TH (A-C) or microautoradiographic detection of [^{3}H]dopamine uptake (D-F) in DIV 6 cultures treated from DIV 0 with NA, NA+ FK, or no additive. NA, 1 μM; FK, 25 μM. Scale bar, 25 μm.

dec et al., 2001). Consistent with this finding, antioxidants such as trolox and pyrocatechol (Ancerewicz et al., 1998) mimicked the neuroprotective action afforded by NA (Troadec et al., 2001). Because FK improved the effects of NA, trolox or pyrocatechol with a comparable efficacy (Fig. 8A), we hypothesized that the diterpene derivative could also act by preventing oxidative stress. To address this point, we used dihydrorhodamine-123 as an indicator of ROS production. As expected, separate treatments with NA, trolox and pyrocatechol, greatly reduced the emission of intracellular reactive oxygen species (Fig. 8B), especially during the period that immediately precedes neuronal death. When NA-, trolox-, or pyrocatechol-treated cultures were concomitantly exposed to FK, no further decreases in ROS production were observed (Fig. 8, B and C). In addition, FK alone failed to significantly diminish the fluorescent signal (Fig. 8, B and C), ruling out the possibility that the diterpene derivative acted as an antioxidant in our system.

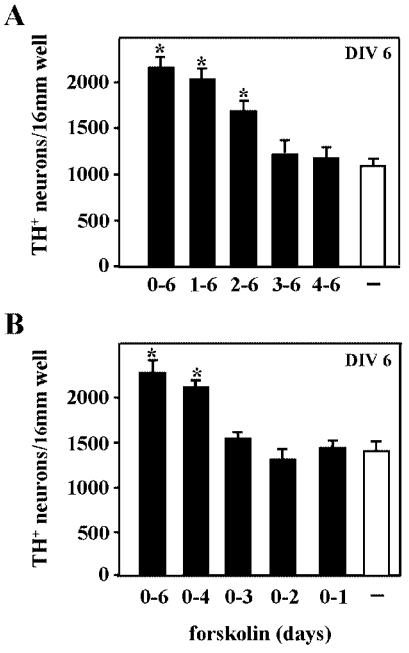

Fig. 3. Effects of delayed or transient treatments with forskolin on NA-treated cultures. A, the protective effects of FK on NA-treated dopaminergic cells decreased progressively when the application of the diterpene derivative was delayed after plating. B, the protective effects of FK remained detectable only for a short period of time after its withdrawal from NA-treated cultures. FK, 25 μM; NA, 1 μM in all conditions. ⋆, $p < 0.05$, versus control cultures maintained with NA for the entire 6-day culture period (open bars).

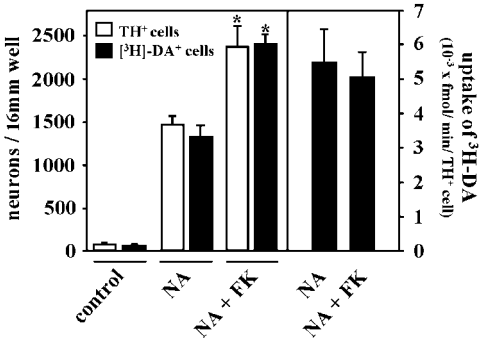

Fig. 4. Dopaminergic neurons treated with NA and forskolin accumulate exogenous dopamine efficiently. Left, the treatment of NA-treated cultures by FK increased to the same extent the number of neurons that express the TH enzyme (□) and those that incorporate [^{3}H]DA (■). Right, the uptake of [^{3}H]DA per TH$^+$ neuron remained unchanged after the exposure to FK. NA, 1 μM; FK, 25 μM. ⋆, $p < 0.05$, versus cultures maintained with NA

The PKA/CREB Signaling Pathway Is Not Involved in the Trophic Effect of Forskolin. Given the probable implication of cAMP in the survival-promoting effects of FK on dopaminergic neurons, we examined subsequently a possible mediating role for CREB, a transcription factor activated by phosphorylation at serine residue 133 in response to the cyclic nucleotide (Mayr and Montminy, 2001). Cultures were immunolabeled with an antiserum that specifically recognizes phosphorylated Ser-133 CREB (*p*-CREB). After a 1-h treatment with NA (1 μM) and FK (25 μM), 89% of TH$^+$ neurons and 94% of the entire population of mesencephalic cells exhibited a positive staining in their nuclei (Fig. 9A). Positively stained nuclei were absent from cultures treated with NA only, attesting to the specific activation of CREB by FK. The archetypal mediator of cAMP-induced CREB phosphorylation is the cyclic AMP-dependent protein kinase (PKA) (Mayr and Montminy, 2001). Accordingly, inhibition of PKA by H-89 (1 μM) prevented CREB activation induced by FK in our system. However, H-89 failed to prevent the trophic effects of FK on TH$^+$ cell survival even when used at a concentration of 5 μM (Fig. 9B). This suggests that the PKA/CREB signaling pathway was not involved in this effect.

The Trophic Effect of Forskolin Results from the Activation of ERK$_{1/2}$. Activation of the MAP kinase (ERK$_{1/2}$) signaling pathway has been shown to promote the survival of certain populations of neuronal cells (Grewal et al., 1999). Activation of this pathway can be achieved by several means, in particular by elevation of intracellular

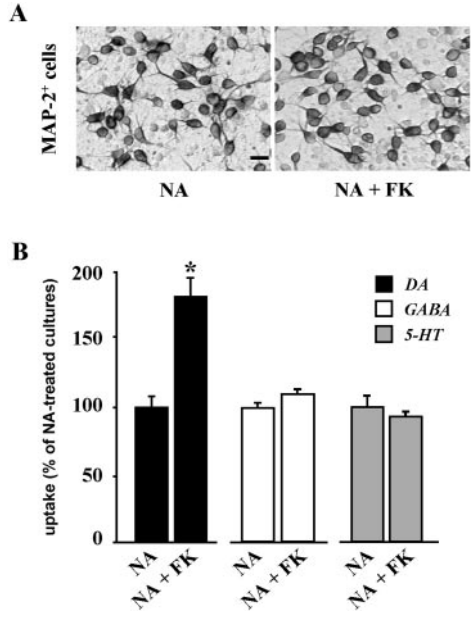

Fig. 5. The trophic action of forskolin is specific to dopaminergic neurons. A, treatment with 25 μM FK from DIV 0 to DIV 6 did not enhance the number of MAP-2$^+$ neurons in NA-treated mesencephalic cultures. B, the same treatment strongly stimulated the accumulation of DA but had no effect on 5-HT or GABA uptakes ⋆, $p < 0.01$, versus corresponding NA-treated cultures. In NA-treated cultures, the number of MAP-2$^+$ neurons per well was 91,200 ± 9,600, and the uptakes of DA, 5-HT, and GABA corresponded to 21.6, 41.9, and 699 fmol/min per well, respectively. Scale bar, 25 μm.

cAMP levels (Grewal et al., 1999). We therefore explored the possibility that the effects of FK occurred through $ERK_{1/2}$ activation using an antibody that specifically recognizes the phosphorylated (active) forms of the two kinases. Cotreatment with FK and NA induced a rapid activation of $ERK_{1/2}$ in mesencephalic cultures as characterized by Western immunoblotting (Fig. 10, A and B). $ERK_{1/2}$ phosphorylation was sustained and lasted for at least 16 h with a single application of FK (Fig. 10B). PD98059 (10 μM), an inhibitor of MAPK kinases (MEKs), the immediate upstream kinases of $ERK_{1/2}$, totally prevented this induction (Fig. 10A). We next studied the expression of the phosphorylated forms of $ERK_{1/2}$ at the cellular level, using the same antibody. Our results revealed that 45% of all mesencephalic neurons and 38% of dopaminergic neurons were immunopositive after cotreatment with FK and NA. At variance, a few cells (<1%) contained a positive label in the presence of NA alone (Fig. 10C). Consistent with the results obtained by Western immunoblotting, no positive cells were found in mesencephalic cultures treated with NA and FK in the presence of PD98059 (Fig. 10C). Interestingly, PD98059 suppressed the trophic action of FK on dopaminergic neurons but did not affect survival promotion elicited by NA (Fig. 10D). $ERK_{1/2}$ activation was not reduced by 1 μM H-89, reinforcing the idea that PKA was not involved in the survival effects of FK. Finally, CREB activation induced by cotreatment with NA and FK was not inhibited by PD98059 (not shown). A summary of these results is shown in Fig. 11.

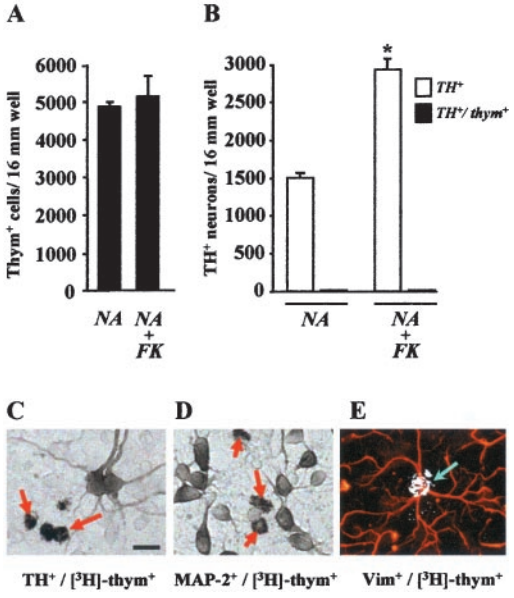

Fig. 6. Neither NA nor forskolin act as mitogens for dopaminergic neurons. A, the total number of thymidine-positive cells was not enhanced by FK treatment. B, TH^+ cells failed to incorporate tritiated thymidine in their nucleus. C, microautoradiographic visualization of thymidine-positive dividing nuclei (red arrowheads in C and D and blue arrow in E) in cultures treated chronically with NA and FK for 5 days. Phenotypic identification of mesencephalic cells was performed using TH, MAP-2, and vimentin immunodetection. Expression of the TH and MAP-2 proteins was revealed with the chromogen diaminobenzidine and that of vimentin by TRITC immunofluorescence. The pulse of tritiated thymidine was carried out between DIV 0 and DIV 1 and the cultures left to recover for 4 more days in the presence of the test treatments before assessment. Scale bar, 20 μm. ⋆, $p < 0.01$, versus corresponding NA-treated cultures.

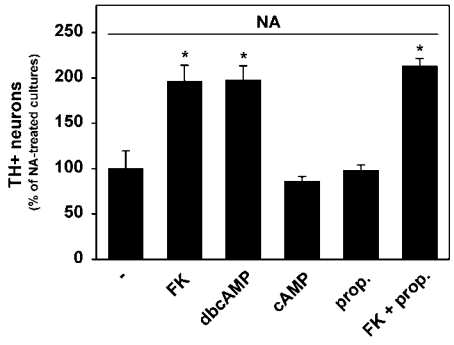

Fig. 7. The potentiation of the effect of NA by FK occurs by a cAMP-dependent mechanism that does not require β adrenoceptor activation The effect of FK in NA-treated cultures was mimicked by dibutyryl cAMP (dbcAMP) but not by its native (nonpermeant) form cAMP. The β adrenoceptor antagonist propranolol failed to reduce TH^+ cell survival in cultures exposed to NA or NA + FK. ⋆, $p < 0.05$, versus cultures exposed to NA alone.

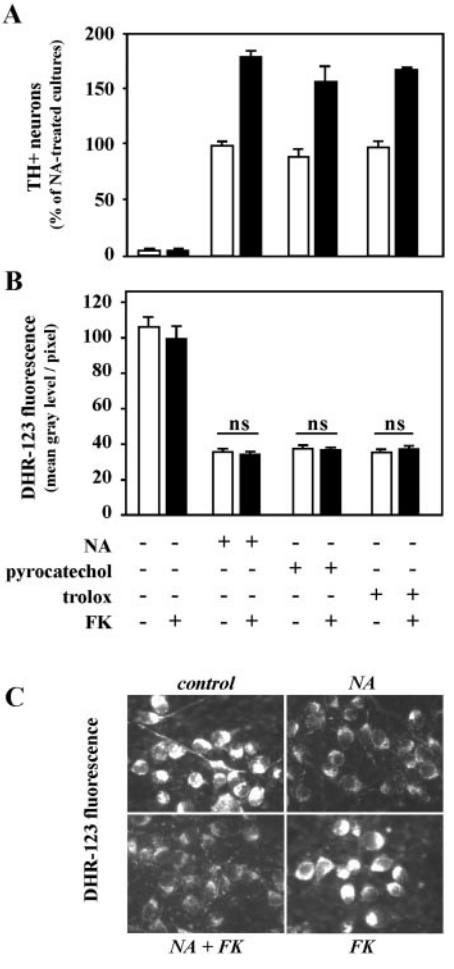

Fig. 8. Forskolin does not behave as an antioxidant. A, comparison of the neuroprotective effects of FK on dopaminergic cells treated with NA, pyrocatechol or trolox. B, detection of intracellular free radical species with the fluorescent dye DHR-123 in DIV 3 mesencephalic cultures exposed to NA, pyrocatechol or trolox, each given separately or in combination with FK. Note that FK alone failed to prevent intracellular ROS emission. C, representative fields showing that FK had no inhibitory effect on ROS production, alone, or in combination with NA. NA and pyrocatechol were each used at 1 μM, trolox at 10 μM, and FK at 25 μM. ns, no significant difference as indicated.

Discussion

The results of the present study demonstrate that the adenylate cyclase activating agent FK powerfully potentiates the neuroprotective activity of the neurotransmitter NA in a culture model of dopamine neuronal cell death. Survival promotion provided by cotreatment with NA and FK did not involve adrenoceptors. Whereas NA seemed to act independently by preventing spontaneously occurring oxidative

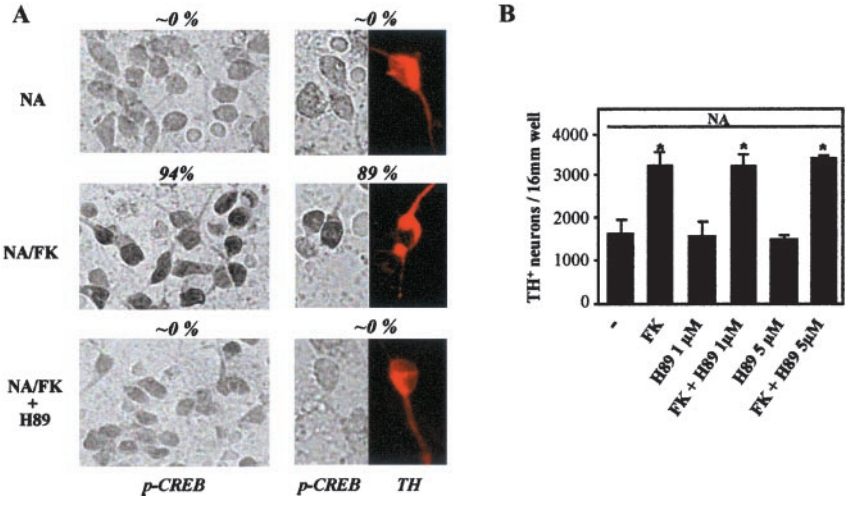

Fig. 9. Survival promotion by FK does not require activation of the PKA/CREB signaling pathway. A, immunodetection of pCREB in DIV1 cultures exposed for 1 h to NA, NA+FK, or NA+FK+H89, using the chromogen diaminobenzidine. Subsequent detection of TH^+ neurons by TRITC immunofluorescence demonstrated that 89% of TH^+ neurons express pCREB after FK exposure. NA, 1 μM; FK, 25 μM; H89, 1 μM. B, survival promotion by FK was not blocked by H-89 (1–5 μM). ⋆, $p < 0.05$, versus cultures exposed to NA alone.

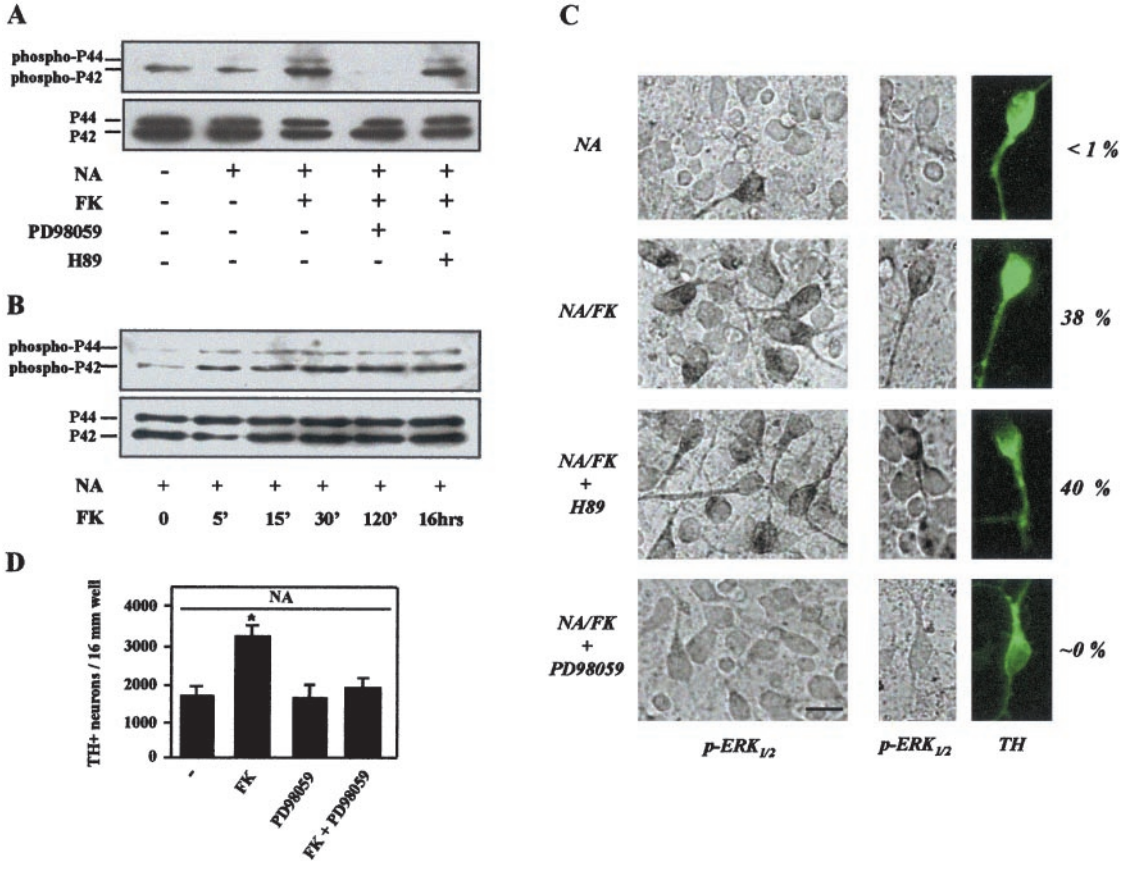

Fig. 10. Forskolin is neuroprotective by activation of the MAP kinase ($ERK_{1/2}$) signaling pathway. A, Western blotting analysis of $pERK_{1/2}$ expression in cultured DIV 1 mesencephalic cells exposed for 15 min to various test treatments. The MEK inhibitor PD98059, but not the PKA inhibitor H89, abolished FK-dependent increase of $ERK_{1/2}$ phosphorylation. Protein loading was controlled by additionally staining blots with a non–phospho-$ERK_{1/2}$ antibody. B, FK induction of $ERK_{1/2}$ phosphorylation was long-lasting. C, immunodetection of $pERK_{1/2}$ using the chromogen diaminobenzidine in DIV 1 cultures exposed for 15 min to NA alone or in the presence of FK, FK+PD98059, or FK+H89. Subsequent visualization of TH^+ neurons by fluorescein isothiocyanate immunofluorescence showing that FK produces an increase in the number of dopaminergic neurons immunopositive for $pERK_{1/2}$. Consistent with the results obtained by Western immunoblotting, PD98059 but not H89 prevented FK-induced $ERK_{1/2}$ phosphorylation at the cellular level. D, promotion of cell survival by FK in NA-treated cultures was suppressed by pharmacological inhibition of MEK using PD98059. PD98059 failed, however, to reduce the neuroprotective action of NA alone. NA, 1 μM; FK, 25 μM; PD98059, 10 μM; H89, 1 μM. ⋆, $p < 0.05$, versus cultures exposed to NA alone.

stress, the contribution by FK seemed to involve the activation of cyclic AMP-dependent MAP kinase ($ERK_{1/2}$). Interestingly, the additional protection afforded by FK was specific to dopaminergic neurons in this model system.

Forskolin Potentiates the Trophic Action of NA by a cAMP-Dependent Mechanism That Does Not Require β Adrenoceptor Activation

Time-course studies revealed that TH^+ neurons die spontaneously and progressively in the course of maturation of mesencephalic cultures. Consistent with previous data, we found that low concentrations of NA added chronically to the cultures can save a significant proportion of these neurons (Troadec et al., 2001). Importantly, the neuroprotective effects of NA that are only partial in our system were improved significantly by pharmacological treatments that elevated the intracellular levels of cAMP. We have shown previously that the protective effects of NA alone were independent of adrenoceptor activation (Troadec et al., 2001). However, because in other models FK has been shown to markedly potentiate the action of β adrenoceptor agonists (Mokhtari et al., 1985; Morin et al., 2000), we hypothesized that blockade of β receptors could possibly interfere with the survival promoting effects produced by a cotreatment with FK and NA. This was not the case, however, because the β adrenoceptor blocker propanolol failed to reduce TH^+ cell survival in the presence of NA and FK.

Forskolin Acts as a True Survival Factor

Among the possible explanations for the potentiation of the trophic action of NA are: (1) a mitogenic action of cAMP analogs (Della Fazia et al., 1997) on TH^+ neuroblasts or their precursor cells; (2) the recruitment of postmitotic neurons not committed initially to the dopaminergic phenotype (Du and Iacovitti, 1997); (3) the maintenance of this phenotype in sick TH^+ neurons; or (4) the rescue of a subpopulation of TH^+ cells for which the NA-dependent mechanism was necessary but insufficient to promote survival. The first possibility is unlikely because TH^+ cells are already postmitotic at the time when the embryos are taken for preparation of the cultures (i.e., at day 15.5 of gestation) (Rothman et al., 1980). Furthermore, we were unable to detect a single [^{3}H]thymidine-positive nucleus in the population of mesencephalic TH^+ neurons after a treatment with NA alone or in combination with FK. The acquisition of a dopaminergic phenotype remains possible because FK has been shown to induce TH^+ neurons in nondopaminergic areas of the brain, in cooperation with other factors such as neurotrophic peptides and catecholamines (Du and Iacovitti, 1997). Yet the fact that the number of TH^+ cells in NA+cAMP-treated cultures was always lower than the number of TH^+ neuroblasts detectable immediately after plating does not support this possibility. Alternatively, given that cyclic AMP elevation is known to increase TH gene expression in various populations of catecholaminergic neurons (Lim et al., 2000), FK could simply restore or help to maintain the expression of the protein in suffering cells that express the enzyme at a very low level in the sole presence of NA. This is not likely, however, because a treatment with FK augmented to the same extent the number of neurons that express the TH enzyme and the number of cells that accumulate dopamine via the dopamine transporter, the latter being not responsive to induction by cyclic AMP (Sacchetti et al., 1999). Taken together, the evidence suggests that cAMP-elevating agents acted as true survival factors in synergy with NA to prevent the death of dopaminergic neurons. Importantly, the fact that the dopaminergic neurons treated with NA and FK were capable of efficiently accumulating exogenous DA suggests that those rescued cells were healthy and functional.

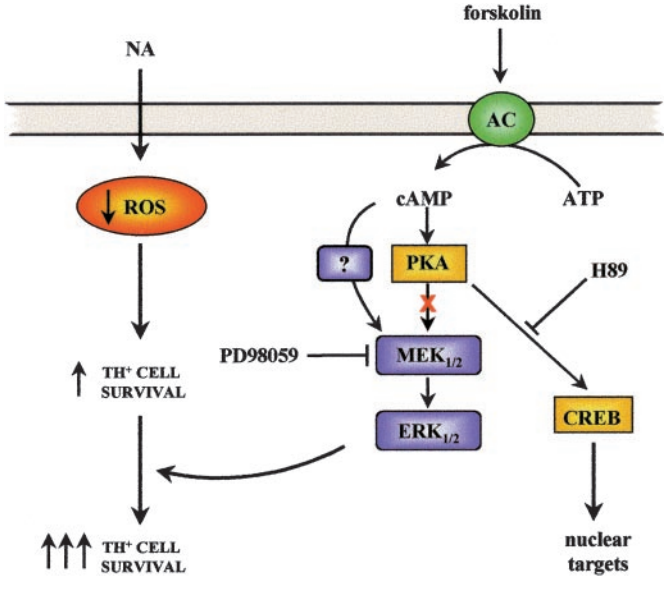

Fig. 11. Schematic representation of the signaling events that might underlie the cooperative effects elicited by FK and NA on the survival of mesencephalic dopaminergic neurons in vitro.

What Signaling Events Are Involved in the Effect of Forskolin?

Forskolin Does Not Enhance the Antioxidant Activity of NA. We have shown previously that NA can reduce the death of dopaminergic neurons by limiting oxidative stress (Troadec et al., 2001). Several arguments suggest that the effect of FK also results from a reduction in ROS production: (1) FK strongly potentiated the neuroprotective effects of several antioxidants; (2) cAMP elevation was found previously to protect against free radical-mediated cell injury (Keller et al., 1998). Such a mechanism, however, cannot account for the neuroprotective effects of FK in our culture model for two reasons: this compound was unable to reduce ROS emission when used alone in mesencephalic cultures and consequently was not protective in itself, and it did not potentiate the antioxidant activity of NA.

Survival Promotion by Forskolin Does Not Require Activation of the PKA/CREB Signaling Pathway. The effects of FK could be possibly explained by activation of the prosurvival transcription factor, CREB. Indeed, phosphorylation of CREB at serine residue 133 has been implicated in the resistance of cells to various insults, and a number of well-established neuroprotective agents are believed to exert their action via pathways that converge on the CREB protein (Bonni et al., 1999; Walton and Dragunow, 2000). In our model, cAMP-elevating agents induced activation of CREB at serine 133 in the vast majority of the mesencephalic neurons including dopaminergic cells, adding further to the notion that CREB participated to the trophic action of FK. The inhibition of PKA, the upstream kinase that controls cAMP-

dependent CREB phosphorylation (Walton and Dragunow, 2000; Mayr and Montminy, 2001), totally abolished CREB activation in dopaminergic neurons. Yet, the survival promoting effects of FK were not reduced in these conditions, suggesting that the PKA/CREB signaling pathway did not contribute to the trophic effect of FK.

Forskolin Is Neuroprotective by Activation of the MAP Kinase Signaling Pathway. MAPKs ($ERK_{1/2}$) are part of a signaling pathway that can be activated by cAMP (Frödin et al., 1994). This pathway seems to be involved in the survival of certain neuronal cell types including cerebellar granule neurons (Villalba et al., 1997; Bonni et al., 1999). In our culture model, FK led to $ERK_{1/2}$ activation in a subpopulation of dopaminergic neurons, an effect that was not prevented by inhibition of PKA. Most importantly, inhibition of $ERK_{1/2}$ by PD98059 totally abolished the trophic action of FK, suggesting that $ERK_{1/2}$ stimulation was required for the trophic function of the adenylate cyclase activator. Interestingly, PD98059 failed to interfere with the neuroprotective action of NA, indicating that the MAP kinase pathway was solely responsible for the effects of FK. These results demonstrate that NA and FK produced their neuroprotective effects by acting via distinct molecular mechanisms.

As discussed above, PKA was not involved in the activation the MAP kinase signaling pathway and for that reason in the neuroprotective action of FK. It has been proposed that two guanine nucleotide exchange factors (GEFs), i.e., cAMP-GEF I and II, (Kawasaki et al., 1998) can stimulate $ERK_{1/2}$ via a PKA independent mechanism (Grewal et al., 1999). Interestingly, Northern blot analysis has revealed that these two proteins are expressed in the human and rat substantia nigra (Kawasaki et al., 1998). Additional studies would be required, however, to determine whether cAMP-GEF-I and –II play a role in the survival-promoting action of FK.

Why Are the Effects of Forskolin Specific to TH^+ Neurons? We have shown that the effects of FK are remarkably specific to TH^+ neurons. This is surprising because $ERK_{1/2}$ activation also occurred in nondopaminergic cells after FK treatment. This suggests that the MAP kinase pathway has a pro-survival function in some but not all neuronal populations of the brain and, in particular, in a subpopulation of TH^+ cells. This function might be transient and correspond to a developmentally regulated process. If this were the case, it may explain why FK had to be added in the early phase of the culture to be effective. Alternatively, MAP kinase activation might be converted into a trophic signal under stressful conditions, reproduced in vitro by the process of trituration that probably mimics some of the aspects of an axotomy in the adult brain (Hartikka and Hefti, 1988). Supporting this view, axotomy-induced cell death of facial motoneurons was completely prevented in transgenic mice in which sustained phosphorylation of $ERK_{1/2}$ was achieved within neurons by means of a constitutive activation of Ras (Heumann et al., 2000). Likewise, persistent activation of $ERK_{1/2}$ was also observed in our cultures after chronic treatment with FK.

In conclusion, our findings demonstrate that cAMP elevation exerts potent neuroprotective effects in cooperation with the neurotransmitter NA. The two molecules seem to operate by distinct molecular mechanisms. Whereas NA seemed to act as a potent antioxidant in our model, the trophic action of FK required activation of the MAPK signaling pathway. Endogenous factors susceptible to reproduce the action of FK on dopaminergic neurons remain to be characterized. K^+-induced depolarization closely mimicked the action of cAMP in NA-treated cultures (results not shown) indicating that molecules, which modulate neuronal excitability, are potential candidates.

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Address correspondence to: Dr Patrick Michel, INSERM 289, Experimental Neurology and Therapeutics, Bâtiment pharmacie, Hôpital de la Salpêtrière, 47 bd de l'hôpital, 75013 Paris, France. E-mail: ppmichel@ccr.jussieu.fr