Modulation of PC12 Cell Viability by Forskolin-Induced Cyclic AMP Levels Through ERK and JNK Pathways: An Implication for L-DOPA-Induced Cytotoxicity in Nigrostriatal Dopamine Neurons

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The intracellular levels of cyclic AMP (cAMP) increase in response to cytotoxic concentrations of L-DOPA in PC12 cells, and forskolin that induces intracellular cAMP levels either protects PC12 cells from L-DOPA-induced cytotoxicity or enhances cytotoxicity in a concentration-dependent manner. This study investigated the effects of cAMP induced by forskolin on cell viability of PC12 cells, relevant to L-DOPA-induced cytotoxicity in Parkinson’s disease therapy. The low levels of forskolin (0.01 and 0.1 μM)-induced cAMP increased dopamine biosynthesis and tyrosine hydroxylase (TH) phosphorylation, and induced transient phosphorylation of ERK1/2 within 1 h. However, at the high levels of forskolin (1.0 and 10 μM)-induced cAMP, dopamine biosynthesis and TH phosphorylation did not increase, but rapid differentiation in neurite-like formation was observed with a steady state. The high levels of forskolin-induced cAMP also induced sustained increase in ERK1/2 phosphorylation within 0.25–6 h and then led to apoptosis, which was apparently mediated by JNK1/2 and caspase-3 activation. Multiple treatment of PC12 cells with nontoxic L-DOPA (20 μM) for 4–6 days induced neurite-like formation and decreased intracellular dopamine levels by reducing TH phosphorylation. These results suggest that the low levels of forskolin-induced cAMP increased dopamine biosynthesis in cell survival via transient ERK1/2 phosphorylation. In contrast, the high levels of forskolin-induced cAMP induced differentiation via sustained ERK1/2 phosphorylation and then led to apoptosis. Taken together, the intracellular levels of cAMP play a dual role in cell survival and death through the ERK1/2 and JNK1/2 pathways in PC12 cells.

Key Words: cyclic AMP; forskolin; cell viability; ERK; JNK; PC12 cells.

L-3,4-Dihydroxyphenylalanine (L-DOPA), the natural precursor of dopamine, is the most frequently prescribed drug for controlling the symptoms of Parkinson’s disease (PD) (Marsden, 1994). L-DOPA also shows neurotrophic and neuroprotective effects on neuronal and PC12 cells and in animal models of PD (Koshimura et al., 2000; Mena et al., 1997; Shin et al., 2009). However, L-DOPA accelerates the progression of PD (Maharaj et al., 2005), and chronic L-DOPA treatment can be accompanied by disabling motor side effects such as L-DOPA-induced dyskinesias that occur after the initial optimal response period in PD patients (Fahn and Cohen, 1992; Lipski et al., 2011). In addition, L-DOPA can lead to cell death through cytotoxicity induced by oxidative stress from reactive oxygen species (ROS) in both dopaminergic neuronal and rat adrenal pheochromocytoma (PC12) cells (Basma et al., 1995; Cheng et al., 1996; Walkinshaw and Waters, 1995).

A large volume of past references indicates that cyclic AMP (cAMP), which is formed by adenyl cyclase mediated by G proteins, modulates cell survival, proliferation, differentiation, and apoptotic induction through the cAMP-dependent protein kinase A (PKA)-extracellular signal-regulated kinase (ERK1/2)-cAMP-response element-binding protein (CREB) signaling pathways in various cell or tissue types, including neuronal and PC12 cells (Stork and Schmitt, 2002).

Intracellular cAMP activates PKA and CREB phosphorylation at Ser133, which leads to dopamine biosynthesis by inducing tyrosine hydroxylase (TH, EC 1.14.16.2) activity and TH gene regulation in neurons and PC12 cells (Kim et al., 1993). PKA-dependent phosphorylation of TH at Ser19 and Ser 40 enhances TH activity by inducing its conformational changes in the regulatory domain, which leads to easier dissociation of catecholamine from the catecholamine-bound inhibited form of TH (Bevilaqua et al., 2001; Haycock, 1990; Ramsey and Fitzpatrick, 1998) and TH binding to the 14-3-3 protein that phosphorylates serine residues in the regulatory domain of TH (Obsilova et al., 2008). In neuronal and PC12 cells, cAMP also induces ERK phosphorylation via the interaction of GTP-loaded Rap1, which is activated by PKA, with B-Raf, a mitogen-activated protein kinase (MAPK) activator, which then causes sustained ERK phosphorylation (Dugan et al., 1999; Stork and Schmitt, 2002; Vossler et al., 1997). In addition, sustained activation of ERKs by nerve growth factor (NGF)
triggers differentiation to form neurite-like processes in PC12 cells (Lambeng et al., 2001; York et al., 1998).

A recent study has shown that L-DOPA, at both nontoxic (20 μM) and toxic (100–200 μM) concentrations, increases L-type Ca\(^{2+}\) channel-mediated intracellular levels of cAMP (Jin et al., 2010), and L-DOPA-induced cAMP levels increase dopamine biosynthesis through PKA and CREB phosphorylation (Jin et al., 2008). At nontoxic concentrations, L-DOPA promotes ERK phosphorylation. Bad phosphorylation at Ser112, and Bcl-2 expression that induces cell survival, whereas at high concentrations, it enhances caspase-3 activity through the c-Jun-N-terminal kinase (JNK) and p38 MAPK signaling pathways and also induces ERK phosphorylation (Jin et al., 2010). However, L-DOPA-induced increased cAMP levels do not protect PC12 cells from L-DOPA-induced cytotoxicity (Jin et al., 2008, 2010).

Forskolin, which increases intracellular levels of cAMP by stimulating adenylyl cyclase activity, stimulates TH activity and TH gene expression (Kim et al., 1993). Forskolin also potentiates the protective effects of noradrenaline via ERK activation on dopaminergic neurons (Troadec et al., 2002) and induces both cell proliferation and differentiation through ERK phosphorylation in PC12 cells (Kiernan et al., 2005). In addition, forskolin reduces dopamine cell loss after 6-hydroxydopamine (6-OHDA) exposure in embryonic cells of rat midbrain (Carrasco et al., 2008). In contrast, it has been shown that forskolin either protects against L-DOPA-induced cytotoxicity or enhances it in a concentration-dependent manner in PC12 cells (Jin et al., 2010).

Considering several reports highlighting the importance of cAMP in either cell survival or death, the roles of cAMP in neuronal cell death and differentiation involving L-DOPA-induced cytotoxicity are unclear. PC12 cells have been widely used as a model to investigate dopamine biosynthesis, L-DOPA-induced cytotoxicity, and neuronal proliferation and differentiation (Sanchez et al., 2004; Tischler et al., 1983; Walkinshaw and Waters, 1995). PC12 cells differentiate toward sympathetic-like neuron by the formation of neurite-like processes and neurite outgrowth in the presence of NGF, cAMP, and forskolin (Sanchez et al., 2004; York et al., 1998). PC12 cells also express the dopamine D\(_1\) and D\(_2\) receptors (Zachor et al., 2000). PC12 cells are, therefore, often applied as an in vitro model of PD. In addition, the index of differentiation by morphological changes has been determined by analyzing the formation of neurite-like processes in PC12 cells (Gunning et al., 1981; Lambeng et al., 2001).

In this study, we investigated whether the intracellular levels of forskolin-induced cAMP play a key role in cell survival and death, using PC12 cells as a model system for L-DOPA-induced cAMP concentration-dependent cytotoxicity in PC12 cells.

**MATERIALS AND METHODS**

**Chemicals.** L-DOPA, dopamine, forskolin, NGF, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St Louis, MO). A cAMP assay kit was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). Primary antibodies against TH, phosphor-TH (Ser 40), ERK1/2, phosphor-ERK1/2 (Thr 202/Tyr 204), JNK1/2, phosphor-JNK1/2 (Thr 183/Tyr 185), cleaved caspase-3 (Asp 175) and β-actin were purchased from Cell Signaling Technology Inc. (Beverly, MA). All sera, antibiotics, and RPMI 1640 used for cell culture were obtained from Gibco BRL (Grand Island, NY). All other chemicals were of reagent grade.

**Cell culture.** PC12 cells were grown in an RPMI medium 1640 supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100μg/ml streptomycin. These cells were placed in a humidified atmosphere with 5% CO\(_2\) and 95% air at 37°C, as previously described (Tischler et al., 1983).

**Measurement of cell viability.** Cell viability was determined by the conventional MTT assay. PC12 cells were treated with forskolin for the designated times, and the MTT solution (final concentration 1 mg/ml) was then added for 2 h in an incubator. The reaction was stopped with acidic isopropanol (0.8M), and the absorbance was measured at 570 nm (Beau Diagnostic Microplate Reader, Molecular Devices Inc., Sunnyvale, CA). Cell viability was expressed as a percentage of apoptotic cells. For the flow cytometry analysis of apoptotic cells, PC12 cells were fixed in 70% ethanol for 30 min at 4°C and suspended in 1 ml staining solution (50 μg/ml propidium iodide, 0.05 mg/mL ribonuclease A, and 0.1mM EDTA, pH 7.4). Cellular DNA content was measured using a flow cytometer (BD Biosciences, San Jose, CA).

**Determination of dopamine levels.** Dopamine levels were determined, as previously described (Jin et al., 2008). Briefly, PC12 cells were treated with forskolin or L-DOPA for the designated time, and trichloroacetic acid (1M, 100 μl) and isoproterenol (200 pmol, internal standard) were added. After centrifuging the mixture of the pellet extract, the mixture was passed through a Toyopak SP-M cartridge (Na\(^+\) resin 1 ml, Tosoh Corporation, Tokyo, Japan), and the cartridge eluate was derivatized with 1,2-diphenylethlenediamine. The final reaction mixture (50μl) was injected into a HPLC system (Tosoh Corporation). The conditions of the HPLC analysis were the same as previously described (Jin et al., 2008).

**Measurement of the formation of neurite-like processes.** The formation of neurite-like processes on PC12 cells, which is used as a marker of differentiation, was observed according to the described method (Gunning et al., 1981; Lambeng et al., 2001). PC12 cells were plated at a relatively low density (1 × 10\(^4\) cells/cm\(^2\)) onto 6-well culture plates coated with poly-L-lysine. After incubation for the designated time, the number of differentiated cells was determined by counting the cells that had at least one neurite with a length of more than 1.5-fold the diameter of the cell body, using a phase-contrast microscope (200X magnification, JP/IX-71-21PH, Olympus Corporation, Tokyo, Japan), and the values were expressed as a percentage relative to the positive control (NGF, 20 ng/ml), which was defined as 100%. For each datum point, the mean values were calculated from six random-field observations of two replicate experiments, and a minimum of 100 cells per field were counted.

**Analysis of the phosphorylation of TH, ERK, JNK, and cleaved caspase-3 by Western blotting.** Activations of the phosphorylation of TH at Ser 40 (phosphor-TH (Ser 40)), ERK1/2 at Thr 202/Tyr 204 (phosphor-ERK1/2 [Thr 202/Tyr 204]), JNK1/2 at Thr 183/Tyr 185 (phosphor-JNK1/2 [Thr 183/Tyr 185]), cleaved caspase-3 at Asp 175, and β-actin were analyzed by Western blot analysis. PC12 cells (ca. 1×10\(^6\) cells/ml) were collected and homogenized at 4°C, and the supernatant was stored at −70°C until use. After the addition of a sample loading buffer, proteins in samples (30μg) were separated using 10–15% sodium dodecyl sulfate-poly acrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane at 300 mA for 1–3 h. The blots were blocked for 1 h at room temperature in a fresh blocking buffer (TBS-T containing 5% bovine serum albumin (BSA)) and then incubated overnight at 4°C using primary antibodies diluted 1:1000 in TBS-T with 5% BSA, and for 1 h at room temperature using secondary antibodies (dilutions, 1:5000 in TBS-T with 5% BSA), according to a standard procedure. The blots were then washed, and the transferred proteins were incubated with ECL substrate solution (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for 5 min, according to the manufacturer’s instructions, and visualized with a radiographic film.
Statistical analysis. All data were presented as the mean ± SEM of at least four independent experiments. Protein amounts were determined with a bicinchoninic acid protein assay kit (Pierce Protein Research Products, Rockford, IL), using BSA. Statistical analysis was performed using ANOVA followed by Dunnett’s for multiple comparisons, and a p value < 0.05 was considered statistically significant.

RESULTS

Intracellular Levels of Dopamine Induced by Forskolin

Treatment of PC12 cells with 0.01, 0.1, 1.0, and 10μM of forskolin resulted in a rapid increase (within 5–20 min) in the intracellular cAMP levels to about 2.3-, 32.3-, 81.2-, and 90.6-fold, respectively, compared with the untreated control groups (261.9 ± 11.4 pmol/mg protein, n = 7). After 3–6 h, forskolin (0.01 μM) increased dopamine levels to 110–115%, compared with control (Fig. 1A) and forskolin (0.1 μM) increased dopamine levels to 130–150% after 1–6 h (Fig. 1B). With these forskolin concentrations, the levels of dopamine were gradually returned to the control levels at 12–24 h (Figs. 1A and B). Forskolin (1.0 μM) also increased dopamine levels to 110–120% after 1–3 h, which was higher than those seen with 0.01 μM forskolin, but the increased levels of dopamine were then gradually decreased to under the control levels at 24 h (Fig. 1C). In contrast, the high concentration of forskolin (10μM) slightly increased dopamine levels relative to the control groups, but it was not significant. And then, the dopamine levels were actually decreased in response to forskolin (10μM) treatment to 73–79% after 6–24 h (Fig. 1D).

Phosphorylation of TH Induced by Forskolin

The pattern of TH phosphorylation at Ser 40 in response to forskolin treatment is shown in Figure 2. After 3–6 h, TH was phosphorylated by 0.01 μM forskolin to 1.3–1.4-fold the control groups. TH phosphorylation was increased by 0.1 μM forskolin to 1.6–2.3-fold the control levels after 0.5–6 h. With 1.0 μM forskolin, TH phosphorylation levels were also increased to 1.2–1.5-fold for 0.5–3 h, and the levels then slightly declined at 6 h. However, TH phosphorylation levels were reduced by the high concentration of 10 μM forskolin to the levels below control levels at 1–6 h (Fig. 2).

Formation of Neurite-Like Processes Induced by Forskolin

The rate of formation of neurite-like processes induced by various concentrations of forskolin treatment was compared.

![Figure 1](image_url)
with that in controls and positive controls treated with NGF (2 and 20 ng/ml) in a normal culture medium in PC12 cells (Fig. 3). Forskolin concentrations of 0.01–0.1 μM had a tendency to induce the formation of neurite-like processes after 3–24 h of treatment, but the rate of formation was not significantly different from that in controls (Figs. 3A and B). However, these forskolin concentrations did induce the formation of neurite-like processes after 2–5 days (Fig. 3A): 15–20% of the cells exhibited neurite-like processes, and this pattern was similar to that seen in the positive control group treated with NGF (2 ng/ml). Treatment with 1.0 μM of forskolin also induced the formation of neurite-like processes in 30–42% of cells after 3–24 h; then, the proportion of cells slightly increased to about 45% after 2–5 days (Figs. 3A and B). Treatment with 10 μM of forskolin for 3 h to 3 days resulted in a steady increase in the number of cells exhibiting neurite-like processes to about 80%, and the same percentage of cells exhibiting neurite-like processes was maintained through the 5 days of treatment. The low levels of cAMP induced by 24 h of treatment with 0.01–0.1 μM of forskolin did not result in neurite formation, but morphological differentiation did occur after 24 h (Figs. 3A and B). In contrast, the relatively higher levels of cAMP induced by treatment with 1.0–10 μM of forskolin resulted in
rapid changes in morphology, observed as early as 3 h after treatment in the normal culture medium (Figs. 3A and B).

**Cellular Viability Induced by Forskolin**

Treatment with 0.01 μM of forskolin did not affect cell viability throughout the 48-h treatment period (Fig. 4A). However, cell death was observed after 48 h of treatment with 0.1 μM of forskolin, after 24 h of treatment with 1.0 μM of forskolin, and after 6 h of treatment with 10 μM of forskolin (Figs. 4B–D). There was rapid decrease in cellular viability following treatment with 10 μM of forskolin, with 88.4% cellular viability seen after 6 h of treatment and only 60.5% cellular viability seen after 48 h of treatment. These results suggest that relatively stronger and faster cytotoxic effects resulted from treatment with 10 μM of forskolin, compared with the other treatment concentrations of forskolin (i.e., 0.01–1.0 μM) (Fig. 4).

In addition, treatment with 0.01 μM forskolin did not induce cellular apoptosis in the flow cytometry analysis at 24 h or 48 h, whereas 0.1 μM forskolin treatment did not induce it at 24 h (Fig. 5A). However, apoptosis was seen after treatment with 1.0 and 10 μM of forskolin at 24 h (percentage of apoptotic cells 21.2 and 35.1%, respectively) and after treatment with 0.1, 1.0, and 10 μM of forskolin at 48 h (percentage of apoptotic cells 23.9,
39.1, and 54.3%, respectively) (Figs. 5A and B). These findings indicate that the forskolin treatment concentrations of 0.01 and 0.1 μM were nontoxic at 24 h, but the forskolin treatment concentrations of 1.0–10 μM were toxic at both 24 and 48 h.

**Phosphorylation of ERK1/2 Induced by Forskolin**

ERK1/2 was phosphorylated by 0.01–10 μM forskolin in a concentration-dependent manner (Fig. 6). Forskolin concentrations of 0.01 and 0.1 μM increased ERK1/2 phosphorylation to about 2.2 (−2.7)-fold and 2.8 (−3.3)-fold after 0.5–1 h, respectively, which then returned to control levels at 3 h. In addition, forskolin at 1.0 and 10 μM rapidly induced ERK1/2 phosphorylation to about 3.6 (−4.7)-fold and 4.8 (−5.1)-fold after 1–30 min, respectively, and the increased phosphorylation of ERK1/2 was sustained for 6 h (Fig. 6).

**Phosphorylation of JNK1/2 Induced by Forskolin**

The phosphorylated levels of JNK1/2 were not affected by 0.01 μM of forskolin after 0.5–6 h (Fig. 7). Forskolin at 0.1 μM did not also induce JNK1/2 phosphorylation at 0.5–6 h. Forskolin at 1.0 μM induced JNK1/2 phosphorylation to about 1.2 (−1.3)-fold after 3–6 h. However, 10 μM of forskolin rapidly induced JNK1/2 phosphorylation to about 1.5-fold after 3 h, and this level was maintained for 3–6 h (Fig. 7). Thus, the higher concentrations of forskolin (1.0 and 10 μM) increased the phosphorylation levels of JNK1/2 from 3 to 6 h.

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**FIG. 6.** Representative blots illustrating the effects of forskolin (0.01, 0.1, 1.0, and 10 μM) on ERK1/2 phosphorylation (p-ERK1/2) in PC12 cells. (A) Immunoblots of lysates from forskolin-treated PC12 cells were probed with phosphor-ERK1/2 antibodies, and then phosphor-ERK1/2 and total ERK1/2 were examined by Western blotting as described under the Materials and Methods section. (B) The values of relative density ratio (p-ERK1/2/ERK1/2) are expressed as one arbitrary unit. The results are expressed as the means ± SEM (n = 4). *p < 0.05 compared with the control values (ANOVA followed by Dunnett’s).
Expression of Cleaved Caspase-3 Induced by Forskolin

Treatment with 0.01–0.1 μM of forskolin did not induce the expression of cleaved caspase-3 after 12–24 h (Fig. 8). However, forskolin at 10 μM induced the expression of cleaved caspase-3 to about 1.6-fold that of controls at 12 h (Fig. 8). In addition, the expression of cleaved caspase-3 was increased by 1.0 and 10 μM of forskolin to about 1.6- to 2.6-fold the control groups at 24 h (Fig. 8). The relatively higher levels of cAMP induced by forskolin treatment of 1.0 and 10 μM enhanced the expression of cleaved caspase-3 (Fig. 8).

Intracellular Levels of Dopamine, Phosphorylation of TH, and Formation of Neurite-Like Processes by Multiple Treatment of L-DOPA

Nontoxic concentration of L-DOPA (20 μM) was repeatedly treated to PC12 cells once a day for 6 days, after changing the culture medium, in order to examine the modulation of both dopamine biosynthesis and neurite-like formation. The intracellular levels of dopamine were increased to 152–160% by multiple treatment with 20 μM L-DOPA for 1–4 days (Fig. 9A). However, the dopamine levels were thereafter gradually decreased by the multiple treatment with 20 μM L-DOPA for 5–6 days (Fig. 9A). TH phosphorylation was also enhanced by multiple treatment of 20 μM L-DOPA at 1–2 days and then significantly reduced for 4–6 days (Fig. 9B), although the levels of β-actin were slightly increased for L-DOPA-treated periods of 4–6 days. In addition, the number of cells showing the formation of neurite-like processes was increased by multiple treatment of 20 μM L-DOPA for 3–6 days, but not by a single treatment (Fig. 9C), which was similar to those by 0.01 μM forskolin (Fig. 3).
FIG. 9. Effects of multiple treatment of nontoxic L-DOPA on the change in intracellular dopamine levels (A), the activation by TH phosphorylation (p-TH) (B), and the formation of neurite-like processes (C) in PC12 cells. PC12 cells were repeatedly treated with 20 μM L-DOPA once a day for 6 days after changing the culture media, and then dopamine levels, TH phosphorylation, and the number of cells that exhibited the formation of neurite-like processes were examined as described under the Materials and Methods section. (A) The mean control levels (0 day) of intracellular dopamine were 6.25 ± 0.19 nmol/mg protein. (B) Immunoblots of lysates from forskolin-treated PC12 cells were probed with phosphor-TH antibodies. The values of relative density ratio (p-TH/TH) are expressed as one arbitrary unit. (C) PC12 cells with at least one neurite with a length longer than 1.5-fold the diameter of the cell body were counted and expressed as a percentage relative to the positive control (NGF, 20 ng/ml; see Fig. 3), which was defined as 100% for 4–6 days. The results are expressed as the means ± SEM (A, n = 6–8; B, n = 4; C, n = 6). Significantly different from control values: *p < 0.05 (ANOVA followed by Dunnett's).

DISCUSSION

cAMP modulation of cell survival, proliferation, differentiation, and apoptotic induction through the PKA-ERK1/2-CREB signaling pathways is not limited to neuronal and PC12 cells (Gunning et al., 1981; Hashimoto et al., 2003; Lambeng et al., 2001; Piiper et al., 2002; Sanchez et al., 2004), but it applies to various other cell or tissue types, including polycystic kidney epithelium, human prostate tumors, cardiac myocytes, granulosa, preadipocytes, and the pituitary (Stork and Schmitt, 2002). In contrast, cAMP inhibits cellular proliferation by inhibiting ERK in adipocytes, bovine brain capillary endothelial cells, NIH3T3 cells, and rat fibroblasts (Stork and Schmitt, 2002). It has been reported that the levels of cAMP in normal human brain (98.8 ± 5.2 pmol/mg protein) are lower than those in intracellular PC12 cells and that the cAMP levels and adenyl cyclase activities in human brain tumors are also related to the degree of the malignancy (Furman and Shulman, 1977). However, PC12 cells have been widely used as a model system for the intracellular functions of cAMP. The functions of cAMP on cellular proliferation, differentiation, survival, and cell death appear to be cAMP-concentration dependent. In addition, cAMP protects both neurons and PC12 cells from oxidative stress caused by ROS (Lambeng et al., 2001). Previously, we have reported that toxic concentrations (100–200 μM) of L-DOPA in PC12 cells result in an increase in cAMP levels mediated by L-type Ca++ channels to about 4.1–5.6-fold (Jin et al., 2008, 2010), which is similar to forskolin (0.01–0.05 μM)-induced cAMP levels, but the increased levels of cAMP do not protect against L-DOPA-induced oxidative cytotoxicity by ROS (Jin et al., 2010). In addition, L-DOPA-induced cytotoxicity is enhanced by cotreatment with forskolin (0.1–10 μM) (Jin et al., 2010). In this study, therefore, the effects of the intracellular levels of cAMP induced by forskolin on cell survival and death were investigated in PC12 cells, in order to elucidate the function of the increased levels of cAMP by L-DOPA.

Forskolin increases intracellular levels of cAMP in PC12 cells. The low levels of forskolin (0.01 and 0.1 μM)-induced cAMP increased dopamine biosynthesis and TH phosphorylation at 3–6 h. However, the dopamine levels and TH phosphorylation, which were increased at 3 h by the high levels of forskolin (1.0 and 10 μM)-induced cAMP, were thereafter reduced at 12–24 h (Figs. 1 and 2). L-DOPA at nontoxic (20 μM) and toxic (200 μM) concentrations increases the intracellular dopamine levels to ca. 230 and 440% (8.03 and 15.4 nmol/mg protein, respectively) at 0.5–4 h in PC12 cells (Jin et al., 2008). Dopamine at 100 μM, but not at 30 μM, showed cytotoxicity in PC12 cells (Koshimura et al., 2000). Therefore, the levels of dopamine (ca. 9.3 nmol/mg protein) induced by forskolin (0.01–10 μM) did not induce cytotoxicity at earlier time periods in PC12 cells, although the basic levels of dopamine were shown with a slight variation by the experimental conditions. The formation of neurite-like processes was also forskolin-induced cAMP concentration-dependent: with low levels of forskolin-induced cAMP, it took 3–5 days for the PC12 cells to differentiate and to form neurite-like processes (Fig. 3). In contrast, it took only 3 h with the high levels of forskolin-induced cAMP, and the number of neurite-like processing cells...
gradually increased and remained at the steady state (Fig. 3). In addition, the high levels of cAMP induced by forskolin (1.0–10 μM) generated ROS to 105–120% within 1 h compared with the control in PC12 cells (data not shown). However, the generated ROS by forskolin did not also induce cytotoxicity in PC12 cells (Fig. 4), and the cells might be moved to the differentiated step before the cell death process by ROS (Fig. 3). These results indicate that both dopamine biosynthesis and TH phosphorylation are closely related to cellular differentiation.

In addition, phosphorylation of ERK1/2 was forskolin-induced cAMP-concentration dependent and showed a biphasic pattern: the low levels forskolin-induced cAMP resulted in transient increase of ERK1/2 phosphorylation within 1 h but sustained increase by the high levels of forskolin-induced cAMP for 15 min to 6 h (Fig. 6). Various evidences in the literature showed the interrelationship between cAMP levels, ERK1/2 activation, proliferation, differentiation, and neurite-like formation of PC12 cells: NGF or epidermal growth factor (EGF) induces transient activation of ERK1/2 that triggers the proliferation of PC12 cells, and NGF or fibroblast growth factor (FGF) determines sustained ERK1/2 that triggers differentiation, including sympathetic-like neurite formation (York et al., 1998). In Schwann cells, the low levels of cAMP activate proliferation through ERK, and the high levels of cAMP induce sustained activation of ERKs, as well as markers of differentiation (Mutoh et al., 1998). cAMP is also responsible for only ERK1/2 phosphorylation and then differentiation, and ERK1/2 activity is required for TH gene expression (Obara et al., 2009). These results support our observation that the high levels of forskolin-induced cAMP lead to differentiation that is mediated by sustained activation of ERK1/2 in PC12 cells. Moreover, our results for increased dopamine biosynthesis through the early ERK1/2 phosphorylation by forskolin-induced cAMP (Figs. 1 and 2) are supported by the finding that cAMP induces neurite-like processes and TH gene expression (Ser 31) through the PKA-ERK1/2 system (Obara et al., 2009), as mentioned above.

Intracellular levels of cAMP are increased by both L-DOPA (Jin et al., 2008, 2010) and 6-OHDA (unpublished data) in a concentration-dependent manner, and several evidences are reported that the activation of ERK signaling cascade induces neurotoxicity. A nontoxic concentration of L-DOPA induces a transient phosphorylation of ERK1/2 in PC12 cells, whereas L-DOPA at toxic ranges exhibits a relatively sustained phosphorylation of ERK1/2, which is biphasic (Jin et al., 2010). 6-OHDA at both nontoxic and toxic ranges also activates ERK1/2 phosphorylation in a biphasic manner in MN9D dopaminergic cells: the rapid activation of ERK1/2 shows a self-protective response, whereas 6-OHDA induces a large increase in ERK1/2 phosphorylation (Lin et al., 2008). However, the sustained activation of ERK1/2 does not protect against either L-DOPA- or 6-OHDA-induced cytotoxicity (Jin et al., 2010; unpublished data). 6-OHDA also induces sustained ERK1/2 activation, leading to cell death in the rat brain-derived B65 cell lines (Kulich and Chu, 2003). A possible mechanism of ERK activation causing neurotoxicity may result from sustained activation of ERK and abnormal subcellular location (Chu et al., 2004; Grewal et al., 2000). It is also reported that dopamine causes sustained activation of ERK mediated by the dopamine D1 receptors, and then the failure of phosphorylated ERK to translocate into the nucleus, which is retained in the cytoplasm, may trigger neurotoxicity rather than a survival in SK-N-MC neuroblastoma cells and rat primary striatal neurons (Chen et al., 2004, 2009). In this study, the high levels of cAMP induced by 1.0 and 10 μM forskolin resulted in apoptotic cell death at 24 and 6 h, respectively (Figs. 4 and 5), and steady and sustained ERK1/2 phosphorylation at 15 min to 6 h (Fig. 6), indicating that the long-term ERK1/2 activation by forskolin-induced cAMP levels led to the cell death process. These results suggest that ERK1/2 phosphorylation has a dual and biphasic function in PC12 cells: a transient activation of ERK1/2 leads to cell survival and proliferation, and a sustained activation of ERK1/2 leads to cell differentiation and death by apoptosis.

We asked then what cell death signaling was activated by forskolin-induced cAMP levels. Literatures show that deprivation of trophic factors, such as NGF, cAMP, and serum in the culture medium in PC12 cells, causes death in cAMP-differentiated cells through an apoptotic process, suggesting that NGF and cAMP can suppress cell death (Lambeng et al., 1999, 2001). Forskolin also increases phosphorylation of p38 MAPK in a PKA-dependent mode in NIH3T3 cells (Delghandi et al., 2005). Our present results showed that the high levels of forskolin-induced cAMP significantly increased JNK1/2 phosphorylation at 1–6 h, cleaved caspase-3 levels at 12–24 h (Figs. 7 and 8), and apoptosis (Fig. 5). These results are strong indications that, after the differentiated state by neurite-like formation in PC12 cells, the high levels of forskolin-induced cAMP lead to the apoptotic cell death process through the JNK1/2 and caspase-3 pathways.

A single treatment with the nontoxic concentration of 20 μM L-DOPA induced dopamine biosynthesis for 0.5–2 days in PC12 cells (Jin et al., 2008) but failed to determine the formation of neurite-like processes for 4–6 days (Fig. 9C). However, multiple treatment with 20 μM L-DOPA once a day for 4–6 days induced the formation of neurite-like processes (Fig. 9C). In these states, the intracellular levels of dopamine were gradually decreased by reducing TH phosphorylation (Figs. 9A and B), indicating that dopamine biosynthesis from L-DOPA was also closely related to the partial cellular differentiation: the modulation of cell survival and death by multiple treatment of L-DOPA is now in progress. In addition, cAMP and other trophic factors and agents, such as NGF, EGF, FGF and dibutyryl cAMP, that induce proliferation and/or differentiation through ERK1/2 activation can protect the cells against oxidative stress in neuronal and PC12 cells (Carrasco et al., 2008; Chiron et al., 1998; Lambeng et al., 2001), and cAMP and NGF are still essential for maintaining neuronal morphology (Lambeng et al., 2003). However, the opposite effects have been also reported to be cell type-specific (Stork and Schmitt, 2002;
Zhou et al., 2005). In an animal model of 6-OHDA-induced PD, the ERK1/2 activity is decreased in the substantia nigra region of the brain (Quesada et al., 2008). It has been also suggested that ERK phosphorylation is linked to cell death in brain cells such as astrocytes, glia, and neurons (Brambilla et al., 2002; Canals et al., 2003; Ferrer et al., 2001) and that the abnormal patterns of ERK activation may contribute to mechanisms of dopamine neuron death relevant to PD (Chen et al., 2009; Kulich and Chu, 2001). ERK1/2 phosphorylation, but not p38 MAPK or JNK phosphorylation, is also found in Lewy bodies in PD (Ferré et al., 2001). Therefore, the changes of ERK1/2 activity via increasing cAMP levels induced by oxidative stress, including those involving L-DOPA and 6-OHDA, using neuroblastoma cells and rat primary brain cell cultures need to be studied further.

In conclusion, the low levels of cAMP induced by forskolin resulted in proliferation that was mediated by transient ERK1/2 phosphorylation, which led to the stimulation of dopamine biosynthesis. However, the high levels of cAMP induced by forskolin resulted in differentiation that was mediated by sustained ERK1/2 phosphorylation, which led to the formation of neurite-like processes, and the cells then exhibited an apoptotic process through JNK1/2 and caspase-3 activation. In these states, a decrease in dopamine biosynthesis was shown. Similarly, multiple treatment of nontoxic concentrations of L-DOPA also showed the formation of neurite-like processes, and L-DOPA-induced cytotoxicity by multiple treatment of L-DOPA might be caused by the similar signal transduction pathways.

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REFERENCES


