Previously, we demonstrated that dopamine D1 receptor (D1R) agonists inhibit epidermal growth factor (EGF)-induced passage of mouse fetal cerebral cortical precursor cells from the G1 phase to the S phase of the cell cycle. Here, we report that this action of D1R agonists may involve regulation of cyclin D, and P27, which respectively promote and suppress the G1 to S transition. Furthermore, regulation of Raf-1, a component of the receptor tyrosine kinase mitogen-activated protein kinase pathway engaged in the mitogenic activity of EGF, may also be involved. Specifically, levels of cyclin D and Raf-1 decrease, whereas those of P27 first increase and then decrease in a dose-dependent fashion in response to the D1R agonist, SKF38393. This agonist also promotes Raf-1 phosphorylation on serine 338 residue, suggesting increased activation of this protein. Only the latter effect can be blocked by adenylyl cyclase (AC) and cAMP-dependent protein kinase A (PKA) inhibitors, and mimicked by agonists of the cAMP signaling pathway. Another D1R agonist, SKF83959, which stimulates phospholipase Cβ (PLCβ) but not AC, reduces levels of Raf-1 and cyclin D similar to SKF38393. However, we detected only down-regulation of P27 by this agonist. Additionally, the concentration-dependent patterns of both SKF38393- and SKF83959-induced alterations in the levels of P27 closely resemble the effects of these ligands on the levels of the D1R-PLCβ-associated second-messenger cascade linker, calycyan. These findings suggest that D1R-induced suppression of the cell cycle progression in EGF-supported fetal cortical precursor cells represents a net effect of competing cell cycle promoting and inhibiting molecular changes, which involve cyclin D, P27 and Raf-1. The data also show that cAMP second messenger cascade is not engaged in the D1R-induced regulation of the levels of these three proteins. Such regulation probably involves PLCβ-associated pathways.

Keywords: adenylyl cyclase–cAMP pathway, calycyan, cell cycle, corticogenesis, D1 dopamine receptor, phospholipase Cβ/inositol triphosphate pathway, receptor tyrosine kinase–Ras–Raf-1–mitogen-activated protein kinase pathway

Introduction

In our previous studies, we demonstrated that cortical precursor cells in the proliferative zones of the fetal cerebral wall express both D1a and D1b subtypes of the D1 dopamine receptor (D1R; Wang et al., 1997; Zhang and Lidow, 2002). Furthermore, since it had been shown that proliferation of different cortical precursor cells requires the presence of either epidermal growth factor (EGF) or fibroblast growth factor (FGF; Tropepe et al., 1999; Yamada et al., 1997; Raballo et al., 2000), we examined the ability of D1R to modulate cell cycle in primary cultures of these cells grown in the presence of the above-mentioned factors. We found that D1R activation prevented cultured cells from passing from the G1 phase into the S phase of the cell cycle (Zhang and Lidow, 2002).

The present work begins to address the molecular mechanism by which D1Rs prevent cerebral cortical precursor cells from entering the S phase of the cell cycle. EGF-supported cultures of cerebral cortical precursor cells were selected as the in vitro model for this study because we found them to be more proliferatively active than those supported by FGF (Zhang and Lidow, 2002). It has been postulated that such cultures represent the subventricular proliferative zone of the fetal cerebral wall (Burrows et al., 1997; Raballo et al., 2000). We have reported (Zhang and Lidow, 2002) that these cultures contain a mixture of multipotential, neuronal, and glial precursor cells, with a slight predominance of the latter cell type. More than 50% of the cells are proliferatively active; this includes all three aforementioned cell phenotypes, although multipotential and glial precursors display slightly higher proliferative activity as compared to neuronal precursors. Virtually all cells express both a and b subtypes of the D1R receptor, and stimulation of D1Rs results in a reversible suppression of cell cycle progression into the S phase for all three cell phenotypes identified in these cultures. This suppression is not accompanied by increases in the rates for either apoptosis or cell differentiation (Zhang and Lidow, 2002).

We focused on examining the ability of D1R to regulate three specific proteins. The first two proteins, cyclin D and P27 (Kip1), are major translators of extracellular influences on the cell progression through the G1-to-S restriction point of the cell cycle, with the promoting and inhibiting activities respectively (Sheaff and Roberts, 1998; Puri et al., 1999). Alterations in the levels of P27 have been described by some researchers as a part of cell differentiation (Tikoo et al., 1997; Perez-Juste and Aranda, 1999). However, detailed analysis showed that this does not reflect some additional activity of this protein, but rather is due its involvement in the suppression of the cell cycle, which is a prerequisite for differentiation of many cell types (Zecula et al., 2001; Munoz et al., 2003). The third protein, Raf-1 (c-Raf), is a component of the receptor tyrosine kinase (Trk)-Ras–Raf-1–MAP kinase (MEK–ERK) pathway involved in EGF-induced elevation of the levels of cyclin D (Denhardt, 1999), a primary cell cycle regulatory target of the mitogenic activity of this growth factor (Bogdan and Klambt, 2001). Our interest in Raf-1 further derives from its role as a factor integrating inputs of a wide range of extracellular signals (Denhardt, 1999; Gomperts et al., 2002). Consequently, Raf-1 is likely to be a part of the D1R-initiated cascade counteracting the EGF-induced up-regulation of cyclin D. In the case of Raf-1, not only its levels but also phosphorylation on the serine 338 (S338) residue were evaluated, since it has been demonstrated that phosphorylation
on this residue reflects Raf-1 activation (Diaz et al., 1997; Mason et al., 1999).

First, we confirmed our earlier finding (Zhang and Lidow, 2002) that the entry of cortical precursor cells in the EGF-supported primary cultures into the S phase of the cell cycle could be reversibly suppressed by agonist-induced activation of D1Rs. Then, we ascertained that this was accompanied by changes in the levels of the three proteins selected for examination in this study. Second, since activation of adenyl cyclase (AC) and elevation of the intracellular levels of cAMP are known to constitute major consequences of the D1R stimulation (Sibley et al., 1993; Missale et al., 1998), we determined whether the AC-cAMP second messenger cascade was involved in the regulation of the above-mentioned proteins. This also included an assessment of the possible role of protein kinase A (PKA), a major cAMP-dependent protein kinase that has the potential to influence Raf-1 activation (Stork and Schmitt, 2002). In addition, we tested the ability of CAMP cascade antagonists to block the D1R agonist-induced suppression of the cell cycle. Third, we evaluated the possible involvement of D1R-induced stimulation of phospholipase Cβ (PLCβ) and inositol triphosphate (DG/IP3) second messenger pathways (Undie, 1999). As a part of the last objective, we examined whether activation of D1Rs results in altered levels of the recently-discovered protein, calcyon, which links D1R to PLCβ-associated second messenger cascades, (Bergson et al., 2005), and whether the pattern of these alterations resembles those seen in the levels of cyclin D, P27, or Raf-1. In this regard, it is notable that a calcyon-like protein isolated from the broad bean Vicia faba is reported to be capable of affecting cell cycle in mammalian cells (Ng and Ye, 2003).

Materials and Methods

Drugs
SKF83959 [1-phenyl-2,3,4,5-tetrahydro-(H)3-3-benzazepine-7-b-diol], a conventional D1R agonist, which stimulates both AC and PLCβ (Roberts-Lewis, 1986; Johansen et al., 1991; Undie and Friedman, 1994; Jin et al., 1998), and the D1R antagonist, SCH23390 [8R+]-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-benzazepine; Lidow et al., 2001], were purchased from Sigma (St Louis, MO). SKF83959 [3-methyl-6-chloro-7,8-dihydroyx-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-benzazepine; Lidow et al., 2001], was also obtained from Sigma (St Louis, MO). The PKA inhibitor, H-89 {3-{methyl-6-chloro-7,8-dihydroxy-1-[3methylphenyl]-2,3,4,5-tetrahydro-1H-benzazepine; Lidow et al., 2001}, was purchased from Sigma (St Louis, MO). EGF-supported primary cultures into the S phase of the cell cycle of bromodeoxyuridine (BrdU)-labeled cells, 82 mM BrdU was added to the cultures for 1 h. After that, the BrdU-containing media was replaced by either dopamine drug-free or D1R agonist-containing media. The cell samples were collected for analysis every 3 h for 27 h. To verify the ability of cells to recover their proliferative activity after exposure to D1R agonist, cells cultures exposed to the agonist for 48 h (between 48 and 96 h after plating) were allowed to grow for additional 4 h in its absence. In the latter case, the control was represented by cultures grown in the absence of D1R drugs for 100 h after plating. To examine the time-course of changes in the levels of specific proteins, cells were collected after 12, 24, or 48 h of drug exposure (60, 72, or 96 h after plating, respectively).

Flow Cytometric Analysis of the Cell Cycle
Cultured cerebral cortical precursor cells were collected, pelleted by 10 min centrifugation at 300 × g on a Clinical Centrifuge (IEC, Austin, TX), and fixed in 70% ethanol for 30 min. The fixed cells were stained for 30 min with 5 µg/ml propidium iodine (PI; Molecular Probes, Eugene, OR) in the presence of 1 mg/ml RNase (Sigma, St Louis, MO). When BrdU labeling also needed to be detected, the PI-labeled cells were re-suspended for 20 min in 0.1 N HCl containing 0.25% triton X-100. They were then washed in 0.1 M NaB4O7, incubated overnight at 4°C with FITC-conjugated anti-BrdU antibodies (Sigma, St Louis, MO) diluted 1:200 in a blocking solution containing 0.5% Tween 20 and 1% BSA, and washed again in phosphate buffered saline (pH 7.4). Flow cytometric analysis was conducted on a FACS Vantage Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). The data acquisition and analyses were performed using CellQuest Pro (Becton Dickinson, Franklin Lakes, NJ) and ModFit LT (Versity, Topsham, ME) software. The percentages of cells in different stages of the cell cycle as well as the percentage of apoptotic cells were calculated based on PI staining. The cell cycle stage of BrdU-labeled cells was also assessed using PI staining of these cells.

PLCβ Activity
Activity of PLCβ in cultured cells was assessed by measuring phosphatidylinositol hydrolysis as described in Undie (1999) and Panchalalingam and Undie (2001). Briefly, 6 µCi/ml 2-[3H]inositol (American Radiolabeled Chemicals, St Louis, MO) was added into the culture media followed by the addition of LiCl (5 mM) 30 min later. At that time, D1R ligands may also be introduced into the media. Incubation continued for 90 min. The cells were collected and pelleted as described above. The concentration of the total protein in the pellet was determined using a modified Lowry Protein Assay kit (Pierce, Upland, IN). The pellet was resuspended in 1.0 ml chloroform/methanol/1 M HCl (100/200/1) mixture, and shaken for 15 min. Aqueous and organic phases were separated by further additions of 0.5 ml chloroform and 0.75 ml deionized water and centrifugation at 1000 × g for 5 min. Aliquots of the upper phase were taken and the content of inositol phosphates analyzed by Dowex anion exchange chromatography.

Cell Blots
Western Blots
Cells were collected and pelleted as described above. They were then homogenized by sonication with a Sonic Dismembrator (Fisher, Suwanee, GA) in 0.5 M Tris-HCl (pH 7.6) containing 0.1 M NaCl, 10 mM phenylmethylsulfonyl fluoride, 2mg/ml aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin (all from Sigma, St Louis, MO), and the concentration of the total protein in each sample was determined using a modified Lowry Protein Assay kit (Pierce, Upland, IN). Sample aliquots containing 40 µg total protein were loaded to the gels and separated by SDS-PAGE. Western Blots were probed for 2 h at room temperature with the following antibodies: 1:2000 rabbit polyclonal anti-PLCβ-1 (Chemicon, Temecula, CA), 1:1000 rabbit polyclonal anti-PKB (Cell Signaling, Beverly, MA), 1:500 mouse monoclonal anti-PLA2 (PharMingen, San Diego, CA), and 1:2000 rabbit polyclonal anti-Raf-1 (Chemicon, Temecula, CA). Blots were washed and probed at room temperature with 1:1000 biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) followed by 1:3000 streptavidin-HRP (Zymed, South San Francisco, CA). Blots were then developed using 3-4-chloro-1,7-naphthoquinone dihydrochloride (Sigma, St Louis, MO) and a Chemiluminescence Kit (Du Pont, Boston, MA). The bands were scanned and quantitated using a NIH Image 1.63 program (Wayne Rasband, National Institutes of Health, Bethesda, MD). Protein levels in the sample were normalized to γ-actin levels determined by Western Blots using anti-γ-actin (1:1500; Sigma, St Louis, MO). The results are expressed as a percentage of the control.
St Louis, MO) and incubated at 70°C for 10 min. The resultant mixtures were loaded onto 4%–15% gradient gels (Bio-Rad, Hercules, CA) and run at 100 mV for 1.5 h. Every gel also included a ‘standard homogenate’ made from the same drug-naive culture. This ‘standard homogenate’ was used to normalize the data between different experimental runs by always designating the densitometric values obtained from blots of this homogenate as equal to 1. Proteins resolved by electrophoresis were transferred onto Hybond ECL nitrocellulose membranes (Amersham, Piscataway, NJ) at 100 mV for 1.5 h. After blocking in 5% dry milk and 0.1% Tween 20 in phosphate buffered saline (pH 7.6), the membranes were processed for immunolabeling overnight at 4°C with one of the following antibodies mouse anti-cyclin D1 (dilution 1:500; Zymed, San Francisco, CA), mouse anti-P27 (dilution 1:500; Upstate, Waltham, MA), rabbit anti-Raf-1 phosphorylated on S338 residue (dilution 1:500; Upstate, Waltham, MA), or rabbit anti-calcyon (1:50; generated in house and described in Lezcano et al., 2000; Koh et al., 2003). Incubation with secondary peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (dilution 1:2000; Sigma, St Louis, MO) was conducted for 1 h at room temperature. The labeled bands were visualized on X-Omat AR Films (Kodak, Rochester, NY) using a SuperSignal Chemiluminescence kit (Pierce, Upland, IN). The uniformity of gel loading and protein transfer was verified by stripping the membranes of the antibodies, used in the initial immunolabeling, and processing them again for immunodetection of β-actin. The latter protein is well suited for these purposes because our previous studies have demonstrated that its levels in the primary cerebral cortical precursor cells remains virtually unchanged for up to 6 days of culturing (Zhang and Lidow, 2003). Mouse monoclonal antibodies for β-actin (1:1000 dilution) and goat anti-mouse peroxidase-conjugated secondary antibodies (1:10000 dilution) were purchased from Sigma (St Louis, MO). Film images of blots were digitized using a UC 1260 flat bed scanner (Umax, Dallas, TX) and subjected to densitometric analysis using IPLab software (Scanalytics, Fairfax, VA). This analysis was performed only on films generated by blots with variability in gel loading/protein transfer of <5% and in which optical densities of images were within the linear range of the relationship between the intensities of chemiluminescent immunosignal and the resultant optical densities of the film brand employed in the present study.

Statistical Analysis

All experiments were repeated five times. Within each repeat, each culture condition was reproduced in triplicate. The statistical analysis of the concentration-dependence of the effects of agonists (which also included the effects of D1R antagonist and AC-cAMP-PKA pathway agonists and antagonists) was performed by a one-way ANOVA followed by a Dunnett’s post hoc test. The ability of cultured cells to recover their proliferative activity after discontinuation of agonist exposure was evaluated using two-tailed Student’s t-test. The temporal effects of drug exposure were examined using a two-way ANOVA, with the presence of drugs and duration of exposure as variables. This was followed by a Tukey’s post hoc test.

Results

SKF38393 Regulation of the Cell Cycle

Prior to examining the capacity of the conventional (AC/PLCβ-stimulating) D1R agonist, SKF38393, to regulate specific cell-cycle-associated proteins, we confirmed the ability of this ligand to prevent EGF-supported fetal cerebral cortical precursor cells from entering the S phase of the cell cycle. For this purpose, we compared the cell cycle progression of BrdU pulse-labeled cells in the presence and absence of 75 μM SKF38393 (the cells were exposed to BrdU for 1 h immediately prior addition of the ligand). We found that in both SKF38393 exposed and unexposed cultures, the highest percentages of BrdU-labeled cells in S phase were detected shortly after BrdU exposure and ~15 h later (Fig. 1A). In addition, in both culture types maximal percentages of BrdU-labeled cells in G2-M phases were observed between 3 and 6 h and between 18 and 21 h after BrdU exposure (Fig. 1A). Finally, the highest percentages of BrdU-labeled cells in G0–G1 phases were observed ~9 and ~24 h after

Figure 1. Effect of the D1R agonist, SKF38393, on cell cycle progression and apoptosis in EGF-supported primary cultures of fetal cerebral cortical precursor cells. (A) Percentage of cells labeled 48 h after plating by 1 h BrdU (82 mM) exposure in G0–G1, S, and G2–M phases of the cell cycle within 27 h after BrdU-containing media was replaced by either ligand-free or 75 μM SKF38393-containing media. The cell samples were taken every 3 h. Each data point represents a mean of five separate experiments. The percentages in SKF38393-exposed cultures significantly different from the corresponding values in ligand-free cultures are marked by asterisks (p < 0.05, Tukey’s post-ANOVA test). (B) Ratio of cells in G0 and G1 phases of the cell cycle to cells in the S, G2, and M phases of this cycle. (C) Percentage of apoptotic cells among the total cells. In both A and C, 0–100 μM SKF38393 was added to the culture medium for 48 h, beginning 48 h after plating. The abilities of the D1R agonist SCH23390, the AC inhibitor SQ 22,536 and the PKA inhibitor H-89 (10 μM each) to block the effects of 75 μM SKF38393 are also presented. Each column shows the mean of the data from five separate experiments ± SEM. The values significantly different from the baseline (the first column in both histograms) are marked by asterisks (p < 0.05, Dunnett’s post-ANOVA test). Note that SKF38393 produced decrease in the percentage of BrdU-labeled cells entering S phase of the cell cycle. Also note a dose-dependent increase in the G0–G1/S–G2–M ratio, which was blocked by SCH23390. SQ 22,536 and H-89 were also able to significantly counteract the SKF38393-induced increase in the aforementioned ratio. At the concentrations tested in this study, none of the drugs caused significant apoptosis.
BrdU exposure independently of the presence or absence of the D1R agonist (Fig. 1A). While the presence of SKF38393 failed to produce notable changes in the maximal percentage of BrdU-labeled cells seen in the G0–G1 phases of the cell cycle, it decreased the percentage of these cells re-entering the S phase, which also resulted in a decreased percentage of the cells passing through the G2–M phases (Fig. 1A). These findings indicate that exposure to SKF38393 did not interfere either with the total length of the cell cycle or with the length of its phases. However, in agreement with our previous studies (Zhang and Lidow, 2002), this D1R agonist suppressed cell entry into the S phase. To further assess this aspect of SKF38393 actions, we also determined the percentages of cells in G0–G1, S, and G2–M phases in cultures exposed for 48 h to 0–100 μM SKF38393. There was a concentration-dependent rise in the percentage of G0–G1-phase cells accompanied by a decline in the percentage of S-phase and G2–M-phase cells. This produced an increase in G0–G1/S–G2–M ratio, which achieved the levels of statistical significance at 50 μM SKF38393 and above (Fig. 1B). Such elevation in G0–G1/S–G2–M provides further support of SKF38393-induced decrease in the percentage of cells entering the S phase of the cell cycle (Zhang and Lidow, 2002). The D1R specificity of SKF38393-induced alterations in G0–G1/S–G2–M was confirmed by the observation that they were absent when the cell cultures were exposed to this agonist (75 μM) in the presence of 10 μM of the D1R antagonist, SCH23390 (Fig. 1B). SCH23390 alone had no effect on the cell cycle (not shown).

We also found that, 4 h after discontinuation of 48 h long exposure to 75 μM SKF38393, the G0–G1/S–G2–M ratio in the recovering cell cultures was significantly higher than G0–G1/S–G2–M ratio obtained from cells cultured for a comparable period of time without this drug ever being present in the media (for recovering and control cultures, these ratios were 2.4 ± 0.5 and 6.3 ± 0.7 respectively, P of t-test > 0.05). This is in agreement with our earlier finding that D1R stimulation largely induces accumulation of proliferating cells in the G1 phase of the cell cycle, rather than forces these cells to exit the cycle and undergo terminal differentiation (Zhang and Lidow, 2002).

Incubation of the cultures with 75 μM SKF38393 in the presence of either 10 μM of the AC inhibitor, SQ 22,536, or 10 μM of PKA inhibitor, H-89, resulted in a small decrease in G0–G1/S–G2–M ratio as compared to that in cultures exposed to the agonist alone. Nevertheless, these ratios remained significantly elevated (Fig. 1B). None of the drug exposures resulted in changes in the levels of apoptosis (Fig. 1C).

**Visualization of the Proteins of Interest**

Immunolabeling of the Western blots generated from our cultures with antibodies to cyclin D and P27 visualized single bands with molecular weights of 36 kD and 27 kD respectively (Figs 2A and 3), which corresponded to the mol. wts of these proteins (Matsushima et al., 1992; Slingerland and Pagano, 2000). Both antibodies to total Raf-1 and to Raf-1 phosphorylated on S338 residue also produced a single band of the appropriate mol. wt of 74 kD (Figs 2A and 3; Stanton et al., 1989). Anti-calcyon antisera showed a 34 kD band (Fig. 7A,B) corresponding to that of calcyon modified by N-linked oligosaccharides (Lezcano et al., 2000; Koh et al., 2003). Antibodies to β-actin, used for verification of the uniformity of gel loading and protein transfer, labeled a protein with the mol. wt of 43 kD (Fig. 3), the weight of β-actin (Otey et al., 1987).

**SKF38393 Regulation of Cyclin D, P27 and Raf-1**

The aim of this part of the study was to determine whether SKF38393-induced suppression of S phase entry in the EGF-supported primary cultures of cerebral cortical precursor cells is accompanied by changes in the levels of cyclin D, P27, and Raf-1 proteins, as well as the level of Raf-1 phosphorylation at S338 residue. The study began with the examination of the effects induced by 12, 24, and 48 h of exposure to 75 μM SKF38393. Agonist-exposed cultures showed a time-dependent decrease in the levels of cyclin D and Raf-1 and an increase in the levels of P27 as compared to those in the age-matched drug-naive cultures (Fig. 2). This increase reached statistical significance at 24 h of SKF38393 exposure. SKF38393-exposed cultures also displayed elevated phosphorylation of Raf-1 on S338 residue, with this effect already achieving statistical significance by 12 h of agonist exposure (Fig. 2). These observations suggest that the D1R-induced alterations in the proteins examined in this study are more robust after 48 h of exposure. Therefore, all future analyses were performed at that time point.

The concentration dependence of the effects produced by 48 h long exposure to SKF38393 was examined at the concentration ranging from 0 to 100 μM. The levels of cyclin D and Raf-1 decreased in a concentration-dependent manner (Figs 3 and 4), with statistical significance being detected at 25 μM of the agonist and above (Fig. 4). In contrast, examination of the changes in the levels of P27 revealed a bell-shaped curve. P27 levels increased steadily when the cultures were exposed to 0–50 μM of SKF38390, but began to decline at higher (75–100 μM) concentrations of this agonist (Figs 3 and 4). The alterations in P27 levels were statistically significant between 25 and 75 μM of SKF38393 (Fig. 4). The extent of Raf-1 phosphorylation on S338 residue underwent yet another pattern of changes. It showed a concentration-dependent increase (Figs 3 and 4), with the statistical significance being achieved at the agonist concentrations of 50 μM and above (Fig. 4). All these SKF38393-induced changes were preventable by co-incubation with 10 μM SCH23390 (tested against 75 μM of SKF38393; Figs 3 and 5). SCH23390 alone produced no detectable effects (not shown).

**Examination of a Possible Involvement of AC-cAMP Second Messenger Pathway**

Activation of AC has been long identified as the most common consequence of D1R stimulation (Missale et al., 1989; Sibley et al., 1993), and the AC–cAMP–PKA cascade has been implicated in inhibiting Raf-1 and suppressing cell cycle progression in several reports (Cho-Chung et al., 1995; Denhardt, 1990; Sorkin and Schmitt, 2002). Therefore, it was reasonable for us to examine whether this cascade was also involved in the molecular and cell cycle suppressing effects observed in the present study.

We found that neither 10 μM of the AC inhibitor SQ 22,536 nor 10 μM of the PKA inhibitor, H-89 were able to counteract the changes in the levels of cyclin D, P27, and Raf-1 produced by 75 μM SKF38393 (Figs 3 and 5A). Furthermore, exposure to 10 μM of the AC activator, forskolin, and 100 μM of the cell-permeable cAMP analog, Sp-8-Br-cAMP also failed to elicit detectable changes in the levels of these proteins (Figs 3 and 5A). In contrast, the SKF38393-induced up-regulation of the S338 residue phosphorylation of Raf-1 was both blockable by SQ 22,536 and H-89 and reproducible by forskolin and Sp-8-Br-cAMP (Figs 3 and 5B).
Figure 2. Time-dependence of changes in the levels of cyclin D, P27, total Raf-1, and Raf-1 phosphorylated on S338 residue (P-S338) induced by 75 μM SKF38393 in EGF-supported primary cultures of fetal cerebral cortical precursor cells. The agonist was added to the cultures 48 h after plating and the data was collected after 12, 24 and 48 h of incubation. Age-matched drug-naive cultures served as controls. (A) Typical examples of immunostaining for cyclin D, P27, total Raf-1, and Raf-1 phosphorylated on S338 residue in Western blots generated from agonist-exposed and corresponding control cultures. (B) The relative levels of these proteins produced by 12-48 h of agonist exposure. The data are expressed as the ratios of the optical densities (ODs) of the film images produced by immunolabeling of a given protein in Western blots generated from drug-exposed cultures to the ODs of the film images produced by immunolabeling of the same protein in Western blots generated from age-matched drug-naive cultures (prior to calculation of these ratios, the ODs from all the experimental samples were normalized to the OD generated by the blots of ‘standard homogenate’ included in every experimental run). Each column shows the mean of the data from five separate experiments ± SEM. The columns representing significant differences between drug-exposed and age-matched drug-naive cultures are marked by asterisks (\( P < 0.05 \), Tukey’s post-ANOVA test). Note that exposure to SKF38393 produced time-dependent changes in all the proteins examined in this study.

Figure 3. Typical examples of immunostaining for cyclin D, P27, total Raf-1 and Raf-1 phosphorylated on S338 residue (P-S338) in Western blots generated from EGF-supported primary cultures of fetal cerebral cortical precursor cells, which 48 h after plating were exposed for additional 48 h to: 0–100 μM of the D1R agonist, SKF38393; 75 μM SKF38393 in combination with either 10 μM of the AC inhibitor SQ 22,536, 10 μM of the PKA inhibitor, H-89, or 10 μM of the D1R antagonist, SCH23390; 100 μM of the cell permeable cAMP analog, Sp-8-Br-cAMP; and 10 μM of the AC activator, forskolin. Examples of the β-actin immunostaining (which was used to verify the uniformity of gel loading/protein transfer) from the blots employed for visualization of cyclin D are also included. The histograms associated with these blots are presented in Figures 4 and 5.
available drugs are incapable of distinguishing between members of the PLC\(\beta\) and PLC\(\gamma\)-initiated intracellular cascades. Therefore, it would be impossible to discern which of the cell-cycle-associated effects of these drugs specifically relate to their interference with the neurotransmitter receptor-PLC\(\beta\) cascade rather than the EGF-PLC\(\gamma\) cascade. We took a different approach by employing SKF83959, a novel D1R agonist that induces receptor-coupled stimulation of PLC\(\beta\) without constitutive activation of AC (Panchalingam and Undie, 2001; Jin et al., 2003).

We began by examining whether activation of D1R in our primary cultures of EGF-supported cerebral cortical precursor cells could lead to stimulation of PLC\(\beta\) and, thus, increase in phosphatidylinositol hydrolysis. We found that application of SKF83959 (10 \(\mu\)M) nearly doubled the levels of \([^{3}H]\)inositol phosphate accumulation (Fig. 6A). This accumulation was absent when SKF83959 was added to the cultures in combination with the D1R antagonist, SCH23390 (0.1 \(\mu\)M, Fig. 6A).

We then assessed the capacity of SKF83959 (concentration 0–100 \(\mu\)M; exposure 48 h) to interfere with the cell cycle in EGF-supported primary cultures of cerebral cortical precursor cells. As in the case of SKF38393, SKF83959 produced a concentration-dependent increase in Go–G1/S–G2–M ratio. This increase reached statistical significance at 10 \(\mu\)M of the agonist (Fig. 6A). The increase in Go–G1/S–G2–M ratio produced by the latter concentration of SKF83959 was blocked by 0.1 \(\mu\)M SCH23390. However, in contrast to SKF38393, SKF83959 produced a significant rise in apoptosis at concentrations >10 \(\mu\)M (Fig. 6B). Consequently, evaluation of the effects of this agonist on the levels of cyclin D, P27, and Raf-1 were examined at 0.3, 3.0 and 10 \(\mu\)M, which did not induce significant apoptosis in our cultures. Exposure to these concentrations of SKF83959 for 48 h resulted in concentration-dependent decreases in the levels of cyclin D, Raf-1 and P27; the effects were statistically significant for 10 \(\mu\)M of the agonist (Fig. 6C). These changes were not observed when 0.1 \(\mu\)M SCH23390 was also added to the media (Fig. 6C).

**Figure 4.** Concentration-dependence of changes in the levels of cyclin D, p27, total Raf-1, and Raf-1 phosphorylated on S338 residue (P-S338) produced by 48 h long exposure of EGF-supported primary cultures of fetal cerebral cortical precursor cells to 0–100 \(\mu\)M of the D1R agonist, SKF38393. The exposure began 48 h after the cells were plated. The data are expressed as the ratios of the optical densities (ODs) of the film images produced by immunolabeling of a given protein in Western blots generated from drug-exposed cultures to the ODs of the film images produced by immunolabeling of the same protein in Western blots generated from drug-naive cultures (prior to calculation of these ratios, the ODs from all the experimental samples were normalized to the OD generated by the blots of ‘standard homogenate’ included in every experimental run). Each column shows the mean of the data from five separate experiments ± SEM. The columns representing significant differences between drug-exposed and drug-naive cultures are marked by asterisks (* \(P<0.05\), Dunnett’s post-ANOVA test). Note concentration-dependent decrease in the levels of cyclin D and total Raf-1, bell-shaped changes in the levels of P27, and increase in the levels of P-S338-Raf-1.

Co-incubation for 48 h of tissue cultures with 75 \(\mu\)M SKF38393 and either 10 \(\mu\)M SQ 22 536 or 10 \(\mu\)M H-89 produced a small statistically insignificant reduction in the Go–G1/S–G2–M ratio as compared to that in cultures exposed to the agonist alone (Fig. 5C).

**Effects of SKF83959**

Since the above-described observations have suggested that the AC–CAMP second messenger pathway is unlikely to be responsible for the D1R stimulation-induced alterations in the levels of the three cell-cycle-controlling proteins examined in this study, we decided to assess possible involvement of D1R-driven PLC\(\beta\)-associated pathways (Undie, 1999; Bergson et al., 2003). Unfortunately, direct agonists and antagonists of these pathways cannot be used under the present circumstances because mitogenic effects of EGF involve PLC\(\gamma\), the activity of which overlaps with that of PLC\(\beta\) (Fedi et al., 2000), and all presently available drugs are incapable of distinguishing between members of the PLC\(\beta\) and PLC\(\gamma\)-initiated intracellular cascades.

Recently, it has been demonstrated that one of the mechanisms by which D1R may stimulate PLC\(\beta\) involves the D1R-interacting protein, calcyon, which acts as a molecular linker between these receptors and G\(_{q}\) proteins coupled to heterologous non-dopamine receptors (Lezcano et al., 2000; Bergson et al., 2003; Dai and Bergson, 2003) In order for calcyon to work it must be primed, which can be achieved in several ways (Bergson et al., 2003). It would not be unreasonable to expect that at least some active calcyon molecules could be present in our cultured cells. We felt that it might be informative to learn whether the levels of this protein in our cultures were regulated by SKF38393 and SKF83959 and whether the observed changes resembled those in the levels of the three cell-cycle-regulatory proteins examined in this study. We found that, indeed, calcyon levels in our cultures were affected by both agonists in a concentration-dependent manner. Furthermore, alterations in calcyon levels produced by 48 h long exposure to 0–100 \(\mu\)M SKF38393 showed a bell-shaped curve. They increased steadily when the cultures were exposed to 0–50 \(\mu\)M of SKF38393, but declined at higher concentrations of this agonist (Fig. 7A,C). The alterations in calcyon levels were statistically significant between 25 and 75 \(\mu\)M of SKF38393 (Fig. 7C). In contrast, 48 h long exposure to
0–10 μM of SKF83959 induced a concentration-dependent decrease in calcyon levels; at 10 μM SKF83959, this decrease was statistically significant (Fig. 7B,D). These effects of SKF8393 (75 μM) and SKF83959 (10 μM) were blocked by 10 and 0.1 μM SCH23390 respectively (Fig. 7). The changes in the calcyon levels produced by both drugs were very similar to the earlier-described changes in the levels of P27.

Discussion

D1R regulation of the cell cycle in EGF-supported cerebral cortical precursor cells is accompanied by alterations in Raf-1, cyclin D, and P27. The present study supports our earlier observations (Zhang and Lidow, 2002) that stimulation of D1Rs is capable of suppressing the passage of EGF-supported cerebral cortical precursor cells from the G1 phase into the S phase of the cell cycle. This was demonstrated by the observation that D1R agonist, SKF8393, reduced the proportion of cells entering the S phase of the cell cycle without significantly affecting the length of the cycle. In addition, we found the G0–G1/S–G2–M ratio to increase in a concentration-dependent manner in response to applications of two different D1R agonists, SKF83893 and SKF83959, with this increase being blocked by the D1R antagonist, SCH23390. Also, as in our previous studies (Zhang and Lidow, 2002), the increase in the G0–G1/S–G2–M ratio was reversible upon discontinuation of agonist exposure. Moreover, shortly after removal of the ligand, this ratio declined to the levels significantly below that seen in never-treated cultures. This points to an overshoot in the number of cells entering the S phase, which is most consistent with the expected aftermath of the D1R stimulation-induced accumulation of cells in the late G1 phase (Stein et al., 1998). Finally, at all SKF83893 concentrations employed in this study and at SKF83959 concentrations up to 10 μM, the inhibition of the cell cycle was not associated with a significant increase in the percentage of apoptotic cells.

A novel finding of this study is that D1R stimulation-induced suppression of the proliferative activity in EGF-supported primary cultures of cerebral cortical precursor cells is accompanied by changes in the levels of cyclin D and P27 and levels and activation of Raf-1. These three proteins are known to be closely associated with regulation of the G1 to S transition of the cell cycle.
cell cycle (Sheaff and Roberts, 1998; Denhardt, 1999; Puri et al., 1999). Therefore, it is reasonable to propose that the mechanism of the observed D1R influence over the cerebral cortical precursor cell progression into the S phase of the cell cycle, at least in part, involves these proteins.

It is also interesting that, based on our observations, the application of D1 agonists to the EGF-supported cerebral cortical precursor cells induces both cell-cycle-suppressing and cell-cycle-promoting molecular changes. Thus, in the case of Raf-1 we observed a combination of a decrease in its levels (which should lead to an inhibition of Trk–Ras–Raf-1–MEK–ERK cascade and consequently a suppression of the EFG-promoted cell cycle; Denhardt, 1999), and an increase in its phosphorylation on S338 residue, which indicates a heightening of its Trk-Ras-dependent activation and consequently a potentiation of the above-mentioned cascade that is associated with promotion of the cell cycle (Díaz et al., 1997; Mason et al., 1999). Also, between the two direct cell-cycle-regulatory factors examined in this study, the cell-cycle-promoting protein, cyclin D, alone showed a persistent dose-dependent D1R agonist-induced down-regulation, which is consistent with the observed cell-cycle-inhibiting action of D1Rs. In contrast, the dose dependent up-regulation of the cell cycle progression-inhibiting protein, P27, was seen only up to 50 μM SKF38393. With further increases in the SKF38393 concentration, the magnitude of the P27 up-regulation declined until this up-regulation completely disappeared at 100 μM of the agonist. Furthermore, SKF83959 concentrations examined in this study produced a decrease in P27 levels suggesting a cell-cycle-promoting activity. This indicates that the D1R-induced suppression of the cell cycle progression in our EGF-supported primary cultures of cerebral cortical precursor cells represents a net effect of competing cell-cycle-promoting and inhibiting molecular changes, with the latter changes being more predominant in this case.

It should be noted that, as was mentioned earlier, our cultures contain a mixture of multipotential, glial, and neuronal precursor cells. The methodology used in this study does not allow us to determine whether the observed protein changes occur in all or only some subpopulations of these cells. We hope that future studies will be able to address this issue.

![Figure 6. Effects of SKF83959.](image)
**cAMP Second Messenger Cascade is not Responsible for D1R-induced Alterations in the Levels of Raf-1, Cyclin D and P27**

The central finding of this study is that the D1R-induced increase in the intracellular levels of cAMP, as well as the resultant activation of the major cAMP-dependent protein kinase, PKA, may not be involved in alterations in any of the three proteins examined in our cultured cells. This was demonstrated by the inability of the AC inhibitor, SQ 22,536, and the PKA inhibitor, H-89, to prevent SKF38393 from inducing such alterations, as well as the failure of the AC stimulator, forskolin, and the cell permeable cAMP analog, Sp-8-Br-cAMP, to reproduce the SKF38393-induced changes. Such lack of regulatory activity by the cAMP cascade agonists and antagonists is unlikely to be due to their application at ineffective concentrations since the same drug applications were fully capable of respectively blocking or replicating the SKF38393-induced increase in the cycle-promoting activation of Raf-1 (seen as up-regulation of its phosphorylation on S338 residue). The observed lack of CAMP and/or PKA participation in cell cycle suppression through Raf-1, cyclin D, and P27 is very surprising since inhibition of Raf-1 activity, down-regulation of cyclin D, and up-regulation of P27 have long been considered among the major cell cycle regulatory actions of the cAMP–PKA intracellular cascade (Hafner et al., 1994; Kato et al., 1994; Cho-Chung et al., 1995; Ward et al., 1996; Cospedal et al., 1999; van Oirschot et al., 2001; Stork and Schmitt, 2002; Shibata et al., 2003). Furthermore, our data suggest that EGF-supported cerebral cortical precursor cells represent one of the relatively rare biological systems in which cAMP second messenger pathway is capable of promoting the activation of Raf-1 (Stork and Schmitt, 2002). As mentioned above, this represents a stimulatory influence on cell proliferation (Diaz et al., 1997; Mason et al., 1999). It is important to note, however, that cAMP-induced inhibition of Raf-1 might be present in other groups of proliferating cerebral cortical neural cells. This is indicated by the reported involvement of this second messenger in deactivation of Raf-1 in cultures of bFGF-supported cortical astrocytes (Kurino et al., 1996).

**Possible Involvement of PLCβ-associated Second Messenger Cascades**

The apparent lack of involvement of the cAMP second messenger cascade in the changes of the levels of Raf-1, cyclin D and P27 seen in our cell cultures does not necessarily exclude this cascade from participating in the cell cycle suppression in these cultures through some other intracellular venues. Indeed, we observed that the addition of antagonists of the cAMP pathway to our SKF38393-treated cultures produced some reduction in the cell cycle suppressing activity of this D1 receptor agonist. However, since exposures of similar cultures to the D1R agonist, SKF83959, which stimulates only PLCβ, resulted in suppression of the cell cycle progression, it is reasonable to propose that activation of the cAMP cascade is not necessary for the cell cycle regulatory actions of D1Rs in EGF-supported cerebral cortical precursor cells. These actions may be conducted via the PLCβ-associated second messenger cascades. Moreover, the ability of SKF83959 to mimic SKF38393-induced concentration-dependent down-regulation of Raf-1 and cyclin D levels suggests that...
these D1R-induced effects may also be driven by PLCβ stimulation. While we are unaware of any studies that have examined PLCβ cascade-induced regulation of Raf-1 levels, the ability of this cascade to affect the levels of cyclin D has been described in several papers (Bianchi et al., 1994; Fukushima et al., 1997; Frey et al., 2000). The concentration-dependent patterns of P27 regulation by SKF83959 did not match those produced by SKF38393. Nevertheless, D1R may still affect the levels of this protein via PLCβ-associated cascades. Such a possibility comes out of our observation of the SKF38393- and SKF83959-induced changes in the levels of calcyon, a protein linking D1Rs to PLCβ stimulation. This protein also was differentially regulated by SKF38393 and SKF83959, with its concentration-dependent changes in response to both agonists resembling closely the concentration-dependent changes induced by these agonists in the levels of P27. It is possible that the observed alterations in the levels of calcyon and P27 represent two independent parallel intracellular processes, but it is tempting to speculate that there is a causal relationship between them, and, since calcyon is a part of the D1R-Gq protein-PLCβ signalling system, this relationship would indicate the PLCβ involvement in P27 regulation by D1Rs. The latter would not be unexpected because the ability of PLCβ-associated pathways to regulate the levels of P27 is well documented (Ashton et al., 1999; Chen et al., 1999; Frey et al., 2000). The fact that SKF38393-induced changes in the levels of calcyon do not match the changes in the levels of Raf-1 and cyclin D may indicate that the D1R-induced regulation of the latter two proteins involves a calcyon-independent pathway. Such a possibility comes out of our observation that directly activate Rap1. Science 282:2275--2279.


References

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