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PKC Activity and PKC-α mRNA Content Are Reduced in Serum-Deprived Human Neuroblastoma Cells without Concomitant Induction of Differentiation

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Protein kinase C (PKC) is a serine/threonine kinase which is thought to play an important role in cellular proliferation and differentiation. PKC activity is stimulated physiologically by diacylglycerol and experimentally by phorbol esters. Long-term exposure of human neuroblastoma cells to phorbol esters results in down-regulation of PKC activity and induction of neuronal differentiation. In this study, we explored the hypothesis that reduced PKC expression is necessary for differentiation of the human neuroblastoma cell line SK-N-SH. PKC activity and PKC-α mRNA levels were assayed in cultured SK-N-SH cells over a period of several days in the presence or absence of serum. These determinants of PKC expression were compared with several known markers of neuroblastoma differentiation, including neurite outgrowth and steady-state levels of c-myc and GAP43 mRNA. We observed steady losses of PKC activity and PKC-α mRNA content after transfer of cells to serum-free or chemically defined media. However, morphological and biochemical differentiation of SK-N-SH cells occurred only in chemically defined medium, perhaps due to the presence of insulin. We conclude that while loss of PKC may be associated with neuroblastoma differentiation, diminished PKC alone is not sufficient to induce or support the differentiation process.

INTRODUCTION

Protein kinase C (PKC) is a lipid-activated kinase which is known to play an important role in a wide variety of fundamental cellular processes, including proliferation and differentiation [1]. The ability of phorbol esters, which directly regulate PKC activity, to induce differentiation of many cell types has prompted a considerable research effort into defining the significance of PKC activity in the differentiation process. Extended incubation of cells with phorbol ester (>24 h) is known to induce down-regulation of protein kinase C activity due to an increased rate of PKC degradation [2, 3]. Åkerman and colleagues were first to report that phorbol ester-mediated down-regulation of PKC may be responsible for the induction of differentiation in neuroblastoma cells [4]. This group demonstrated that long-term incubation with the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), but not diacylglycerol, induced neurite outgrowth in the human neuroblastoma cell line, SH-SY5Y, while short-term incubation with either TPA or diacylglycerol activated both PKC activity and c-fos expression [4]. They also showed that the time course of TPA-induced down-regulation was well correlated with the TPA-induced neurite outgrowth and the changes in several markers of differentiation [5]. Girard and Kuo extended this work by showing that a decrease in phosphorylation of the endogenous 80-kDa PKC substrate (MARCKS protein) correlated with morphological differentiation [6]. Several groups have also reported that kinase inhibitors, such as 1-(isoquinolinylsulfonyl)-2-methylpiperazine (H7) and staurosporine, which are partially selective for PKC, induced neurite outgrowth in neuroblastoma [7, 8] and PC12 cells [9, 10]. Furthermore, loss of PKC activity has also been found to be associated with retinoic acid-induced neuroblastoma differentiation [6, 11]. These studies have led to the idea that PKC may act as a negative effector in neuronal differentiation.

To investigate further the relationship between loss of PKC activity and the onset of differentiation, we have compared PKC activity and PKC-α mRNA content with the state of differentiation of a human neuroblastoma cell line maintained in a variety of growth media. We report here that serum deprivation induced loss of PKC activity and PKC-α mRNA content without a concomitant onset of differentiation. Therefore, although loss of PKC activity may play a role in the differentiation process in neuroblastoma, diminished PKC activity alone may not be sufficient to induce differentiation.

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MATERIALS AND METHODS

Cell culture. Human SK-N-SH neuroblastoma cells were kindly provided by Dr. Stephen Fisher (University of Michigan Medical School, Ann Arbor, MI) and grown at 37°C in a humidified atmosphere of 10% CO_2. Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL) was the normal growth medium. To test effects of medium other than FBS, SK-N-SH cells were passaged and grown for 2 days in FBS, washed with DMEM, and then incubated in test medium (or fresh FBS to serve as a control). The chemically defined medium (CD) used was a modification of the N2 medium of Bottenstein and Sato [12], containing 5 μg/ml insulin (Boehringer Mannheim, Indianapolis, IN), 100 μg/ml transferrin (human, partially iron saturated, Boehringer Mannheim), 20 n*M* progesterone, 100 μ*M* putrescine (Sigma, St. Louis, MO), 30 n*M* sodium selenate, and 0.1% (w/v) fatty acid-free BSA (Calbiochem, San Diego, CA) in DMEM.

cDNA probes. Human PKC-α cDNA was obtained from American Type Tissue Culture (Washington, DC). The cDNA was supplied as an insert in a pUC12 plasmid in *Escherichia coli.* Plasmid was purified as described by Krieg and Melton [13], and the 1.3-kb PKC-α cDNA insert was removed by digestion with *Eco*R1 (GIBCO BRL), and purified by electrophoresis and eluted from gel slices by the method of Vogelstein and Gillespie [14]. Rat c-myc cDNA was generously provided by Dr. C. D. Logsdon (University of Michigan Medical School) from stock originally supplied by Dr. E. V. Prochownik [15]. Rat GAP43 cDNA [16] was generously provided by Dr. M. B. Willard (Washington University School of Medicine, St. Louis, MO). Chicken β-actin was kindly provided by Dr. Paul Killen (University of Michigan Medical School, Ann Arbor, MI) from stock originally provided by Dr. D. W. Cleveland [17].

Northern analysis. Northern analysis was performed as previously described [18] with minor modifications. Total RNA was isolated from SK-N-SH cells using a guanidinium thiocyanate–chloroform–phenol extraction procedure [19]. RNA samples (20–25 μg) were electrophoresed in gels containing 1% agarose, 2.2 *M* formaldehyde, 0.02 *M* Mops, 1 m*M* EDTA, and 5 m*M* sodium acetate. Electrophoresed RNA was blotted by capillary transfer onto Nytran membranes (Schleicher and Schuell, Keene, NH) following the supplier's guidelines. For hybridization, membranes were incubated for 18 h at 42°C in 10% dextran sulfate, 1 *M* NaCl, and 1% SDS with random-primer-labeled cDNA (random primer labeling kit, Promega, Madison, WI; [α-^{32}P]dCTP, ICN, Irvine, CA) of specific activity greater than 1×10^9 cpm/μg at concentrations from 0.1 to 0.5 ng/ml. Membranes were washed successively, twice each for 15 min in 2× SSC (0.1 *M* NaCl, 0.15 *M* sodium citrate) and 1% SDS at room temperature, in 0.5× SSC and 1% SDS at 65°C, and in 0.1× SSC and 0.1% SDS at 65°C. X-ray film was exposed to washed membranes for from 5 h to 7 days at −70°C. An RNA ladder (GIBCO-BRL) was electrophoresed with samples for use in determining transcript sizes.

Densitometry. Imaging software (MCID, Imaging Research Inc., St. Catherines, Ontario, Canada) was used for densitometric analysis of digitized images of the autoradiographs. At least three exposures, including brief (2–24 h) and long (several days) exposures, were produced for each blot. Excepting very short exposures (<6 h), the variation in the relative optical density (ROD) of any given band relative to the appropriate internal control band (from RNA of untreated cells) was less than 10% over a broad range of exposures (6 h to several days). To determine the average change in mRNA content for a given condition, the ROD of a band was first normalized to the ROD of the corresponding β-actin band to control for differences in gel sample loading, and then this normalized ROD was expressed as a percentage of the ROD of the band from internal control sample.

PKC activity. The PKC assay method was modified after House *et al.* [20]. Cells were broken by probe sonication in homogenization buffer (0.05 *M* Tris-HCl, pH 7.5) containing 5 m*M* EDTA, 10 m*M* EGTA, 0.6 g/liter β-mercaptoethanol, 10 m*M* benzamidine, and 1 m*M* phenylmethylsulfonyl fluoride. Nuclei and unbroken cells were removed by a low-speed spin, and the resulting supernatant was spun at 100,000*g* for 30 min. The 100,000*g* supernatant was termed the soluble or cytosolic fraction. The 100,000*g* pellet was washed with homogenization buffer, suspended by probe sonication in homogenization buffer containing 0.1% Triton X-100, incubated on ice for 1 h, and spun again at 100,000*g*. The resulting supernatant was termed the particulate or membrane fraction. The soluble and particulate fractions were diluted with 0.05 *M* Tris–HCl (pH 7.5) and 25 μl of each was added to equal volumes of reaction solution consisting of 0.05 *M* Tris–HCl containing 1.5 m*M* $CaCl_2$, 7.5 m*M* dithiothreitol, 45 μ*M* PKC peptide substrate ([ser25 PKC(19-31)], GIBCO-BRL), ±82 μ*M* dioleoylphosphatidylserine (Sigma), and 3 μ*M* dioleoylglycerol (Sigma). Then 25 μl of 150 μ*M* [^{32}P]ATP (sp act 50–100 mCi/mmol) in 0.05 *M* Tris–HCl with 45 m*M* $MgCl_2$ was added to initiate the reaction. After an incubation of 20–40 min, 100 μl of 10% TCA was added to quench, and 125 μl of this mixture was blotted on Whatman P81 paper discs. The blotted discs were washed twice in 5% acetic acid and the bound radioactivity was counted using liquid scintillation spectroscopy. Activity specific to PKC was defined as the difference in the activity [pmol (of phosphate in bound substrate)/min/mg protein] in the presence or absence of PS and DG. Protein content in the soluble and in the particulate fractions was measured using a modification of the Lowry method [21].

RESULTS

Cell Morphology

SK-N-SH cells grown in DMEM supplemented with 10% FBS developed short neurites between 1 and 2 days after seeding, and these neurites did not appear to elongate further over successive days (Fig. 1a). When SK-N-SH cells were transferred to CD, these short neurites progressively elongated and became more dense over successive days (Fig. 1b). In contrast, such changes in neurite elongation and density were not observed after transfer from FBS to DMEM or to CD without added insulin (Figs. 1c and 1d). Clustering of cell bodies precluded a quantitative assessment of the neurite outgrowth.

PKC Activity

PKC activity was assayed selectively in soluble and in particulate cellular fractions using a peptide analog of the pseudosubstrate prototope of the PKC regulatory domain, modified to function as a specific PKC substrate [22]. In the presence of calcium, an increased phosphorylation of this substrate was observed after addition of lipid micelles composed of PS and DG. Such lipid-mediated stimulation is diagnostic of PKC activity. No PKC activity was detectable in the absence of calcium. Within 24 h after transfer from FBS to CD, PKC activity was decreased in both the soluble and the particulate fractions. Thereafter, in the soluble fraction, PKC activity continued to decrease, comprising a loss of 66% at Day 7 relative to cells maintained in FBS (Fig. 2). In the particulate fraction, the activity decreased nearly 40% relative to FBS after 2 days in CD and remained at that decreased level through to Day 7. Growth of cells in DMEM alone for 7 days resulted in a loss of PKC activity in both the soluble and particulate

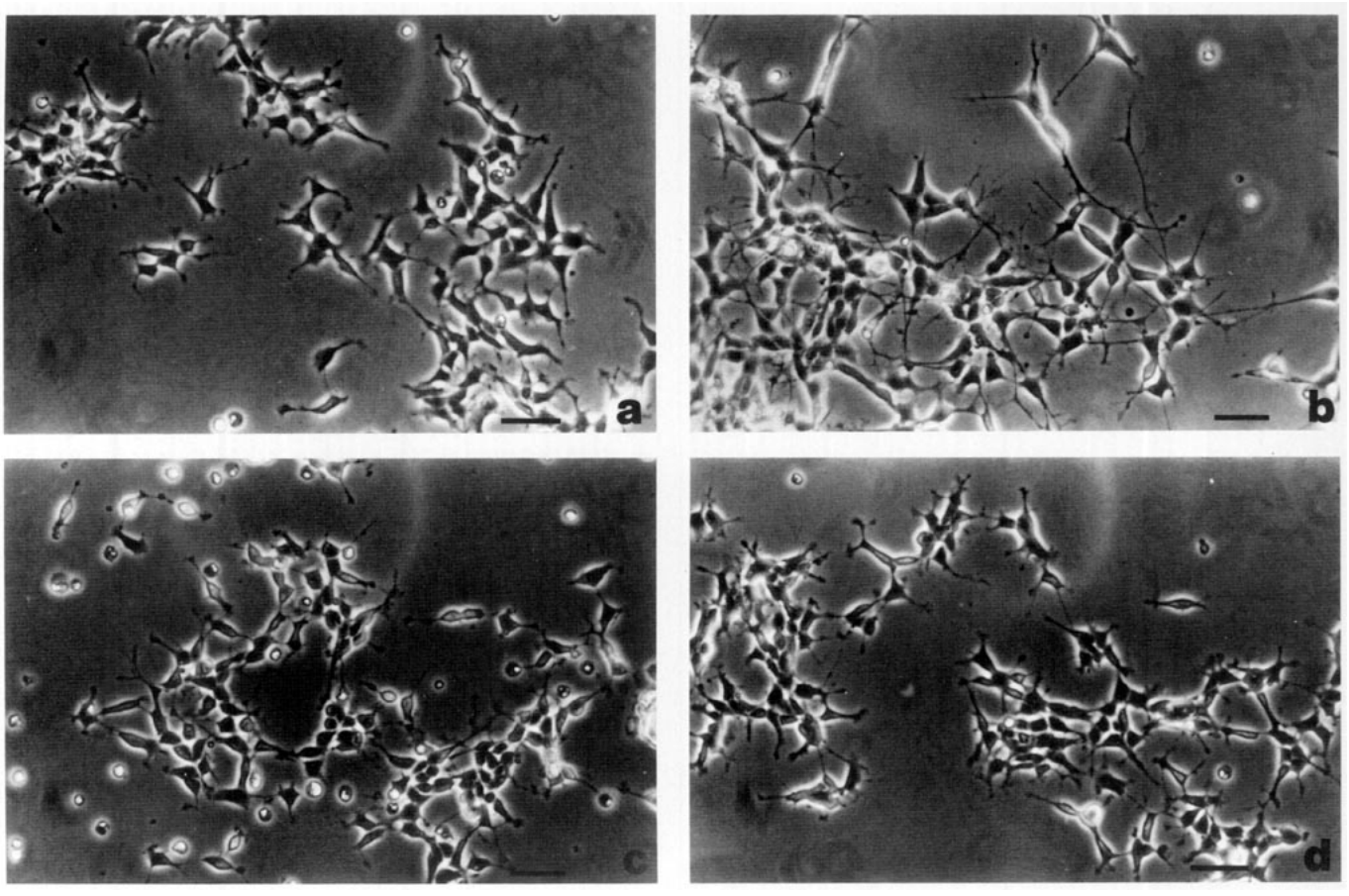


FIG. 1. Representative photographs of SK-N-SH human neuroblastoma cells maintained for 4 days in 10% FBS (A), CD (B), DMEM (C), or CD without insulin (D). Bar, 50 μm. Similar morphology was observed and photographed in other experiments after 4 days of growth in FBS ($N = 8$), CD ($N = 5$), DMEM ($N = 6$), or CD without insulin ($N = 3$) and at other time points over a period of 7 days after the change of medium, except that in CD there was a progressive increase in length and density of neurites (data not shown).

fractions of SK-N-SH cells comparable to that measured for CD (Fig. 2).

Steady-State PKC-α mRNA Levels

Because PKC activity was reduced in both soluble and particulate fractions from serum-deprived cells, we tested the hypothesis that such a reduction may result from changes in PKC gene expression. To test this hypothesis, we used Northern analysis of total RNA to measure the steady-state levels of PKC-α mRNA in SK-N-SH cells. PKC-α mRNA was easily detectable in total RNA extracts of SK-N-SH cells grown in FBS or serum-free media (Figs. 3, 4, and 5). Transcript sizes of 9.0 and 4.0 kb were observed, consistent with previous reports in neuroblastoma [23] and other neural tissues [24, 25]. The 9.0-kb transcript was much more abundant than the 4.0-kb transcript; therefore, changes in the content of the 9.0-kb mRNA were used as a measure of PKC-α mRNA levels.

The content of the 9.0-kb transcript was found to decrease progressively in SK-N-SH maintained in CD (Figs. 3A and 3B). After 7 days of growth in CD, the 9.0-kb PKC-α mRNA content was reduced 44% relative to cells maintained in FBS (Fig. 3B). The magnitude and time course of this loss of PKC-α mRNA correlated with the loss of soluble PKC activity (Fig. 2).

We next sought to determine whether this loss of PKC-α mRNA, like that of PKC activity, was due to removal of serum. Cells were grown in FBS, CD, or DMEM for 4 days, after which the PKC-α mRNA content was found to be decreased in cells grown in either CD or DMEM relative to cells grown in FBS (Fig. 4). In addition, PKC-α mRNA levels were consistently lower in cells grown in DMEM than in CD. After a 4-day period in DMEM, PKC-α mRNA was reduced to about 20% compared to FBS-treated cells, and in CD, PKC-α mRNA was reduced to about 50% (Fig. 4). PKC-α mRNA content was further reduced after 6 days in DMEM to about 10% of that in cells grown in FBS ($n = 2$; data not shown). As shown in Fig. 3, PKC-α mRNA levels in CD were reduced only to about 40% of that in FBS.

We speculated that a supplement or supplements present in CD were responsible for the significantly greater content of PKC-α mRNA in cells grown in CD

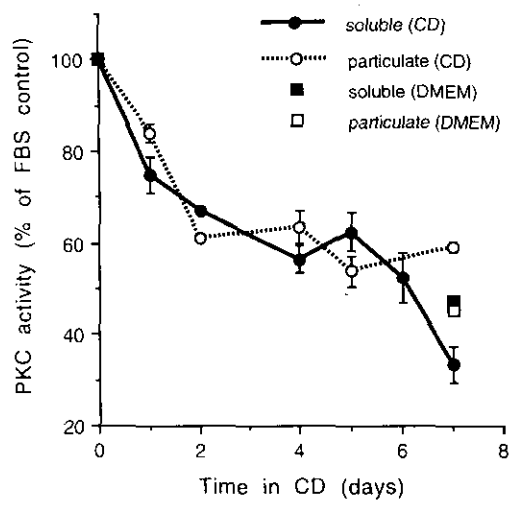


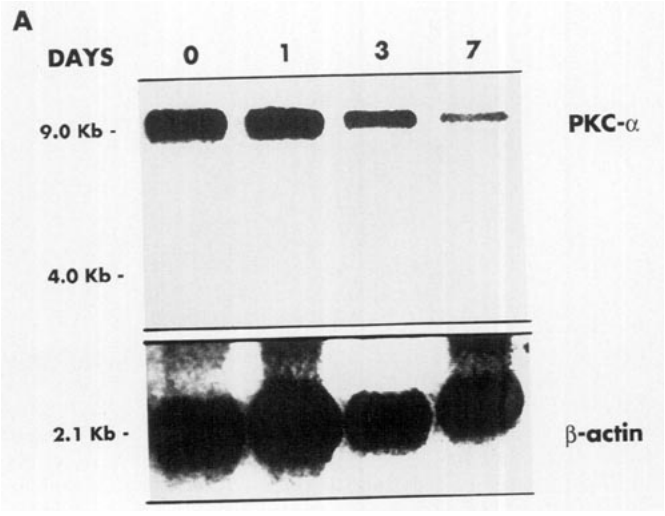
FIG. 2. PKC activity was measured in soluble and particulate fractions of SK-N-SH cells grown in 10% FBS, CD, or DMEM over a period of 7 days. Activity in CD or DMEM is depicted as the percentage of the FBS control [from the same passage and day(s) in culture]. The activity from cells in FBS did not change as a function of days in culture. The mean specific activity of SK-N-SH cells grown in FBS was 4000 ± 560 pmol/min/mg for the soluble fraction and 1376 ± 283 pmol/min/mg for the particulate fraction. Each point represents the mean activity (±SD) in CD relative to FBS for at least two experiments in which activity in samples were measured in triplicate. Each point after Day 0 was different from the same-day FBS control at $P < 0.01$ as determined by paired t test.

relative to cells grown in DMEM. To identify which components of CD were responsible for this difference, SK-N-SH cells were maintained in CD without insulin or without progesterone. After 4 days of growth in CD without insulin, the level of PKC-α mRNA was found to be commensurate with that of cells maintained in DMEM alone (Fig. 4). In contrast, PKC-α mRNA in cells grown in CD without progesterone was equivalent to that of cells grown in CD ($n = 2$; data not shown). Therefore, the presence of insulin in CD is likely necessary for the greater content of PKC-α mRNA in cells grown in CD relative to cells grown in DMEM.

c-myc and GAP43 mRNA

To investigate further the correlation between changes in PKC and the state of SK-N-SH differentiation, we measured the mRNA content for two genes, c-myc and GAP43. Changes in c-myc expression have been associated with the differentiation of many cell types, typically involving reduced expression [26, 27]. In a subclone of SK-N-SH, the SH-SY5Y cell line, Hammerling *et al.* reported virtually complete loss of c-myc mRNA content in association with phorbol ester or retinoic acid-induced differentiation [28]. Synthesis and axonal transport of GAP43 has been associated with neuronal regeneration [29, 30], and there is evidence of localization in neurites [31, 32]. Also, an increase in GAP43 mRNA in conjunction with phorbol ester-induced differentiation of neuroblastoma has been reported [33, 34].

Both c-myc and GAP43 were easily detectable in total RNA from cells grown in 10% FBS (Figs. 5 and 6). The transcript sizes detected for both were consistent with previous reports on SH-SY5Y cells (Figs. 5 and 6) [28, 33]. As previously described for SH-SY5Y cells [33], a doublet of 1.8 and 1.6 kb GAP43 mRNA transcripts was resolved in some experiments. However, because this doublet was not routinely resolved, the combined content of these transcripts was used to estimate changes in GAP43 mRNA. Profound changes in both c-myc and GAP43 mRNA were measured in cells grown in CD, further suggesting that a commitment to differentiation was occurring. C-myc mRNA was found to decrease sig-



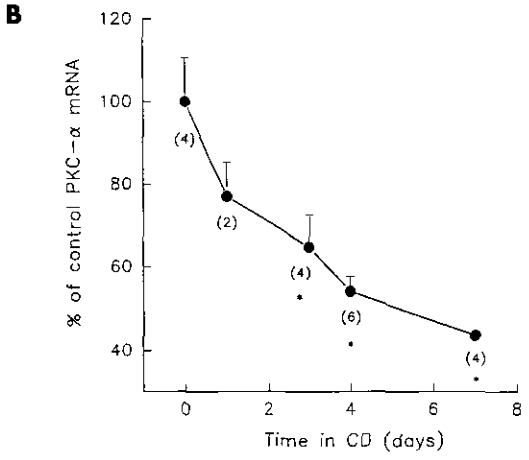


FIG. 3. (A) Representative autoradiograph of a Northern blot of total RNA probed with PKC-α cDNA (top) and subsequently with β-actin cDNA (bottom). Total RNA was extracted from SK-N-SH cells after 0, 1, 3, and 7 days in CD. (B) Densitometric analysis of PKC-α mRNA content over successive days of growth in CD, expressed as the percentage mean ± SEM of identically treated FBS controls. Numbers of observations averaged for each data point are in parentheses. *$P < 0.01$ compared to same-day FBS control by unpaired t test.

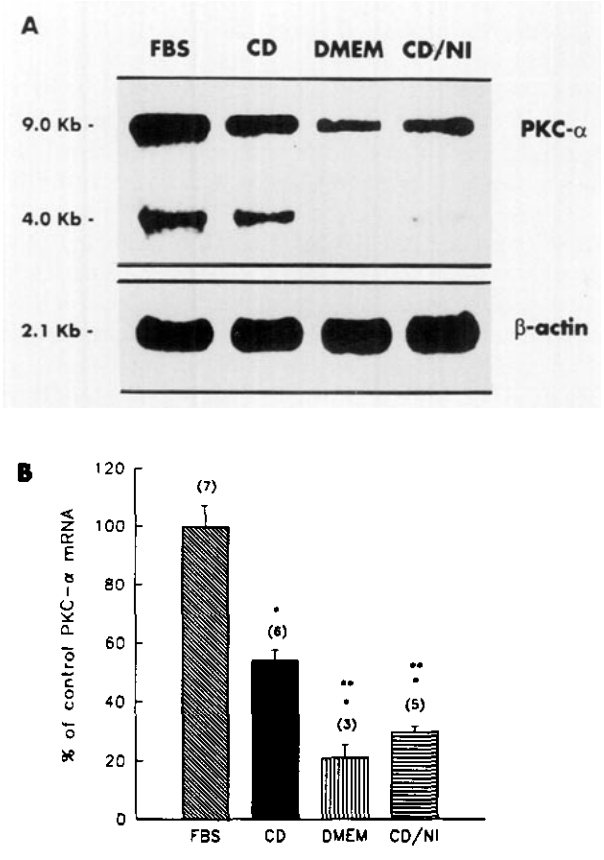


FIG. 4. (A) Representative autoradiograph of a Northern blot of total RNA probed with PKC-α cDNA (top) and subsequently with β-actin (bottom). Total RNA was extracted from SK-N-SH cells after 4 days in FBS, CD, DMEM, or CD without insulin (CD/NI). (B) Densitometric analysis of PKC-α mRNA content after 4 days in FBS, CD, DMEM, or CD without insulin (CD/NI), expressed as the percentage mean ± SEM of identically treated FBS controls. Numbers of observations averaged for each bar are in parentheses. $^{*}P < 0.01$ compared to FBS by unpaired t test; $^{**}P < 0.01$ compared to CD by unpaired t test.

nificantly within 3 days after transfer from FBS to CD and to return nearly to control levels by Day 7 (Fig. 5B). GAP43 mRNA content rose to a plateau of approximately 50% above control for the first 4 days following transfer to CD and then increased dramatically to over 400% relative to the FBS control at Day 7 (Fig. 5C). In contrast, cells grown in either DMEM alone or CD without insulin did not exhibit similar changes in c-myc or GAP43 mRNA levels, but rather exhibited increases in c-myc mRNA and decreases in GAP43 mRNA after 4 d (Fig. 6).

DISCUSSION

PKC Expression

PKC has been implicated to play an important role in neuronal proliferation and differentiation [1–4]. In this study, we observed steady decreases in PKC activity and PKC-α mRNA levels over a 7-day period in serum-deprived SK-N-SH neuroblastoma cells. Cells cultured in CD or DMEM exhibited similarly decreased PKC activity, whereas PKC-α mRNA was lower in DMEM than in CD. Given our finding that PKC-α mRNA levels were similar in the presence of DMEM or CD without insulin, it seems likely that the insulin in CD is necessary for maintaining higher PKC-α mRNA levels. CD contains 700 n*M* insulin, which is well in excess of saturation of the insulin or the insulin-like growth factor-I (IGF-I) receptor [35]. If insulin were the only positive regulator of PKC-α mRNA under these conditions, one might expect maximal levels of PKC-α mRNA in CD. However, we observed the highest PKC-α mRNA levels in FBS, suggesting that serum factors in addition to insulin are necessary for maintaining high PKC expression, and that removal of these factors results in down-regulation of PKC. This observation is in agreement with studies by Parrow and colleagues, who found that PKC-α mRNA levels remain high in SH-SY5Y cells after treatment with serum and phorbol ester [36].

To date, at least six and possibly nine isozymes of PKC have been identified and divided into two subgroups, Group A (α, βI, βII, and γ) and Group B (δ, ϵ, ζ, η, and L). This subdivision is based primarily on the absence of the C2 region in the regulatory domain and the lack of calcium dependence of the Group B isozymes [1]. Since the PKC activity we measured was strictly calcium dependent, one or more of the Group A isozymes were most likely involved. Of the Group A isozymes, detection of mRNA for the α isozyme has been reported in mouse, rat, and human neuroblastoma [32, 37] including SH-SY5Y cells [36], while mRNAs for the β and γ isozymes were undetectable [36, 37]. Therefore, loss of PKC activity with serum deprivation may be solely due to PKC-α expression. It is not yet known whether PKC protein levels or PKC-α mRNA transcription and stability rates are also regulated with serum removal.

Serum Deprivation and Differentiation

Treatment of SK-N-SH cells with DMEM alone was not sufficient to induce neurite outgrowth or elongation or to promote changes in c-myc and GAP43 which are consistent with a commitment to differentiation. However, SK-N-SH cells cultured in CD did exhibit decreased c-myc and increased GAP43, suggesting that the factors in CD (insulin, progesterone, transferrin, putrescine, sodium selenate, and BSA) are necessary for differentiation to occur. It is therefore necessary to distinguish the effects of "serum-free" and "chemically defined" media on neuronal differentiation. Other researchers have demonstrated that serum deprivation of induces morphological differentiation other neuroblastoma cell lines [38–40]. In agreement with the present study, Påhlman and colleagues have shown that treatment of SH-SY5Y cells with serum-free media does not induce spontaneous neurite outgrowth [34] or result in changes in c-myc expression [28]. Moreover, serum ap-

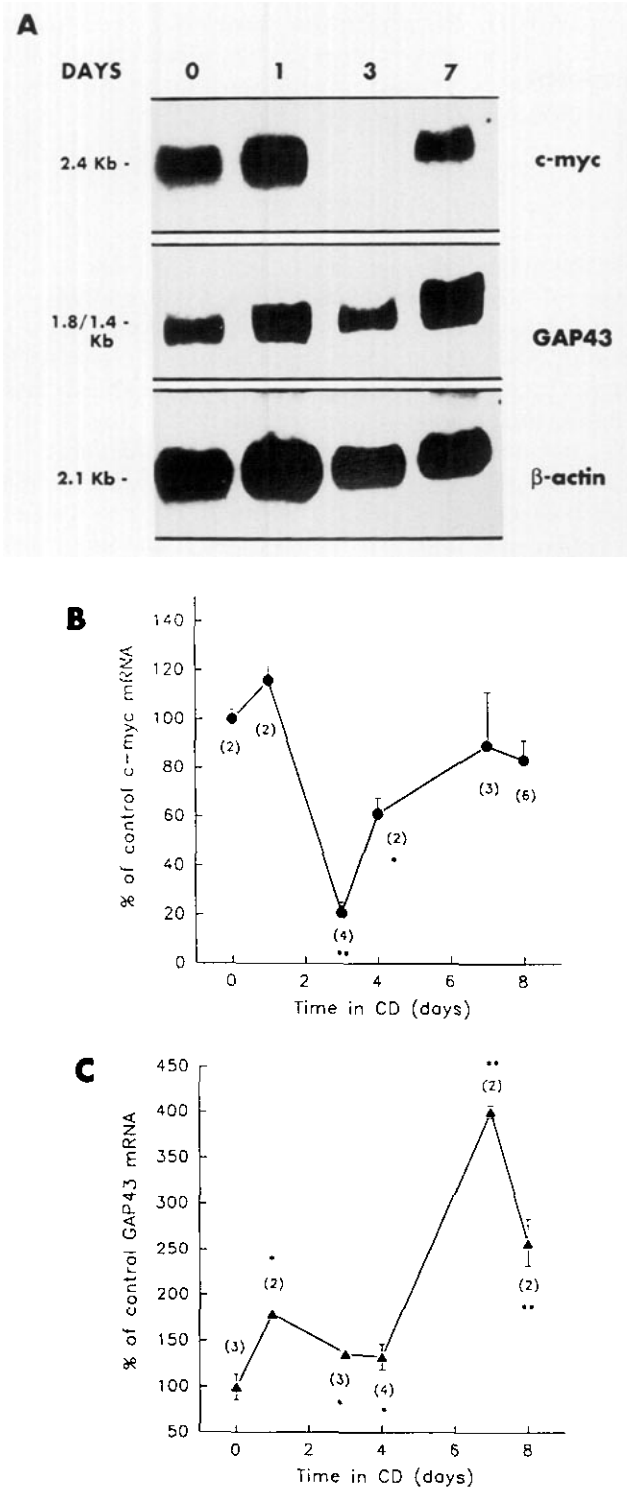


FIG. 5. (A) Representative autoradiograph of a Northern blot of total RNA probed (separately) with c-myc cDNA (top), GAP43 cDNA (middle), and β-actin cDNA (bottom). Total RNA was extracted from SK-N-SH cells after successive days in CD (or fresh FBS). This is the same blot used for PKC-α hybridization in Fig. 3. Densitometric analysis of c-myc (B) and GAP43 (C) mRNA content over successive days of growth in CD, expressed as the percentage mean ± SEM of identically treated FBS controls. Because the content of GAP43 mRNA was measured as the sum of these two transcripts, we cannot rule out the possibility of differential changes in the content of these two transcripts. Numbers of observations averaged for each data point are in parentheses. $^*P < 0.05$, $^{**}P < 0.01$ compared to same-day FBS control by unpaired t test.

pears to be necessary for phorbol ester-induced differentiation [34].

Since a dramatic reduction of PKC activity and of PKC-α mRNA content resulted from serum deprivation without concomitant onset of differentiation, we conclude that loss of PKC is not sufficient to induce differentiation of SK-N-SH cells. It is possible that DMEM lacks factors that are present in CD and serum which could act in synergy with reduced PKC activity to implement or to facilitate initiation of the differentiation process. Indeed, Påhlman and colleagues recently showed that TPA-induced neurite outgrowth and increased GAP43 mRNA are serum dependent [34].

Whereas Påhlman and colleagues found that TPA reduces c-myc mRNA in SH-SY5Y in the presence or absence of serum [34], we found that c-myc levels are not reduced in DMEM alone. This result indicates that reduced PKC-α mRNA in serum-deprived cells is not associated with loss of c-myc. If PKC is directly involved in the regulation of c-myc expression, then the residual PKC activity in SK-N-SH cells after several days of serum-deprived growth may be sufficient to prevent a reduction in c-myc mRNA levels. Down-regulation of this residual PKC activity with phorbol ester could be used as a test for the role of PKC in regulating c-myc.

Our results showing that PKC is reduced with or without differentiation of SK-N-SH cells and previous studies showing that induction of neuroblastoma differentiation occurs after down-regulation of PKC [4–6] and after treatment with putative inhibitors of PKC [7–10] collectively suggest that reduced PKC may be necessary but not sufficient for differentiation to occur. Wada and colleagues have reported that various differentiating agents, including ganglioside GM1, 8-bromo-cAMP, or α- or β-alkyl glycerol ether induce losses of PKC-α, -ε, and -ζ mRNA in murine Neuro2a neuroblastoma cells [37]. Retinoic acid-induced differentiation also has been associated with loss of PKC activity in neuroblastoma cells [6, 11]. In contrast, Parrow and colleagues recently demonstrated that PKC remains functionally active in neuroblastoma cells after differentiation by phorbol ester. It is therefore likely that reduced PKC may be associated with specific differentiating agents and not with others.

Analyses of the content of PKC enzymes and of PKC mRNAs in developing brain support the possibility that diminished levels of PKC are necessary for normal brain development. PKC protein in rat brain [41, 42] and PKC mRNA in mouse brain [24] for the Group A isozymes is detectable only several days prior to birth, and both protein and mRNA levels for PKC increase dramatically during postnatal development to reach a plateau in adulthood. A similar profile for PKC mRNAs is apparent during human brain development [24]. Ap-

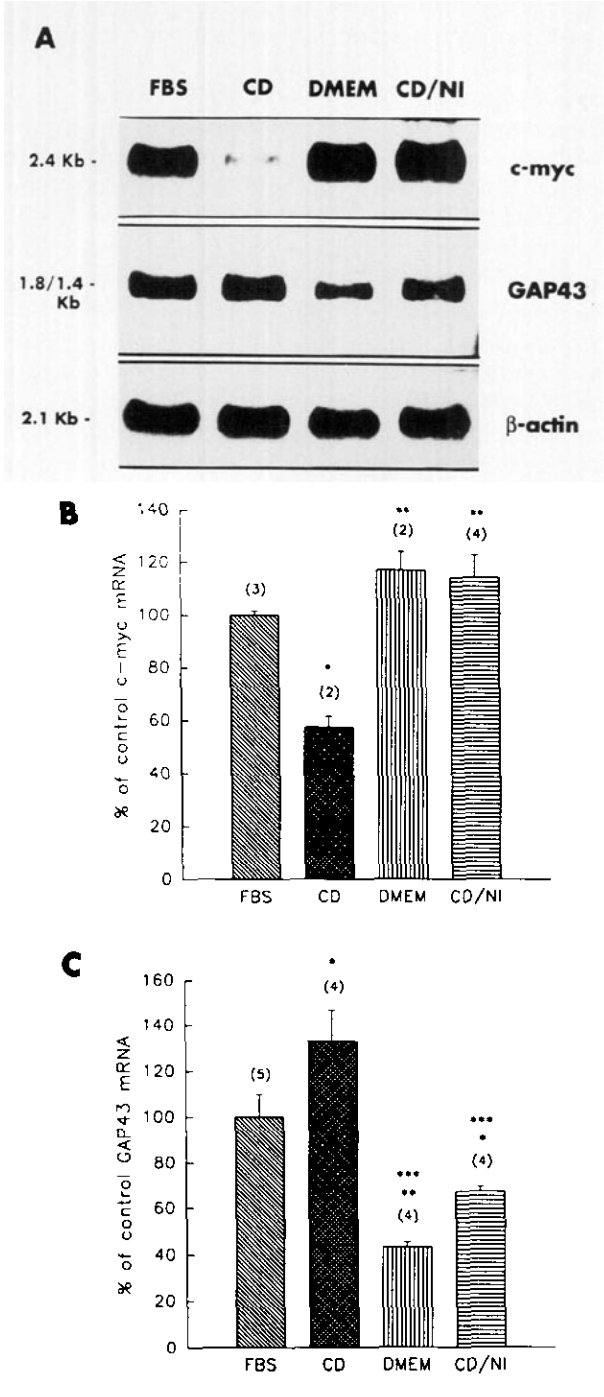


FIG. 6. (A) Representative autoradiograph of a Northern blot of total RNA probed (separately) with c-myc cDNA (top), GAP43 cDNA (middle), and β-actin cDNA (bottom). Total RNA was extracted from SK-N-SH cells after 4 days in FBS, CD, DMEM, or CD without insulin (CD/NI). This blot is the same as that used for PKC-α hybridization in Fig. 4. (B) Densitometric analysis of c-myc (B) and GAP43 (C) after 4 days in FBS, CD, DMEM, or CD without insulin (CD/NI), expressed as the percentage mean ± SEM of identically treated FBS controls. Numbers of observations averaged for each bar are in parentheses. $^{*}P < 0.01$ compared to same-day FBS control by unpaired t test; $^{**}P < 0.05$, $^{***}P < 0.01$ compared to CD by unpaired t test.

parently, PKC content is low during the most active period of brain development, suggesting that diminished PKC activity may be an important factor, allowing neuronal differentiation to proceed.

Insulin and Neuronal Differentiation

SK-N-SH cells grown in CD exhibited a decrease in c-myc mRNA and an increase in GAP43 mRNA, two changes known to be consistent with a commitment to differentiation. In contrast, cells cultured in either CD without insulin or DMEM alone did not exhibit these changes, suggesting that the insulin in CD may be partially responsible for differentiation. Considered together with the observed ability of insulin to induce neurite elongation, it is possible that insulin acts as a differentiating agent in SK-N-SH neuroblastoma cells. Indeed, insulin and IGF-I have been reported to induce both neurite outgrowth [44, 45] and proliferation [46, 47] of SH-SY5Y cells. Påhlman and colleagues have suggested that the shift from induction of proliferation to promotion of differentiation which is mediated by IGF-I in SH-SY5Y may be dependent upon down-regulation of PKC [34]. Insulin has also been reported to permit nerve growth factor-induced differentiation of SH-SY5Y in serum-free medium, while anti-insulin antiserum reduced nerve growth factor-stimulated neurite outgrowth in the presence of serum [42]. In light of these findings, it will be interesting to determine if insulin or IGF-I acts synergistically with reduced PKC to promote differentiation. We also found that insulin is likely responsible for the increased level of PKC mRNA in SK-N-SH cells cultured in CD relative to those cultured in DMEM alone. PKC activity was equal after 4 days in cells grown in CD or DMEM, suggesting that insulin may be important only during the early stages of differentiation.

In conclusion, we have demonstrated that reduced PKC activity and PKC-α mRNA can occur in the presence or absence of neuronal differentiation. Therefore, in spite of a growing body of evidence of the importance of diminished PKC to the neuronal differentiation process, this event does not appear to be sufficient for differentiation to occur. Nevertheless, this does not rule out an important supporting role for reduced PKC in differentiation, nor does it decrease the need for future work regarding the elucidation of the role of constitutive PKC activity in regulating neuronal maturation.

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