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Regulation of Protein Kinase C Activity in Neuronal Differentiation Induced by the N-*ras* Oncogene in PC-12 Cells

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Expression of the N-*ras* oncogene under the control of the glucocorticoid-responsive promoter in the pheochromocytoma cell line UR61, a subline of PC-12 cells, has been used to investigate the differentiation process to neuronal cells triggered by *ras* oncogenes (I. Guerrero, A. Pellicer, and D. E. Burstein, Biochem. Biophys. Res. Commun. 150:1185–1192, 1988). Using *ras*-inducible cell lines, we observed that expression of the oncogenic N-*ras* p21 protein interferes with the ability of phorbol esters to induce downregulation of protein kinase C. This effect was associated with the appearance of immunologically detectable protein kinase C as well as the activity of the enzyme as analyzed either by binding of [^{3}H]phorbol-12,13-dibutyrate in intact cells or by in vitro kinase activity. These results indicate a relationship between *ras* p21 and protein kinase C in neuronal differentiation in this model system. Comparison to the murine fibroblast system suggests that this relationship may be functional.

The family of *ras* genes is a normal subset of the genome of all eucaryotic cells (for a review, see references 3 and 24). Three different members have been found in mammalian cells, designated as Harvey-, Kirsten-, and N-*ras*. *ras* genes are highly conserved during evolution, with homologous members found in almost every species analyzed. Despite intensive investigation at both the genetic and biochemical levels, the actual function of the *ras* gene products, designated as *ras*-p21 proteins, remains elusive. Initial findings related the yeast RAS proteins to the regulation of the adenylate cyclase system both in vitro and in vivo (7, 36). However, similar experiments utilizing mammalian adenylate cyclase components did not reproduce these results (5, 25).

RAS proteins are essential for viability in a number of species (3, 24). Their biochemical properties are similar to those of regulatory G proteins (15, 33). Evidence which implicates *ras*-p21 proteins in both proliferation and differentiation processes has been provided (3, 24).

Protein kinase C (PKC) is a ubiquitous kinase whose activity depends on phospholipids and Ca^{2+} (28). The enzyme can be activated by tumor promoters such as phorbol esters, which substitute for the natural activator, 1,2-diacylglycerol (DAG). It is well established that PKC is the endogenous receptor for phorbol esters (1). The enzyme is actually a family of isoenzymes with similar biochemical properties and different cell specificities (6, 12, 29, 30). PKC activity has been related to a number of functions, including regulation of cell proliferation and neuronal differentiation (28), functions in which *ras*-p21 proteins are also thought to play an important regulatory role (3, 24).

In a previous study, we investigated the functional relationship between RAS proteins and PKC in 3T3 cells, the model system for *ras*-induced malignant transformation of fibroblasts (22). Our results demonstrated the functional requirement of PKC for the mitogenic activity of H-*ras* p21 protein, since removal of endogenous PKC by pretreatment of the cells with phorbol-12,13-dibutyrate (PDBu) for 48 to 72 h inhibited more than 80% of the mitogenic activity of microinjected *ras*-p21 proteins. Comicroinjection of *ras*-p21 and PKC restored the mitogenic activity induced by *ras*-p21. In the present study, we investigated the possible functional relationship between *ras*-p21 and PKC in a model system for neuronal differentiation of PC-12 cells carrying the oncogenic N-*ras* p21 gene under the control of the glucocorticoid responsive promoter. Our results demonstrate that expression of N-*ras* p21 in these cell lines affects PKC activity.

MATERIALS AND METHODS

Cells and morphometric assays. Cells were grown at a density of 1×10^6/35-mm plate on collagen-treated plastic plates in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum as previously described (16). When indicated, cells were analyzed under the microscope, and the percentage of cells bearing neurite extensions was estimated in a total of 200 to 300 cells. Only those with at least one extension equal to or greater than the cell diameter were scored as positive.

***ras*-p21 expression levels.** Cells were treated with the indicated concentrations of dexamethasone in dimethylsulfoxide (DMSO) or equivalent volumes of solvent alone. At indicated times, the medium was changed to RPMI 1640 without methionine, containing 1 mCi of L-[^{35}S]methionine per ml and DMSO alone or 1 μM dexamethasone in DMSO. After 2 h of incubation at 37°C, monolayers were washed twice with phosphate-buffered saline (PBS) and cells were lysed and added to inositol phosphate (IP) buffer (100 mM phosphate [pH 7.6], 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 100 mM NaCl). Extracts containing equal amounts of trichloroacetic acid-precipitable counts were immunoprecipitated with monoclonal antibody Y13-259, and proteins were resolved in a 12.5% polyacrylamide gel as previously described (23).

Western blot (immunoblot) analysis. Cells were grown under normal conditions and treated as indicated in each experiment with 200 nM PDBu or 1 μM dexamethasone or

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both. After the indicated times, cells were washed twice with PBS and lysed by the addition of IP buffer. Protein concentrations were estimated in each sample by the BCA procedure (Pierce Chemical Co.). Portions of 100 μg from each sample were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at a constant voltage of 60 V for 18 h. Gels were transferred to nitrocellulose membranes by using a Hoeffer Transblot apparatus in buffer A (20 mM Tris hydrochloride, 0.15 M glycine, 20% methanol) for 4 h at a constant current of 500 mA. Nitrocellulose filters were blocked for 2 h in buffer B (50 mM Tris hydrochloride [pH 7.5], 10 mM EDTA, 1% Triton X-100, 2% nonfat milk), and incubated for 2 h in the same buffer containing a 1:200 dilution of anti-PKC monoclonal antibody MC5 (Amersham Corp.). Filters were washed three times in buffer B and incubated for 1 h in the same buffer containing a 1:200 dilution of affinity-purified rabbit anti-mouse immunoglobulin G (Jackson Laboratory). After being washed three times for 10 min in buffer B, filters were incubated in the same buffer containing 2×10^5 cpm of ^{125}I-protein A (Amersham) per ml for 20 min, washed three more times, air dried, and exposed to Kodak autoradiographic film. ^{14}C-labeled, prestained markers were utilized to check for migration, transfer, and molecular weight determinations. Actual estimates for PKC quantizations were carried out by cutting ^{125}I-protein A bands from nitrocellulose filters and counting in a gamma counter.

[^{3}H]PDBu-binding analysis. Cells were grown in 12-well plates as described above and treated as indicated with either 200 nM PDBu or 1 μM dexamethasone or both. At indicated times, cultures were washed five times with 1 ml of PBS and incubated in 0.5 ml of RPMI 1640 medium without serum. [^{3}H]PDBu (Amersham; 12.4 Ci/mmol) was added to each plate to a final concentration of 8 pM (4×10^6 cpm per plate), and unlabeled PDBu was added to a final concentration of 100 nM. The experiment was performed in triplicate. To establish nonspecific binding, three plates were incubated with the same total radioactivity but with a final concentration of 50 μM unlabeled PDBu. After 30 min of incubation at 37°C, cells were washed five times with 1 ml of PBS containing 1 mg of bovine serum albumin (Sigma Chemical Co.; fatty acid free) per ml. Cells were washed two times with PBS alone to remove bovine serum albumin and dissolved in 200 μl of 0.5 N NaOH. Protein concentrations were estimated by the Bio-Rad or BCA methods, and radioactivity was determined by scintillation counting in 10 ml of Aquasol. Total counts were converted into pmol of [^{3}H]PDBu by standard procedures and referred to the total protein content in each sample. Specific binding was determined by subtracting the radioactivity of the samples incubated in 50 μM PDBu from the values obtained in the samples incubated at 100 nM PDBu. One picomole of [^{3}H]PDBu was equivalent to 8×10^4 cpm; nonspecific binding was 40 to 60% of the total binding in control (untreated) cells.

RNA extractions and Northern (RNA) blots. Total mRNA was isolated from U7 and UR61 cells in the presence of guanidinium thiocyanate (11). Portions of 20 μg of total denatured RNA were run in 1% agarose–2.2 M formaldehyde gels and blotted onto nitrocellulose membranes (26). Oligonucleotide probes were designed from the specific V3 region of the different PKC isoforms. Probes were labeled to a specific activity of 1×10^9 cpm/ng by standard procedures (26) and were used for hybridization at a concentration of 1 ng/ml. The same blots were stripped out and rehybridized with the complete coding sequence of the glucose 6-phosphate dehydrogenase gene as an internal control.

PKC assay. UR61 cells were grown as indicated and treated for 72 h at 37°C with 400 nM PDBu in DMSO or with DMSO alone. Cells were washed twice with 5 ml of low-salt sucrose buffer (0.33 M sucrose, 20 mM Tris hydrochloride, pH 7.5), as described by Thomas et al. (35). The last wash was completely aspirated off, and the cells were scraped with a rubber policeman into 1.5 ml of the same buffer and transferred to an Eppendorf tube. Cells were rapidly pelleted by spinning in a microfuge for 10 s, the supernatants were discarded, and the tubes were placed on ice. Ice-cold distilled water (200 μl) was added, and the cells were disrupted on ice by three consecutive 5-s bursts with a Kontes Cell Disrupter at maximum power output. A total of 1.3 ml of ice-cold buffer B (2 mM EDTA, 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.1 mM phenylmethylsulfonyl fluoride, 20 mM Tris hydrochloride, pH 7.5) was added to the sonic extract, and tubes were vortexed briefly. Samples were centrifuged at 100,000 × *g* for 30 min at 4°C. Supernatants were carefully removed and designated as crude soluble fractions. Pellets were suspended by brief (2-s) sonication at low power in 600 μl of ice-cold buffer B, and then 600 μl of buffer B containing 1% (vol/vol) Nonidet P-40 was added. Samples were kept on ice for 30 min with occasional vortexing and centrifuged at 100,000 × *g* for 15 min at 4°C. Supernatants (1.2 ml) were quantitatively removed, transferred to a borosilicate test tube and designated as crude particulate fractions. Both crude soluble and crude particulate fractions were applied to 0.5-ml packed bed volume CELLEX D (Bio-Rad) columns poured previously in 11-ml disposable polypropylene columns (Bio-Rad no. 731-1550) and preequilibrated with buffer B at 4°C. Columns were then washed twice with 3 ml of buffer B, and bound PKC was eluted with 1.5 ml of buffer B containing 100 mM NaCl. The protease inhibitor leupeptine was immediately added to the eluants to a final concentration of 25 μg/ml. Eluted fractions were immediately assayed for PKC activity by using histone III-S (Sigma) as the substrate. PKC activity was expressed as picomoles of ^{32}P incorporated per total milligrams of protein in 3 min.

Pulse-chase experiments of PKC. Control cells and cells treated for 24 h with 1 μM dexamethasone were labeled for 4 h in methionine-free medium supplemented with 0.5 mCi of L-[^{35}S]methionine (Dupont, NEN Research Products; 1,083 Ci/mmol) per ml. Cells were then washed twice and incubated in regular medium supplemented with a 10-fold excess of unlabeled methionine. Equal amounts of proteins from control and dexamethasone-treated cells were immunoprecipitated with monoclonal antibody MC5 at different times after the addition of 200 nM PDBu. Immunoprecipitates were then analyzed by standard polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Neuronal differentiation induced by N-*ras* p21 proteins in PC-12-derived cells. In previous studies, we have shown that the N-*ras* oncogene can trigger PC-12 neuronal differentiation (17), and we subsequently developed a system for the inducible expression of neuronal differentiation in a PC-12 subline by the *ras* oncogene. The activated N-*ras* oncogene was under the control of the dexamethasone-inducible long terminal repeat promoter from the mouse mammary tumor virus. The resulting plasmid (pMMTV/m-N-*ras*) was introduced into the PC-12 subline U7, and several independent

clones were isolated for further characterization (17). U7 cells divide at a faster rate than PC-12 cells and do not extend neurites in response to neural growth factors (NGF) under normal growth conditions (8). However, under conditions of nonproliferation, such as in the presence of hydroxyurea, U7 cells fully respond to the NGF-triggered differentiation program (9). As has been shown previously (17), within a few hours after addition of different amounts of dexamethasone, one of the isolated clones, UR61, readily showed the morphological changes associated with neuronal differentiation. Concentrations of dexamethasone as low as 0.01 μM were sufficient to activate neuronal differentiation in at least 70 to 80% of the cells after 24 h of treatment.

Analysis of the levels of expression of the N-*ras* p21 protein in the absence of dexamethasone indicated that the rates of p21 synthesis were comparable to those of the parental U7 cell line (17). In the presence of dexamethasone, a 10- to 20-fold increase in the rate of p21 synthesis was observed, with a maximum effect 6 h after the addition of dexamethasone (J. C. Lacal, unpublished observations). No alteration in the levels of p21 synthesis were observed at equivalent concentrations of dexamethasone in control parental U7 cells. These results were in agreement with previous observations when total mRNA for N-*ras* or p21 protein content was analyzed in both U7 and UR61 cells under similar conditions (17). At later times, the synthetic rates for the N-*ras* p21 protein decreased slowly, as expected from the feedback mechanisms of regulation of the glucocorticoid-responsive elements.

Effects of downregulation of PKC on neuronal differentiation of UR61 cells induced by *ras*. Treatment of a number of cell lines with phorbol esters induces activation of PKC in a mechanism thought to be associated with translocation from the cytosol to the plasma membrane (28). Upon activation and translocation, the enzyme is rapidly inactivated by degradation by specific proteolysis (21). Prolonged treatment of a variety of cells with phorbol esters induces downregulation of the enzyme (10) without apparent effect on de novo synthesis (38).

Since downregulation of PKC in 3T3 cells impairs the mitogenic activity of *ras*-p21 proteins (22), we investigated the effects of PKC downregulation in the neuronal differentiation of UR61 cells after *ras*-p21 induction. After 24 or 48 h of treatment with 200 nM PDBu, a complete downregulation of PKC was observed when analyzed by Western blot with the anti-PKC monoclonal antibody MC5 (Fig. 1). This monoclonal antibody was raised against a peptide sequence (residues 312 to 323) from the α-PKC from bovine brain (Amersham). It can recognize both α and β forms from bovine brain but does not interact with the γ-PKC (see Amersham description).

Under conditions of complete downregulation, expression of *ras*-p21 was induced by dexamethasone treatment, and the effects on neuronal differentiation were analyzed at different times. Contrary to the results observed in the 3T3 cells, removal of endogenous PKC did not interfere with *ras*-p21 function in the differentiation process (Table 1). By contrast, in PKC-downregulated UR61 cells, *ras*-p21 expression resulted in a more efficient induction of neuronal differentiation. Both the rate of appearance and number and length of neurites were increased. The effect of treatment of UR61 cells with phorbol esters on *ras* action prior to *ras*-p21 induction has been studied in detail and will be reported elsewhere (R. Trotta, J. C. Lacal, A. Pellicer, and D. E. Burstein, J. Cell. Physiol., in press). Thus, removal of

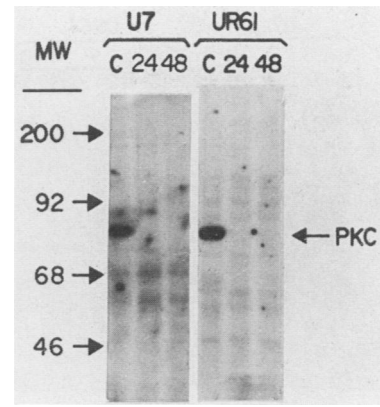

FIG. 1. Downregulation of PKC in U7 and UR61 cells by treatment with PDBu. Both U7 and UR61 cells were treated with 200 nM PDBu for 24 or 48 h or incubated under identical conditions without PDBu for 48 h (lanes C). Cells were then washed with PBS and lysed into IP buffer. Similar amounts of total proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and processed for Western blot analysis as described in the text. Immunologically detectable PKC and molecular weight standards are shown with arrows. The experiment was repeated at least three times with similar results.

detectable PKC by chronic treatment of the cells with PDBu apparently enhanced *ras*-p21 function.

PKC activity in downregulated UR61 cells after induction of *ras*-p21 expression. A number of studies have established the functional relationship of *ras*-p21 proteins to the activity of growth factors. By elegant microinjection experiments, Stacey and co-workers have been able to demonstrate the requirement of normal *ras*-p21 for the mitogenic activity of serum, since a monoclonal antibody against p21, Y13-259 (14), was able to block DNA synthesis induced by serum (27). In PC-12 cells, microinjection of Y13-259 blocked differentiation induced by NGF (18). A role of PKC has also been described for NGF-induced differentiation (19), although this is a matter of controversy since it has been speculated also that PKC is not required for NGF-triggered differentiation (32). Our results showing enhancement of differentiation by *ras* in PKC-downregulated UR61 cells prompted us to further investigate the effects of *ras*-p21 expression on the PKC activity in this system.

TABLE 1. Neurite extension induced in UR61 cells by dexamethasone treatment after downregulation of PKC[a]

PDBu pretreatment	Dexamethasone (μM)	% Cells with neurites at (h):		
		10	24	48
−		1.3	6.6	6.5
+		2.1	4.4	5.2
−	0.01	8.7	46	50
+	0.01	19.4	70	90
−	0.1	19.2	55	70
+	0.1	24	90	95
−	1	23	70	95
+	1	35	95	97

[a] UR61 cells were treated or not treated with 200 nM PDBu for 48 h to induce downregulation of PKC. Then cells were exposed to different concentrations of dexamethasone in the presence or absence of 200 nM PDBu. The numbers of cells extending neurites were estimated by microscopic visualization at the indicated times after addition of dexamethasone. Results are expressed as the percentage of cells exhibiting at least one extension equal to or greater than the cell diameter in a total of at least 200 cells.

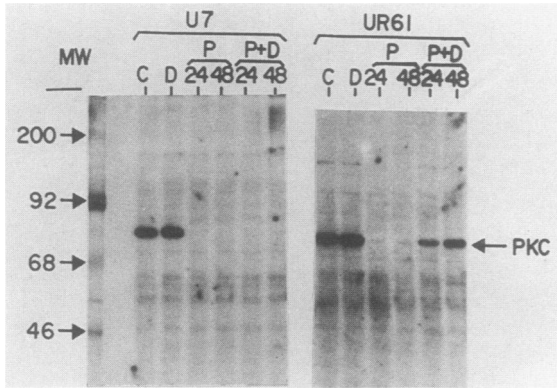

FIG. 2. Effect of expression of *ras*-p21 on PKC downregulation induced by PDBu. U7 and UR61 cells were incubated under normal conditions for 48 h (lanes C) or in the presence of 1 μM dexamethasone for 48 h (lanes D), 200 nM PDBu for 24 or 48 h (lanes P), or both 200 nM PDBu and 1 μM dexamethasone for 24 or 48 h (lanes P + D). At the indicated times, cells were washed and lysed as described in text. Western blots were performed by using a monoclonal antibody against PKC (MC5), as described in the text. The band corresponding to PKC and those for molecular weight standards are indicated with arrows. This experiment was repeated five times with similar results.

When UR61 cells were treated with 200 nM PDBu for 24 or 48 h, all immunodetectable PKC was lost from the cells. Treatment with 1 μM dexamethasone alone induced a slight increase in the total level of detectable PKC (Fig. 2). Combined treatment of the cells with PDBu and dexamethasone showed total levels of immunologically detectable PKC at about 50% the levels found in control (untreated) cells. In contrast with this observation, parental U7 cells showed no alteration in the downregulation pattern with or without dexamethasone treatment. Table 2 shows PKC values detected under each condition.

The above results indicated that PKC protein was not downregulated by phorbol esters after dexamethasone treatment. However, to establish whether the immunodetectable PKC was functional, we investigated PKC activity under the above conditions by its ability to bind [^{3}H]PDBu and by its in vitro kinase activity, following standard procedures. Table 3 shows the results of specific [^{3}H]PDBu binding in both U7 and UR61 intact cells after treatment with 200 nM PDBu alone, 1 μM dexamethasone alone, or combined treatment for 24 or 48 h. While UR61 cells challenged with PDBu alone showed a loss of total binding sites, dexamethasone alone slightly increased total binding, and the combined treatment showed binding levels comparable to those of control (untreated) cells. In contrast with these results, in U7 cells treated under identical conditions, no binding of PDBu was observed when the cells were incubated in the presence of both PDBu and dexamethasone, although a similar PKC downregulation was observed. It has been established that activation of PKC by phorbol esters is associated with proteolytic cleavage of the enzyme. As a net result, two subunits are generated, one of which carries the phorbol ester-binding domain while the other one carries the catalytic activity (for a review, see reference 28). This observation could explain the lack of a complete downregulation of PDBu binding after PDBu treatment in both U7 and UR61 cells but complete downregulation of the immunodetectable enzyme.

The enzymatic activity of PKC was also analyzed by the in vitro standard assay of histone phosphorylation. Table 4 shows the results obtained when cytoplasmic extracts from UR61 cells were analyzed (since the majority of the activity was localized in the cytosolic fraction). No significant alteration of the total distribution of PKC was observed after dexamethasone treatment in UR61 cells.

Similar results to those of the PDBu-binding assay were found, with a substantial reduction of enzymatic PKC activity in both U7 and UR61 cells after 24 h of treatment with PDBu. Treatment with dexamethasone alone had little effect on UR61 cells. However, when PDBu treatment was combined with dexamethasone treatment, the levels of total enzymatic activity were elevated in UR61 cells specifically

TABLE 2. Quantization of PKC levels in U7 and UR61 cells after downregulation induced by PDBu treatment[a]

Cell line	Treatment time (h)		PKC (dpm)
	Dexamethasone	PDBu	
U7			499
	48		470
		24	43
		48	5
	24	24	6
	48	48	20
UR61			600
	48		850
		24	38
		48	5
	24	24	113
	48	48	308

[a] U7 and UR61 cells were treated with either 1 μM dexamethasone or 200 nM PDBu or both for 24 or 48 h. Cells were washed with PBS and processed as described in the text for Western blot analysis with MC5. After exposure of the blots to MC5, rabbit anti-mouse immunoglobulin G, and ^{125}I-protein A, blots were exposed to Kodak autoradiographic film. The corresponding bands in the nitrocellulose papers were cut, and radioactivity was estimated in a gamma counter.

TABLE 3. Ability of PKC to bind [^{3}H]PDBu in U7 and UR61 cells after treatment with dexamethasone and/or PDBu[a]

Cell line	Dexamethasone	PDBu	Time (h)	Mean ± SD [^{3}H]PDBu (fmol/μg of protein)
U7	−	−	24	4,265 ± 89
	+	−	24	4,010 ± 101
	−	+	24	1,458 ± 54
	+	+	24	1,167 ± 77
	−	−	48	4,431 ± 126
	+	−	48	4,099 ± 39
	−	+	48	1,239 ± 25
	+	+	48	583 ± 23
UR61	−	−	24	4,921 ± 201
	+	−	24	5,541 ± 189
	−	+	24	2,005 ± 57
	+	+	24	4,921 ± 86
	−	−	48	4,885 ± 177
	+	−	48	7,692 ± 244
	−	+	48	1,203 ± 48
	+	+	48	3,354 ± 99

[a] U7 or UR61 cells were grown in 12-well plates as described in the text and treated with dexamethasone (1 μM), PDBu (200 nm), or both for 24 or 48 h. Then cells were incubated in the presence of [^{3}H]PDBu as described in the text for the determination of total specific PDBu-binding levels. Counts were converted into femptomoles of PDBu per protein content in each sample. Each datum is the mean ± standard deviation of three samples.

TABLE 4. Enzymatic activity of PKC after treatment with dexamethasone of downregulated UR61 cells[a]

Treatment	Preincubation	PKC (sp act [cpm])
Control	DMSO	1,324
Dexamethasone	DMSO	1,049
Control	PDBu	1
Dexamethasone	PDBu	1,118

[a] UR61 cells were grown as described in the text and treated with 400 nM PDBu for 72 h to induce downregulation of PKC. Dexamethasone was added to a final concentration of 1 μM, and cells were incubated for an additional 16 h. Cells were then processed for PKC enzymatic activity, as described in the text. PKC activity is reported as counts per minute of ^{32}P incorporated into histone III-S (Sigma) after 3 min of incubation at 30°C per milligram of protein. Only the activity of the soluble fraction is reported, as the particulate fraction accounted for less than 4% of total cellular kinase activity.

to those found in control (untreated) cells. Thus, expression of the N-*ras* p21 protein in UR61 cells chronically treated with phorbol esters induced the protection of fully functional PKC from phorbol ester downregulation.

PKC activity in PDBu-treated UR61 cells is mediated by protection of degradation. The level of PKC activity found in PDBu-treated UR61 cells after induction of *ras*-p21 protein could be mediated by at least two different mechanisms. Activation of the de novo synthesis of the enzyme would involve an increase of the transcription rate of PKC genes. Alternatively, p21 could directly or indirectly protect PKC from proteolytic degradation by physical interaction with PKC or inhibition of proteolytic activity.

To evaluate whether increased levels of PKC were a consequence of de novo protein synthesis, we investigated the rate of downregulation by PDBu treatment in the presence of cycloheximide, a potent inhibitor of protein synthesis. UR61 cells were treated with 1 μM dexamethasone for 48 h to allow expression of the RAS protein and then incubated with 10 μg of cycloheximide per ml in the presence or absence of 200 nM PDBu for 12 or 24 h. At different times, cells were analyzed by immunoblotting with monoclonal antibody MC5 (Fig. 3). No significant difference was observed in the rate of PKC degradation in the absence or presence of cycloheximide, suggesting that PKC was not elevated by stimulation of de novo protein synthesis. Similar conclusions were reached when pulse-chase experiments were performed. Prelabeled PKC was still detectable by immunoprecipitation with MC5 in UR61 cells pretreated with dexamethasone 20 h after the addition of PDBu (data not shown). No prelabeled PKC could be detected 5 h after the addition of PDBu in control UR61 cells. These results confirmed that after expression of oncogenic N-*ras* protein, PKC is protected from degradation of phorbol esters.

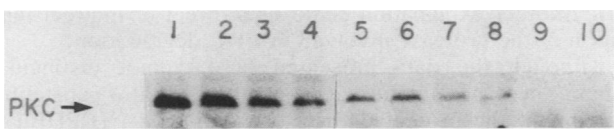

FIG. 3. Downregulation of PKC in cycloheximide-treated UR61 cells. UR61 cells were grown as indicated in the text for 48 h in the presence of 1 μM dexamethasone and then treated with 200 nM PDBu in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 10 μg of cycloheximide per ml. Cells were processed as indicated in the text, and samples were analyzed for PKC levels by immunoblot analysis with monoclonal antibody MC5. Shown is the resulting autoradiogram. Treatment was for 0 (lanes 1 and 2), 8 (lanes 3 and 4), 16 (lanes 5 and 6), 24 (lanes 7 and 8), or 36 (lanes 9 and 10) h. The experiment was repeated three times with similar results.

In order to complete these studies, we also investigated the levels of PKC mRNA after PDBu treatment. An increase in the transcript level of PKC genes should be detected by Northern blot analysis with specific probes for each isoform. We designed probes specific for the V3 variable region of the α, β, and γ forms of PKC on the basis of known sequences (for a review, see reference 30). When the α-specific probe was utilized, two different messages corresponding to the 3.5- and 8.5-kilobase mRNA species (data not shown) were detected, in agreement with previous observations (6). The total mRNA content was normalized by the corresponding blots with a probe specific for the glucose 6-phosphate dehydrogenase gene. After normalization, no significant alteration in the levels of the α-form mRNAs was observed. Moreover, no detectable messages were found for the β and γ forms (results not shown). Thus, the level of PKC activity observed in dexamethasone-treated UR61 cells was not a consequence of an increased level of the transcript from PKC genes.

Differential rate of PKC downregulation induced by *ras*-p21 expression. We then analyzed the possible effects of p21 expression on the downregulation rates of PKC after the addition of PDBu. For this purpose, we performed parallel cultures of UR61 cells, untreated or treated with 1 μM dexamethasone, for 48 h. At this time, PDBu was added to a final concentration of 200 nM, and the amounts of total PKC detectable by Western blotting with monoclonal antibody MC5 was determined at different times of incubation. In control (untreated) UR61 cells, PKC was rapidly downregulated with a 50% degradation rate of 2.1 ± 0.1 h (mean ± standard deviation, $n = 3$) (Fig. 4A). In these cells, no PKC could be detected after 16 h of treatment. In contrast with these results, in UR61 cells previously treated with 1 μM dexamethasone for 48 h to allow the expression of the N-*ras* p21 protein, the downregulation rate of PKC was substantially reduced to 50% degradation in 8.3 ± 0.2 h (mean ± standard deviation, $n = 3$). Even at 24 h of PDBu treatment, a significant level of PKC activity could still be detected. Therefore, the level of PKC activity found in UR61 cells treated with PDBu and dexamethasone is a consequence of the protection of degradation induced under normal conditions by PDBu.

The levels of PKC were also estimated by immunorecognition by MC5 at different times after dexamethasone addition in UR61 cells previously treated with phorbol esters for 48 h. Induction of *ras*-p21 produced detectable levels of PKC after dexamethasone addition (Fig. 4A). The range of detectable enzyme at this point was around 10 to 20% total PKC levels in control (untreated) cells. Thus, expression of the activated N-*ras* protein protected PKC from degradation by phorbol ester treatment.

To further characterize the specificity of PKC protection by *ras*-p21 induction, we extended our analysis to other U7 derivatives carrying the same construction as the UR61 clone. We tested the ability of PDBu to downregulate as well as the ability of dexamethasone treatment to protect PKC degradation in T5H and UR17a clones. Both clones were proven to carry the MMTV/m-N-*ras* plasmid. However, T5H cells express constitutively high levels of the N-*ras* p21 protein, which are not sufficient to fully induce the differentiation process but which are sufficient to induce some morphological alterations reminiscent of the initial stages of neuronal differentiation (T. Thomson and A. Pellicer, unpub-

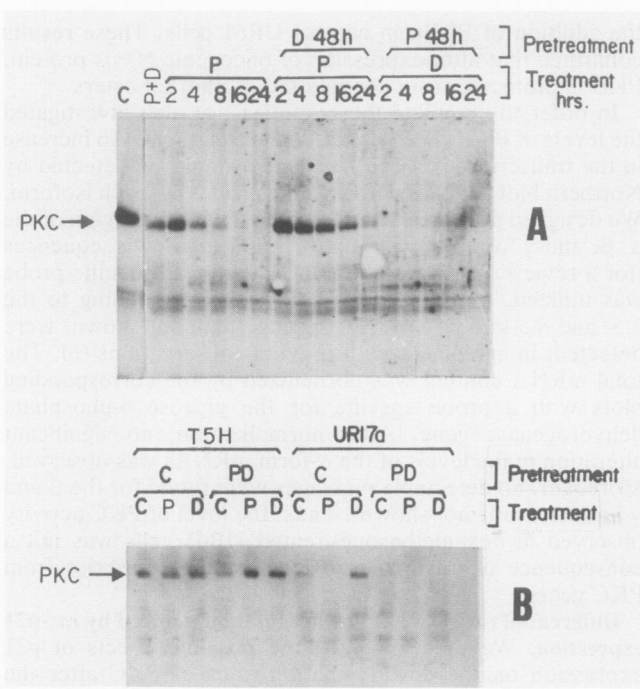

FIG. 4. Levels of PKC in different PC-12 derivatives after dexamethasone or PDBu treatment. (A) UR61 cells were grown under normal conditions with no treatment (lane C) or were pretreated with either 1 μM dexamethasone (lanes D) or 200 nM PDBu (lanes P) or both dexamethasone and PDBu (lane P + D). After 48 h of incubation, cells were treated with either 200 nM PDBu or 1 μM dexamethasone for 2, 4, 8, 16, or 24 h. Cells were then washed and processed for Western blot analysis, as indicated in the text, with monoclonal antibody MC5 against PKC. Bands corresponding to PKC are shown by an arrow. (B) T5H and UR17a cells were incubated as described in the text under normal conditions (lanes C) or in the presence of 200 nM PDBu and 1 μM dexamethasone (lanes PD). After 48 h of incubation, both sets of cells were treated for an additional 24 h with either PDBu (lanes P) or dexamethasone (lanes D) alone or carried as controls with no further addition (lanes C). Cells were then washed and processed as indicated in the text for Western blot analysis. The band corresponding to PKC is shown by an arrow.

lished observations). Clone UR17a is a cell line whose response to dexamethasone is weak, both in terms of *ras*-p21 expression and neuronal differentiation, and only after long exposure to the inducer does it undergo morphological changes associated with the differentiation process. When UR17a cells were treated with 200 nM PDBu for 48 h, PKC was not detectable, nor was it detectable when this treatment was combined with 1 μM dexamethasone (Fig. 4B). In contrast, when T5H cells were treated with PDBu for 48 h, only partial downregulation was achieved, and the levels of PKC were increased when both PDBu and dexamethasone or dexamethasone alone was added to the cells. Therefore, there is a good correlation between induction of the *ras*-p21 protein and the protection of PKC from downregulation and degradation induced by phorbol esters.

PKC downregulation in NGF-induced differentiation of PC-12 cells. The effects on PKC downregulation observed in the above experiments can be explained by either a direct effect of *ras*-p21 expression or by a nonspecific effect on PKC mediated by the differentiation process itself. To distinguish between these two possibilities, we analyzed PKC levels in PC-12 cells triggered by NGF treatment. Cells were first treated with 50 ng of NGF per ml for 48 h and then with 200 nM PDBu for 24 or 48 h. At the indicated times, analysis of PKC levels was performed by immunoblotting with monoclonal antibody MC5 as previously described. No difference was observed in the rate of downregulation induced by PDBu in control or NGF-treated cells (Fig. 5). These results indicate that protection from phorbol ester-induced downregulation of PKC in PC-12 cells is not a common mechanism of neuronal differentiation.

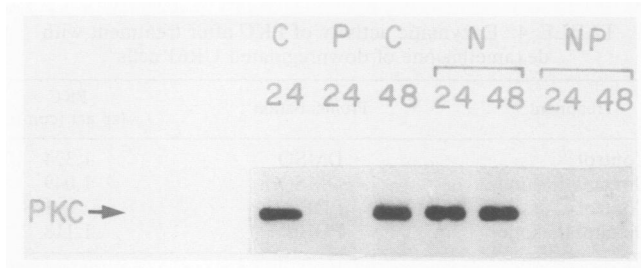

FIG. 5. PKC levels in PC-12 cells treated with NGF. PC-12 cells were grown as described in the text under normal conditions with no treatment (lanes C) or were treated with either 200 nM PDBu (lane P), 50 ng of NGF per ml (lanes N), or both (lanes NP) for 24 or 48 h. Samples were collected and treated as indicated for immunoblot analysis of PKC levels with monoclonal antibody MC5. The experiment was repeated twice with similar results.

DISCUSSION

Using an inducible cell system in which the levels of oncogenic N-*ras* p21 protein can be modulated by treatment with dexamethasone, we investigated the functional relationship between *ras*-p21 and PKC during neuronal differentiation. We observed that after induction of the *ras*-p21 protein, treatment of the cells with phorbol esters did not induce complete downregulation of PKC. This effect was not a consequence of increased levels of the transcripts of PKC genes but of protection of the degradation of the enzyme itself, an effect which results from activation of the kinase (10).

These results can be interpreted as a direct interaction of the *ras*-p21 protein with PKC. Evidence for such interaction has been provided both in vitro and in vivo, since purified PKC can phosphorylate *ras*-p21 proteins and addition of phorbol esters to intact cells induces phosphorylation of K-*ras* p21 (2, 20). These results can alternatively be explained by an indirect mechanism such as production of a second messenger, induced by *ras*-p21, which could in turn interact directly with PKC, making it less susceptible to degradation. One of the candidates for this activity is DAG, shown to be increased in *ras*-transformed cells (13, 23, 31, 37). Another explanation could be a direct or indirect inhibition of the protease involved in PKC degradation.

Although the data presented here cannot distinguish among these possibilities, it is significant that a substantial amount of the protected PKC can still bind [^{3}H]PDBu in intact cells. This result suggests that PKC is not occupied by a competitor ligand of phorbol esters such as DAG. Therefore, other metabolites different from DAG could be responsible for this effect. In agreement with these findings, it has been postulated that *ras*-p21 can activate phospholipase A2, with the consequent generation of lysophospholipids and free fatty acids (4). Thus, the altered membrane-associated phospholipids or cytosolic fatty acids might interact with PKC, leading to its stabilization, without affecting its ability to bind phorbol esters.

In *ras*-induced UR61 cells, preincubation with or addition of PDBu potentiated neurite outgrowth, one of the hallmarks of neuronal differentiation. A simple explanation could be that *ras*-p21 proteins require functional PKC for their neurite-promoting activity. Under conditions of *ras*-p21 induction in the presence of phorbol esters, protection of PKC by p21 and activation of PKC by phorbol esters might lead to an overall increase in PKC activity, thus enhancing *ras*-p21 action. This may not be the case, since p21-induced neurite outgrowth has been seen as early as 12 h after exposure of PDBu-treated UR61 cells to dexamethasone, a time point which precedes *ras*-mediated protection from PKC downregulation (Trotta et al., in press). Furthermore, NGF-mediated neurite outgrowth, which has been shown to be p21 dependent (18), occurs in PDBu-treated PC-12 cells, despite their inability to be protected from phorbol ester-mediated downregulation.

It has been postulated that activation of the v-H-*ras* gene in PC-12 cells under the control of the glucocorticoid-inducible promoter may involve simultaneous activation of PKC- and cAMP-dependent kinase pathways (34). This hypothesis is based on the findings that induction of v-H-*ras* p21 by dexamethasone generated a sustained elevation of the levels of cAMP as well as those of IPs and DAG. However, while the *ras* protein was already expressed at high levels 6 h after dexamethasone addition, cAMP levels were elevated only after 24 h, and DAG and IP levels were elevated after 48 h of treatment. These results suggest that an early effect, distinct from cAMP or DAG and inositol phosphate production, might be responsible for the onset of *ras*-triggered neuronal differentiation programs in PC-12 cells. Such an effect could be related to modulation of PKC by a mechanism independent of DAG production. Our results indicate that there is a functional relationship between *ras*-p21 proteins and PKC during neuronal differentiation. The surprising observation that PKC is specifically protected in UR61 cells as a consequence of *ras*-p21 induction even after downregulation with phorbol esters deserves special attention. Whether this effect on PKC is crucial for *ras*-mediated differentiation awaits further investigation.

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