JOURNAL OF VIROLOGY, Oct. 1992, p. 6093–6098
0022-538X/92/106093-06$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 66, No. 10

Adenovirus E1A Proteins Stimulate Inositol Phospholipid Metabolism in PC12 Cells

KAZUKO SHIROKI,[1]* AKIO YAMAKAWA,[2] MASAO SHIBATA,[2] TADAOMI TAKENAWA,[2] SUMIO SUGANO,[1] AND AKIO NOMOTO[1]

Department of Microbiology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108,[1] and Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Sakaecho, Itabashi-ku, Tokyo 173,[2] Japan

Received 4 May 1992/Accepted 10 July 1992

To study the influence of nuclear oncogenes on inositol phospholipid metabolism, we examined the various parameters of inositol phospholipid metabolism in PC12 cells expressing adenovirus type 12 or adenovirus type 5 E1A. Although the inositol 1,4,5-trisphosphate content was increased only slightly, the diacylglycerol content was 2.4-fold higher in E1A-expressing PC12 cells. Furthermore, we found that the activity of phospholipase C, one of the key enzymes in inositol phospholipid metabolism, was increased at least five- to eightfold. Diacylglycerol kinase activity in the membrane fraction was 10 to 15% of that in parental PC12 cells. Overall protein kinase C activities in E1A-expressing PC12 cells were decreased, but the activity of membrane-bound protein kinase C was significantly increased. These observations clearly indicate that inositol phospholipid metabolism is stimulated in cells producing E1A and suggest that nuclear oncogene E1A has the ability to stimulate inositol phospholipid metabolism.

The effect of nerve growth factor (NGF) on the PC12 pheochromocytoma cell line provides a good model for neuronal differentiation, since NGF can induce neurite outgrowth from this cell line (10, 13). The mechanisms underlying this effect, especially the signal transduction processes, have been studied in detail. The effects of NGF on signal transduction processes in PC12 cells include changes in inositol phospholipid metabolism, tyrosine phosphorylation, cyclic AMP levels, and intracellular Ca^{2+} levels (20, 24, 38, 45). The PC12 cell line is also unique compared with other differentiation-inducible cell lines because neuronal differentiation of PC12 cells is affected by efficient transfection of several oncogenes. *ras* and *src* induce neurite outgrowth from PC12 cells (1, 4, 34), while *fos*, *fra-1*, *myc*, and E1A block the induction of neurite outgrowth by NGF (12, 17, 18, 29, 34). This property allowed us to investigate how various oncogenes interfere with neuronal differentiation, with particular emphasis on the signal transduction processes.

Nuclear oncogenes, such as *myc* and E1A, are thought to act within the cell nuclei, and their effects on the signal transduction processes, which occur mainly within the cell membrane, have not been extensively studied. Recently, we found that E1A can induce significant changes in the cell membrane of a fibroblast cell line (42). This finding led us to use PC12 cells to examine the effect of E1A on the signal transduction processes. In this report, we show evidence that inositol phospholipid metabolism is greatly increased in E1A-expressing PC12 cells.

MATERIALS AND METHODS

Cells. PC12(10), PC12h, and E1APC12 (12E1APC12 and 5E1APC12) cells were grown in a mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (1:1) supplemented with 10% fetal calf serum and 5% horse serum. In addition to the regular culture medium, the following agents were included, depending on the growth experiment: NGF (7S) at 50 to 100 ng/ml and dibutiryl cyclic AMP at 0.5 mM (both purchased from Sigma Chemical Co.).

Plasmid DNAs. pNeo12E1A (pSV2Neo with adenovirus type 12 [Ad12] E1A nucleotides 12 to 1596), pNeo5E1A (pSV2Neo with Ad5 E1A nucleotides 1 to 1772), and pSV2Neo plasmid DNAs (42) were used for introducing E1A genes into PC12 cells. For analysis of cellular mRNA expression, fragments from the following plasmid DNAs were used as probes: pBR322 with a 110K-type isozyme of diacylglycerol (DG) kinase (47a), pPLC-β, pPLC-γ1, pPLC-γ2, pPLC-δ, and pUC18 with phospholipase C-β (PLC-β), PLC-γ1, PLC-γ2, and PLC-δ (14).

Preparation of PC12 cells expressing Ad12 E1A and Ad5 E1A genes. PC12 cells were transfected with pNeo12E1A, pNeo5E1A, and pSV2Neo by the calcium phosphate precipitation method (42). Neomycin-resistant clones were selected in medium containing 300 μg of G418 per ml for 7 to 10 days. Cells in isolated G418-resistant colonies were picked, cultured, and established as cell clones.

Preparation and blot analysis of mRNA. RNA was prepared from whole cells by the guanidine HCl-CsCl gradient method. Poly(A)-rich RNA was prepared with an oligo(dT)-cellulose column. The RNA was denatured, mixed with ethidium bromide, and electrophoresed on a 1.0 to 1.4% agarose gel containing formaldehyde. After electrophoresis, the RNA was transferred to a nylon filter, immobilized, and hybridized with ^{32}P-labeled nick-translated DNA probes.

Assays of PI, PI-4P, and DG kinase activities and phospholipase C activity. Subconfluent cultures were washed three times with cold phosphate-buffered saline (PBS) and scraped off with a rubber policeman. The cell pellet was suspended in extraction buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 10 mM 2-mercapthoethanol, and 100 μM phenylmethylsulfonyl fluoride. After sonication, membrane and cytosol fractions were separated by centrifugation at 100,000 × *g* for 1 h. The activities of phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PI-4P) kinases were measured in 50 μl of a reaction mixture containing 20 mM Tris-HCl (pH 7.4), 2 mM ethylene glycol-

* Corresponding author.

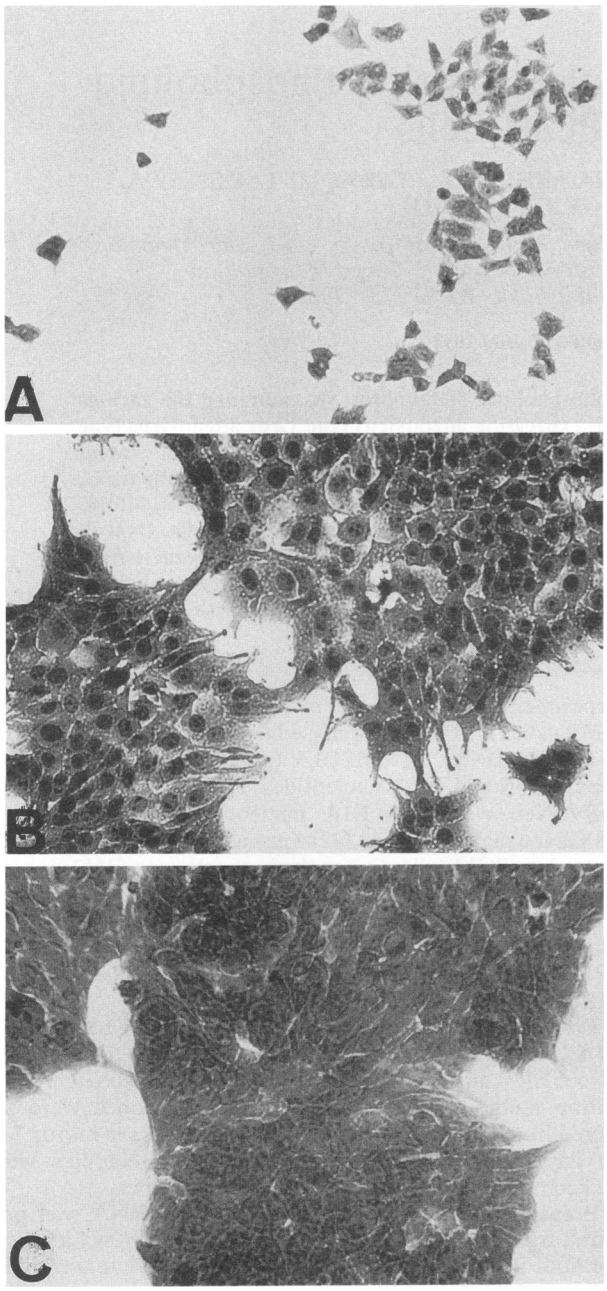

FIG. 1. Morphology of PC12 (A), 12E1APC12 4 (B), and 5E1APC12 1 (C) cells. The plates were fixed with 3.7% formaldehyde and stained with Giemsa stain.

bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 20 mM $MgCl_2$, 0.4% Triton X-100, 50 mg of PI or PI-4P, 200 μM [γ-^{32}P]ATP (1 to 2 μCi), and the enzyme sample (5 to 60 μg of protein). The reaction proceeded for 10 min at 30°C (48). The activity of DG kinase was measured in 50 μl of 50 mM 2-mercaptoethanol–20 mM NaF–1 mM sodium cholate–50 mg of 1,2-diolein–200 μM [γ-^{32}P]ATP (1 to 2 μCi)–enzyme sample (5 to 60 mg of protein) (22). Formed ^{32}P-labeled PI-4, phosphatidylinositol 4,5-bis-phosphate (PIP2), and phosphatidic acid were separated by two-phase partitioning and thin-layer chromatography, and enzyme activities were determined by scraping the corresponding spots and measuring their radioactivity in a liquid scintillation counter. PIP2-phospholipase C activity was measured as described previously (33). In brief, the reaction was performed with a 50-μl mixture containing 50 mM morpholineethanesulfonic acid (MES)-NaOH buffer (pH 6.5), 20 mM $CaCl_2$, 1 mg of bovine serum albumin per ml, 100 μM [^{3}H]PIP2 (25,000 dpm), 40 μM phosphatidylethanolamine, and the enzyme sample (5 to 60 μg of protein).

Measurement of the steady-state level of DG. Subconfluent cells were washed with PBS, 1.0 ml of ice-cold methanol was added, and the cells were scraped off into 0.5 ml of chloroform. The samples were sonicated for 3 min, 0.25 ml of 1 M NaCl was added, and the samples were centrifuged. The lower phases were washed with the upper phases of a mixture of chloroform, methanol, and NaCl. The resultant lower phases were dried under vacuum and used for DG analysis with a DG assay kit (Amersham Corp.) (9) and for phosphate determination (2).

Analysis of IP3. Subconfluent cultures were washed with PBS, suspended in PBS, and immediately added to a one-half volume of 30% trichloroacetic acid. After the samples were kept on ice for 20 min, the extracts were centrifuged at 3,000 × *g* for 15 min at 4°C. The resulting supernatants were extracted with ether three times and adjusted to pH 7.4 with 7.5% $NaHCO_3$. The samples were used for inositol 1,4,5-triphosphate (IP3) analysis with an IP3 assay kit (Amersham) (9) and for phosphate determination (2).

Phosphate determination. Samples (10 to 50 μl) were mixed with 10% $Mg(NO_3)_2 \cdot 6H_2O$ in ethanol (50 μl), evaporated to dryness over a strong flame with rapid shaking, and further heated in the flame until the brown fume disappeared. After the tube was cooled, 0.3 ml of 1 N HCl was added. The tube was heated in a boiling water bath for 15 min to hydrolyze any PP_i formed in the ashing procedure to P_i. The P_i was mixed with 0.7 ml of an ascorbic acid-molybdate mixture, kept at 45°C for 20 min, and measured by reading the A_{820}. The ascorbic acid-molybdate mixture, which was made daily, contained 1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate · $4H_2O$ in 1 N H_2SO_4. An A_{240} was obtained from 0.01 μmol of phosphate (2).

Assay of protein kinase C activity. Subconfluent cultures were washed three times with cold PBS and scraped off with a rubber policeman into PBS. The cell pellet was resuspended in extraction buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM 2-mercaptoethanol, sonicated, and centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was used as cytosol protein kinase C. The precipitated membrane fraction was solubilized in extraction buffer containing 1% Triton X-100 for 30 min on ice and centrifuged at 100,000 × *g* for 1 h. The resulting supernatant was subjected to DE-52 column chromatography, and the fraction eluted with 0.3 M NaCl was used as membrane-bound (membrane) protein kinase C. The activity of protein kinase C was assayed with a protein kinase C assay kit (Amersham). The protein contents of the cytosol and membrane fractions were measured with a Bio-Rad protein assay.

RESULTS

Establishment and characterization of E1A-expressing PC12 cells. To establish PC12 cells constitutively expressing the E1A gene, we introduced pNeo12E1A (pSV2Neo with Ad12 E1A nucleotides 12 to 1596) and pNeo5E1A (pSV2Neo

TABLE 1. Expression of cellular genes in PC12, 12E1APC12, and 5E1APC12 cells

PC12 cells[a] or plasmid	Neurite outgrowth induced by[b]:		Expression of the following mRNA[c]:					
	NGF	EGF	E1A	DG kinase	PLC-β	PLC-γ1	PLC-γ2	PLC-δ
Parental	+	−	−	1.0	1.0	1.0	1.0	1.0
12E1APC12								
1	++	−	+	1.4	1.5	4.5	1.8	0.7
2	−	+	++	2.5	4.1	4.7	2.1	0.9
4	−	+++	+++	4.2	4.0	4.8	2.3	1.3
11	−	++	+++	3.5	3.8	4.6	1.8	1.5
5E1APC12								
1	−	−	+++	4.8	4.5	2.9	2.1	0.9
2	−	−	+++	1.8	2.1	1.7	1.4	0.7
pSV2Neo	+	−	−	0.9	1.4	1.2	1.1	1.0

[a] PC12 cells were transfected with pNeo12E1A, pNeo5E1A, and pSV2Neo plasmid DNAs. Colonies resistant to G418 were independently isolated and established as cell clones.

[b] The cell clones were cultured on plastic plates for 2 to 4 days in medium with NGF (50 ng/ml) and EGF (100 ng/ml), and neurite formation was observed. The percentages of cells with processes were more than 50% (+++), 30 to 50% (++), 1 to 30% (+), and less than 1% (−).

[c] Total RNA was prepared from PC12 cells by the guanidine HCl-CsCl centrifugation method. Poly(A)-rich RNA was selected and electrophoresed on a 1% agarose gel containing 3% formaldehyde. After electrophoresis, the RNA was transferred to a nylon filter, immobilized, and hybridized with ^{32}P-labeled probes. As probes, Ad12 E1A from pNeo12E1A, Ad5 E1A from pNeo5E1A, pBR322 with a 110K-type isozyme of DG kinase, pPLC-β, pPLC-γ1, pPLC-γ2, pPLC-δ, and pUC18 with PLC-β, PLC-γ1, PLC-γ2, and PLC-δ were used. The amount of mRNA was measured with an image analyzer and normalized on the basis of the intensities of 28S and 18S rRNAs. The amount of each mRNA is expressed relative to that (1.0) in PC12 cells.

with Ad5 E1A nucleotides 1 to 1772) (42) into PC12 cells by the calcium phosphate precipitation method. After cultivation for 2 weeks in medium containing G418, several colonies resistant to G418 were independently isolated and cultures of cell clones 12E1APC12 (12 clones) and 5E1APC12 (6 clones) were established. 12E1APC12 cells grew well and showed increased cell substratum adhesiveness. 5E1APC12 cell clones also grew well and were round and flattened. 12E1APC12 and 5E1APC12 cells could be distinguished by those features (Fig. 1). These E1APC12 cell clones and parental PC12 cells were tested to examine whether NGF and dibutiryl cyclic AMP prompted neuronal differentiation. Morphological differentiation prompted by NGF was blocked in PC12 cells expressing Ad12 or Ad5 E1A (Table 1 and data not shown) but not in parental PC12 cells. 12E1APC12 1 cells were an exception: they expressed a low level of Ad12 E1A, and NGF was able to induced neurite outgrowth. Epidermal growth factor (EGF) induced neurite outgrowth from 12E1APC12 cells. This interesting result will be described in another paper.

Activities of PI-4, PI-4P, and DG kinases and phospholipase C in E1A-expressing PC12 cells. The activities of PI-4 kinase (48), PI-4P kinase (48), DG kinase (22), and phospholipase C (33) were examined. There were no differences in PI-4 (Table 2) and PI-4P (data not shown) kinase activities among 12E1APC12, 5E1APC12, and PC12 cells in both cytosol and membrane fractions. Phospholipase C activity was increased severalfold in both membrane and cytosol fractions of 12E1APC12 or 5E1APC12 cells, compared with that in parental PC12 cells (Table 2). In contrast, the activity of membrane DG kinase in 12E1APC12 or 5E1APC12 cells was reduced to 10 to 14% of that in parental PC12 cells (Table 2). However, the DG kinase activity in the cytosol was not significantly different among 12E1APC12, 5E1APC12, and PC12 cells. The results of experiments involving other cell clones of 12E1APC12 and 5E1APC12 were essentially the same as those shown in Table 2 (data not shown).

Elevated steady-state levels of DG and IP3 in E1A-expressing PC12 cells. Increased activity of phospholipase C and decreased activity of DG kinase in the membrane fraction

TABLE 2. Phospholipase C, PI-4 kinase, and DG kinase activities of parental and E1A-expressing PC12 cells[a]

Cells	Activity, pmol/min/mg of protein, of:		
	Phospholipase C	PI-4 kinase	DG kinase
Cytosol			
PC12	51.3 ± 0.7 (100)	10.7 ± 0.19 (100)	19.7 ± 1.02 (100)
12E1APC12 4	412.7 ± 24.0 (804)	12.2 ± 0.14 (114)	14.1 ± 1.20 (73)
5E1APC12 1	435.5 ± 16.0 (849)	10.7 ± 0.2 (100)	20.3 ± 2.48 (103)
Membrane			
PC12	8.62 ± 0.3 (100)	212.2 ± 3.49 (100)	17.8 ± 1.22 (100)
12E1APC12 4	39.5 ± 2.5 (458)	155.4 ± 1.47 (73.2)	1.9 ± 0.13 (10.7)
5E1APC12 1	32.6 ± 1.6 (378)	222.7 ± 5.37 (105)	2.5 ± 0.24 (14)

[a] Subconfluent cell cultures were suspended in extraction buffer and lysed by sonication. The suspension was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was used as a cytosol fraction. The resultant pellet was resuspended in extraction buffer, sonicated, and used as a membrane fraction. The enzyme activities of both membrane and cytosol fractions were measured in triplicate as described in Materials and Methods. Results are expressed as the mean ± standard error of the mean in triplicate assays. Values in parentheses show the activities relative to that (100%) in PC12 cells.

TABLE 3. Contents of IP3 and DG in PC12, 12E1APC12, and 5E1APC12 cells

Cells or plasmid	Content (pmol/nmol of phosphate) of:	
	IP3[a]	DG[b]
PC12	0.05	1.4
12E1APC12		
4	0.072	3.5
11	0.0674	2.8
5E1APC		
1	0.103	3.7
2	0.067	3.4
pSV2Neo 1	0.058	1.8

[a] Cells were collected into trichloroacetic acid and prepared for assays as described in Materials and Methods. IP3 in samples was analyzed with an IP3 assay kit (Amersham), and phosphate in samples was assayed as described in Materials and Methods. The data represent the mean of triplicate determinations. The IP3 content was expressed as a fraction of total phospholipid content.

[b] Samples from each type of cell were extracted with chloroform-methanol as described in Materials and Methods. DG in samples was analyzed with a DG assay kit (Amersham), and phosphate in samples was assayed as described in Materials and Methods. The data represent the mean of triplicate determinations. The DG content was calculated on the basis of the total phospholipid content.

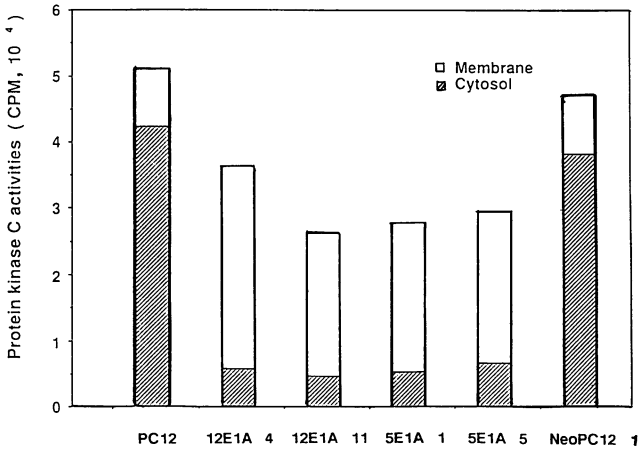

FIG. 2. Activities of protein kinase C in the membrane fraction (shaded bars) and the cytosol fraction (open bars), measured in triplicate as described in Materials and Methods.

may result in the accumulation of DG and IP3 in E1APC12 cells. As confirmation, DG and IP3 contents in subconfluent 12E1APC12, 5E1APC12, and PC12 cells were examined (Table 3). The IP3 content in 12E1APC12 and 5E1APC12 cells was 1.5-fold higher than that in PC12 cells. The DG content in 12E1APC12 and 5E1APC12 cells was 2.4-fold higher than that in PC12 cells (Table 3). The IP3 content in 12E1APC12 and 5E1APC12 cells may not have been as high as the DG content because of a quick turnover of IP3. The results suggest that E1A proteins modulate inositol phospholipid metabolism in PC12 cells.

Activation of protein kinase C in E1A-expressing PC12 cells. DG is an endogenous activator of protein kinase C and causes the intracellular translocation of protein kinase C from the cytosol to the membrane. Cytosol and membrane fractions from subconfluent 12E1APC12, 5E1APC12, and PC12 cells were assayed for protein kinase C activity. As expected from the increased levels of DG in 12E1APC12 or 5E1APC12 cells, most of the activity (70 to 80%) was found in the membrane fraction, whereas in PC12 cells, only 10% of the activity was recovered in the membrane fraction (Fig. 2). In these E1APC12 cells, the protein kinase C activity of the membrane fraction was 2.3- to 3.2-fold higher than that in parental PC12 cells. However, the total activities of protein kinase C, the sum of the activities in the cytosol and membrane fractions, were lower in E1APC12 cells than in parental PC12 cells (Fig. 2). This result may have been due to down-regulation of protein kinase C in E1APC12 cells. These results suggest that the expression of the E1A protein (encoded by the E1A nuclear oncogene) activates protein kinase C in PC12 cells. This observation appears to be the same as those made for rat 3Y1 cells transformed with the Ad12 E1A, Ad5 E1A, *src*, or *ras* gene (data not shown).

Transcription of the cellular genes in 12E1APC12 or 5E1APC12 and PC12 cells. For confirmation of the stimulation or reduction of the key enzyme activity for inositol phospholipid metabolism, the expression of cellular genes encoding the key enzymes and receptors was analyzed in 12E1APC12 or 5E1APC12 cell clones by Northern (RNA) blot analysis (Table 1). The expression of the 110K-type isozyme of DG kinase, PLC-β, and PLC-γ1 was higher in 12E1APC12 or 5E1APC12 cells than in parental PC12 cells. There was no difference in the expression of PLC-γ2 and PLC-δ between E1APC12 and PC12 cells. These results supported the suggestion of enhanced phospholipase C activity in E1APC12 cells. It is of interest that the stimulation of PLC-γ1 expression may be dependent on the activation of tyrosine kinase (27, 30, 44).

DISCUSSION

In the present study, inositol phospholipid metabolism and protein kinase C activity were examined in Ad12 and Ad5 E1A-expressing PC12 cells. We found that the expression of the Ad12 or Ad5 E1A gene, one of the nuclear oncogenes (31, 39), could stimulate inositol phospholipid metabolism, leading to the accumulation of DG and the activation of protein kinase C in PC12 cells. Increased protein kinase C activity and a change in inositol phospholipid metabolism have been reported in *erbB*-transformed chicken embryo fibroblast cells (23) and *ras*-transformed cells (8, 15, 22, 23, 25, 26, 36, 47). There has been no report of inositol phospholipid metabolism in nuclear oncogene-expressing cells. In c-*myc* or simian virus 40 T antigen-expressing cells, inositol phospholipid metabolism was not changed. The activation of inositol phospholipid metabolism by adenovirus E1A has not yet been reported.

There have been several reports on inositol phospholipid metabolism in *ras*-transformed cells (8, 15, 22, 25, 36, 47). Kato et al. (22) reported that the DG content in *ras*-transformed NIH 3T3 cells increased 1.5-fold compared with that in nontransformed NIH 3T3 cells and that the increased DG content was at least partly due to the defect in DG kinase translocation. In *ras*-transformed cells, DG kinase activity in the membrane fraction was significantly lower (22). Huang et al. (15) reported the accumulation of DG, the decreased activity of membrane DG kinase, and the activation of protein kinase C in *ras*-transformed rat fibroblast cells. In their report, PI-4P and PIP2 kinase activities were increased, but phospholipase C activity was not changed. Meanwhile, it was reported that the increased DG content in *ras*-trans-

formed cells was dependent not on inositol phospholipase C but on phospholipase D (7). The mechanism of the increased DG content in *ras*-transformed cells was not clear. In this study, the change in inositol phospholipid metabolism in E1APC12 cells was different from that in *ras*-transformed cells in that the activities of PI-4P and PIP2 kinases were not changed. DG kinase activity in the membrane fraction was decreased, although the cytosol fraction DG kinase activity was not changed. PIP2-phospholipase C activity was drastically increased. We suggest that the E1A protein induced increased DG production in the membrane fraction, the activation of protein kinase C, and the translocation of protein kinase C, although it is not known whether these changes were due to the direct effect of the adenovirus E1A protein. Judging from the increased IP3 content, the increased DG content was mostly dependent on the stimulation of PI metabolism. Since PC12 cells expressing membrane or cytosol *ras* or *src* oncogene products are differentiated and form neurites (1, 4, 34), it is impossible to use these PC12 cells in experiments similar to those described in this work. Therefore, PI metabolism was studied with rat 3Y1 cells expressing Ad12 E1A, Ad5 E1A, *src*, or *ras* protein. Preliminary results indicate that the protein kinase C activities in 3Y1 cells expressing Ad12 E1A, Ad5 E1A, or *src* protein were similar to those in PC12 cells expressing Ad12 E1A or Ad5 E1A protein (data not shown). Thus, adenovirus E1A protein seems to have an influence in stimulating PI metabolism similar to that of *ras*. The molecular mechanisms of similar changes in PI metabolism resulting from the function of nuclear and membrane oncogene E1A and *ras* products are currently under investigation.

There are several isozymes of phospholipase C (37). In PC12 cells, PLC-β, PLC-γ1, and PLC-δ were expressed (14). Among these, PLC-β is activated by the δ subunit of the Gq class of G proteins (43). PLC-γ is phosphorylated by receptor tyrosine kinases, such as that of the platelet-derived growth factor receptor, and physically associates with the receptor protein through an SH2 domain structure (44). The mechanism of activation of PLC-δ is not clear. Northern analysis revealed the increased expression of PLC-β and PLC-γ1 in E1APC12 cells. Since PLC-β is localized in the membrane and cytosol fractions, the increased expression of PLC-β was correlated with increased phospholipase C activity in the membrane and cytosol fractions of E1APC12 cells.

It is particularly interesting that phospholipase C activity is higher in E1A-expressing PC12 cells than in PC12 cells. In transformed cells or tumors, an elevated level of PLC-γ1 was first reported by Artega et al. (3). They showed that a high percentage of mammary carcinomas displayed an increased level of receptor tyrosine kinases and a direct tyrosine phosphorylation substrate (PLC-γ1). This result suggests that PLC-γ1 is functionally important for receptor-dependent cell proliferation.

The activation of inositol phospholipid metabolism by E1A seems to be a novel phenomenon, but there are a number of reports that membrane characteristics are drastically changed in E1A-transformed cells (6, 16, 32, 35, 40, 41). Shimura et al. reported the selective cytotoxicity of phospholipids and DGs in 3Y1 cells transformed by the Ad12 E1A gene (40, 41). Recently, we reported that the altered membrane structures in E1AY cells, 3Y1 cells expressing the Ad12 E1A gene, may be responsible for the high-efficiency focus formation by Rous sarcoma virus (42). Adenovirus E1A proteins have been localized in the cell surface membrane (5, 21, 46). The localization of E1A proteins on the cell surface may be important for the activation of inositol phospholipid metabolism in 12E1APC12 or 5E1APC12 cells and for the changes in membrane characteristics mentioned above.

There are several hypotheses concerning signal transduction after the exposure of PC12 cells to NGF, such as changes in inositol phospholipid metabolism, activation of tyrosine kinase by a *trk* proto-oncogene, or changes in cyclic AMP and Ca^{2+} levels (20, 24, 38, 45). Traynor et al. reported that inositol phospholipid composition and metabolism in PC12 cells were increased after exposure of PC12 cells to NGF (45). In E1APC12 cells, inositol phospholipid metabolism is constitutively activated, making these cells insensitive to NGF. Further analysis of E1APC12 cells may provide a better understanding of signal transduction in PC12 cells following exposure to NGF.

ACKNOWLEDGMENTS

We are grateful to H. Hatanaka for PC12h cells, to Y. Emori for pPLC-β, pPLC-γ1, pPLC-γ2, and pPLC-δ plasmid DNAs, and to J. Fujisawa and K. Kataoka for PC12 cells. We thank H. Shimojo for critical reading of the manuscript. We are grateful to K. Nemoto, K. Maruyama, Y. Fukui, H. Iba, and N. Yamaguchi for invaluable advice and discussions. We thank E. Wakabayashi, M. Kubo, and M. Shirato for expert technical assistance.

This work was supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Alema, S., P. Casalbore, E. Agostini, and F. Tato.** 1985. Differentiation of PC12 phaeochromocytoma cells induced by v-*src* oncogene. Nature (London) **316**:557–559.
2. **Amens, B. N., and D. T. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**:769–775.
3. **Artega, C. L., M. D. Johnson, G. Todderud, R. J. Coffey, G. Carpenter, and D. L. Page.** 1991. Elevated content of the tyrosine kinase substrate phospholipase C-1 in primary human breast carcinomas. Proc. Natl. Acad. Sci. USA **88**:10435–10439.
4. **Bar-Sagi, D., and J. R. Feramisco.** 1985. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell **42**:841–848.
5. **Bellgrau, D., T. A. Walker, and J. L. Cook.** 1988. Recognition of adenovirus E1A gene products on immortalized cell surfaces by cytoxic T lymphocytes. J. Virol. **62**:1513–1519.
6. **Chen, M. J., B. Holskin, J. Stricker, J. Gorniak, M. A. Clark, P. J. Johnson, M. Mitcho, and D. Shalloway.** 1987. Induction by E1A oncogene expression of cellular susceptibility to lysis by TNF. Nature (London) **330**:581–583.
7. **Fink, D. W., and G. Guroff.** 1990. Nerve growth factor stimulation of arachidonic acid release from PC12 cells: independence from phosphoinositide turnover. J. Neurochem. **55**:1716–1726.
8. **Fleischman, L. F., S. B. Chahwala, and L. Cantley.** 1986. *ras*-transformed cells: altered levels of phosphatidylinositol-4, 5-bisphosphate and catabolites. Science **231**:407–410.
9. **Fukami, K., and T. Takenawa.** 1989. Quantitative changes in polyphosphoinositides 1,2-diacylglycerol and inositol 1,4,5-trisphosphate by platelet-derived growth factor and prostaglandin F_2. J. Biol. Chem. **264**:14985–14989.
10. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73**:2424–2428.
11. **Heasley, L. E., S. Benedict, J. Gleavy, and G. L. Johnson.** 1991. Requirement of the adenovirus E1A transformation domain 1 for inhibition of PC12 cell neuronal differentiation. Cell Regul. **2**:479–489.
12. **Heasley, L. E., and G. L. Johnson.** 1989. Regulation of protein kinase C by nerve growth factor, epidermal growth factor, and phorbol ester in PC12 pheochromocytoma cells. J. Biol. Chem.

264:8646–8652.
13. **Hempstead, B. L., D. Martin-Zanca, D. R. Kaplan, L. F. Parada, and M. V. Chao.** 1991. High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. Nature (London) **350:**678–683.
14. **Homma, Y., T. Takenawa, Y. Emori, H. Sorimachi, and K. Suzuki.** 1989. Tissue- and cell type-specific expression of mRNAs for types of inositol phospholipase C. Biochem. Biophys. Res. Commun. **164:**406–412.
15. **Huang, M., K. Chida, N. Kamata, K. Nose, M. Kato, Y. Homma, T. Takenawa, and T. Kuroki.** 1988. Enhancement of inositol phospholipid metabolism and activation of protein kinase C in *ras*-transformed rat fibroblasts. J. Biol. Chem. **263:** 17975–17980.
16. **Ikezaki, K., S. Akiyama, C. Miyazaki, G. Kimura, and M. Kuwano.** 1984. Imidazol-resistant phenotype and virus transformation in cultured rat cells. Cancer Res. **44:**1791–1795.
17. **Ito, E., J. L. Sonnenberg, and R. Narayanan.** 1989. Nerve growth factor-induced differentiation in PC12 cells is blocked by fos oncogene. Oncogene **4:**1193–1199.
18. **Ito, E., L. A. Sweterlitsch, P. Bui-Vinh Tran, F. J. Rauscher III, and R. Narayanan.** 1990. Inhibition of PC-12 cell differentiation by the immediate early gene *fra*-1. Oncogene **5:**1755–1760.
19. **Johnson, D., A. Lanaha, C. R. Buck, A. Sehgal, C. Morgan, E. Mercer, M. Bothwell, and M. Chao.** 1986. Expression and structure of the human NGF receptor. Cell **47:**545–554.
20. **Kalman, D., B. Wong, A. E. Horvai, M. J. Cline, and P. H. O'Lague.** 1990. Nerve growth factor acts through cAMP-dependent protein kinase to increase the number of sodium channels in PC12 cells. Neuron **2:**355–366.
21. **Kast, W. M., R. Offringa, P. J. Peters, A. C. Voordouw, R. H. Meloen, A. J. van der Eb, and C. J. M. Melief.** 1989. Eradication of adenovirus E1A-induced tumors by E1A-specific cytotoxic T lymphocytes. Cell **59:**603–614.
22. **Kato, H., S. Kawai, and T. Takenawa.** 1988. Disappearance of diacylglycerol kinase translocation in *ras*-transformed cells. Biochem. Biophys. Res. Commun. **154:**959–966.
23. **Kato, M., S. Kawai, and T. Takenawa.** 1987. Altered signal transduction in erbB-transformed cells. J. Biol. Chem. **262:** 5696–5704.
24. **Klein, R., S. Jing, V. Nanduri, E. O'Rourke, and M. Barbacid.** 1991. The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell **65:**189–197.
25. **Lacal, J. C., J. Moscat, and S. A. Aaronson.** 1987. Novel source of 1,2-diacylglycerol elevated in cells transformed by Ha-*ras* oncogene. Nature (London) **330:**269–271.
26. **Macara, I. G., G. V. Marinetti, and P. C. Balduzzi.** 1984. Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: possible role in tumorigenesis. Proc. Natl. Acad. Sci. USA **81:**2728–2732.
27. **Margolls, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. Cell **57:**1101–1107.
28. **Martin-Zanca, D., S. H. Hughes, and M. Barbacid.** 1986. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature (London) **319:** 743–745.
29. **Maruyama, K., S. C. Schiavi, W. Huse, G. L. Johnson, and H. E. Ruley.** 1987. *myc* and E1A oncogenes alter the responses of PC12 cells to nerve growth factor and block differentiation. Oncogene **1:**361–367.
30. **Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell **57:**1109–1122.
31. **Mudryj, M., S. W. Hiebert, and J. R. Nevins.** 1990. A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. EMBO J. **9:**2179–2184.
32. **Nakakuma, H., Y. Sanai, K. Shiroki, and Y. Nagai.** 1984. Gene-regulated expression of glycolipid: appearance of GD3 ganglioside in rat cells on transfection with transforming gene E1 of human adenovirus type 12 DNA and its transcriptional subunits. J. Biochem. **96:**1471–1484.
33. **Nakanishi, O., Y. Homma, H. Kasaki, Y. Emori, K. Suzuki, and T. Takenawa.** 1988. Purification of two distinct types of phosphoinositide-specific phospholipase C from rat liver. Biochem. J. **256:**453–459.
34. **Noda, M., M. Ko, A. Ogura, D. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma virus carrying *ras* oncogenes induces differentiation-associated properties in a neuronal cell line. Nature (London) **318:**73–75.
35. **Onodera, K., A. Okubo, K. Yasumoto, T. Suzuki, G. Kimura, and K. Nomoto.** 1986. ^{31}P nuclear magnetic resonance analysis of lung cancer: the perchloric acid extract spectrum. Jpn. J. Cancer Res. **77:**1201–1206.
36. **Preiss, J., C. R. Loomis, W. R. Bishop, R. Stein, and R. M. Bell.** 1986. Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells. J. Biol. Chem. **261:**8597–8600.
37. **Rhee, S. G., P.-G. Suh, S.-H. Ryu, and S. Y. Lee.** 1989. Studies of inositol phospholipid-specific phospholipase C. Science **244:** 546–550.
38. **Schanen-King, C., A. Nel, L. K. Williams, and G. Landreth.** 1991. Nerve growth factor stimulates the tyrosine phosphorylation of MAP2 kinase in PC12 cells. Neuron **6:**915–922.
39. **Shenk, T., and J. Flint.** 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. Adv. Cancer Res. **57:**47–85.
40. **Shimura, H., A. Matsuzaki, K. Shiroki, M. Ohtsu, K. Fujinaga, K. Onodera, and G. Kimura.** 1988. The high sensitivity of cells transformed by E1a gene of adenovirus type 12 to diacylglycerol-mediated cell killing. Cancer Lett. **44:**143–149.
41. **Shimura, H., M. Ohtsu, A. Matsuzaki, T. Mitsudomi, K. Onodera, and G. Kimura.** 1988. Selective cytotoxicity of phospholipids and diacylglycerols to rat 3Y1 fibroblasts transformed by adenovirus type 12 and its E1A gene. Cancer Res. **45:**578–583.
42. **Shiroki, K., M. Hamaguchi, and S. Kawai.** 1992. Highly efficient focus formation by Rous sarcoma virus on adenovirus type 12 E1A-transformed rat 3Y1 cells. J. Virol. **66:**1449–1457.
43. **Taylor, S. J., H. Z. Chae, S. G. Rhee, and J. H. Exton.** 1991. Activation of the B1 isozyme of phospholipase C by δ subunits of the G_q class of G proteins. Nature (London) **350:**516–518.
44. **Todderud, G., M. I. Wahl, S. G. Rhee, and G. Carpenter.** 1990. Stimulation of phospholipase C-γ1 membrane association by epidermal growth factor. Science **249:**296–298.
45. **Traynor, A. E., D. Schubert, and W. R. Allen.** 1982. Alterations of lipid metabolism in response to nerve growth factor. J. Neurochem. **39:**1677–1683.
46. **Urbanelli, D., Y. Sawada, J. Raskova, N. C. Jones, T. Shenk, and K. Raka, Jr.** 1989. C-terminal domain of the adenovirus E1A oncogene product is required for induction of cytotoxic T lymphocytes and tumor-specific transplantation immunity. Virology **173:**607–614.
47. **Wolfman, A., and I. G. Macara.** 1987. Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in *ras*-transformed fibroblasts. Nature (London) **325:**359–361.
47a. **Yamakawa, A., et al.** Unpublished data.
48. **Yamakawa, A., and T. Takenawa.** 1988. Purification and characterization of membrane-bound phosphatidylinositol kinase from rat brain. J. Biol. Chem. **263:**17555–17560.