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Intracellular delivery of protein kinase C-α OR -ε isoform-specific antibodies promotes acquisition of a morphologically differentiated phenotype in neuroblastoma cells

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The protein kinase C (PKC) family participates in a ubiquitous cell signalling system utilizing increased turnover of phosphoinositides. Because down-regulation of total PKC activity has been implicated in the acquisition of a morphologically differentiated phenotype in SH-SY5Y neuroblastoma cells, we aimed to identify the specific PKC isoforms in this process. Here we report that intracellular delivery of PKC-α and -ε, but not -β_I, -γ or -δ isoform-specific antibodies is sufficient to induce acquisition of a morphologically differentiated phenotype in SH-SY5Y neuroblastoma cells.

Differentiation; Isoform; Neuroblastoma; Neuritogenesis; Protein kinase

1. INTRODUCTION

Our understanding of the complexity of intracellular signal transduction pathways has been increased greatly in the recent years by the discovery of eight isoforms of protein kinase C (PKC) [1–7], a phospholipid-dependent serine/threonine kinase [8] that constitutes one of the arms of the bifurcating signalling system involving enhanced phosphoinositide turnover [9]. PKC isoforms differ in their sensitivity to activators and inhibitors [1,10,11] and have discrete subcellular distribution and tissue specific expression [1]. The α, β_I, β_{II} and γ isoforms are Ca^{2+}-dependent [1], but the newly described subspecies δ, ε, (ζ) and η/L are Ca^{2+}-insensitive [2,6,7,10,11]. Because the diversity in the PKC family suggests specialization, it has been proposed that the different PKC isoforms may be involved in discrete cellular functions [1,2]. In addition, it is well documented that PKC can be down-regulated through an increase in proteolysis [12,13]. However, the biological importance of this phenomenon is unclear.

Neuroblastoma cells in culture are a model of neuronal differentiation [14,15]. The purely neuroblastic adrenergic subclone SH-SY5Y [16] extends neurites and expresses neuronal markers when treated for prolonged periods of time with the co-carcinogenic phorbol ester TPA [17,18] and early after exposure to PKC inhibitors such as staurosporine [19]. Differentiation of SH-SY5Y cells induced by TPA is almost complete [15], involving down-regulation of the expression of c-*myc* and increase of the expression of c-*fos* [20], followed by appearance of voltage-dependent Ca^{2+}-channels [21], neuron-specific enolase [18,21], norepinephrine [22], and neurites [18], containing stable cytoskeleton [23].

A decrease in PKC activity has been proposed to be a triggering event in neuritogenesis in SH-SY5Y neuroblastoma cells [17]. Thus PKC inhibitors such as staurosporine or H-7 induce extension of neurites [17,19], and treatment with TPA, sufficient to induce down-regulation of the kinase, also results in neuritogenesis [17,24]. The processes induced by these treatments contain neurofilaments, microtubules and neuromediator vesicles (U. Leli et al., in preparation).

We have previously demonstrated the presence in SH-SY5Y cells of PKC-α, -β_I, -γ, -δ and ε [24]. We have also reported differential down-regulation of the α and ε isoforms during SH- SY5Y cell differentiation induced by long-term exposure both to TPA or staurosporine [24]. The downregulation in PKC-α and -ε expression could be a phenomenon secondary to differentiation. However, the fact that the inhibitor staurosporine also caused downregulation of the same PKC isoforms affected by prolonged TPA treatment, suggests that the decrease in PKC-α and -ε could play an active role in the process of differentiation. In this study we attempted to explore this second hypothesis by interfering selectively with each isoform, using isoform-specific antibodies delivered intracellularly.

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2. EXPERIMENTAL

SH-SY5Y cells were obtained from Dr. June Biedler (Memorial Sloan-Kettering Center, NY) and maintained in RPMI 1640 with L-glutamine, containing 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, in a 95%/5% (v/v) atmosphere of air/CO_2 [17]. For the permeabilization experiments described in this paper cells were grown in Lab-Tek (8-chamber) culture slides (Nunc, Naperville, IL) to 75% confluency, and equilibrated for 24 h in the same medium containing 7% of fetal bovine serum before use.

Polyclonal antibodies against synthetic peptides representing a variable region at the carboxyl-terminal of PKC(V5) were raised in rabbit and characterized as described ([11,25,26], and P.J.P., unpublished). Antibodies were introduced into SH-SY5Y cells after permeabilization with lysolecithin as described [27] with minor modifications. Briefly, after 24 h equilibration with 7% fetal bovine serum-containing medium, cultures were washed with serum-free medium and 100 μl of 1.2 M glycerol in phosphate-buffered saline at 37°C was added. After 6 min on ice, 4 μl of a 1 mg/ml lysolecithin (Sigma Chemical Co., St. Louis, MO) solution in water was added, and the incubation continued for an additional 5 minutes. Cells were then brought to 37°C and 100 μl of a dilution of antibody was added. After 10 additional minutes at 37°C, during which the cells reseal [27], 100 μl of medium containing 21% fetal bovine serum was added. This was called t_o. After 30 min equilibration a change of medium was effected, and the cells brought back to the 7% serum-containing medium. Incubations were continued as indicated. At the end of the incubation cells were fixed and examined by phase-contrast microscopy [23]. When antibodies were pre-absorbed with antigen peptides, the peptides in three-fold excess were mixed with the antibodies at least 5 min before use. For visualization of antibodies in the treated cultures, cells were immunostained with a secondary biotinylated goat anti-rabbit antibody, using the ABC system as described [23].

Extension of neurites was quantitated by counting 100±10 cells in ten random fields per culture, and scoring as positive the cells containing one or more than one neurite, longer than the major cell body diameter [19]. Values were expressed as neurite-bearing cells, as percentage of the total cells counted. Statistical analysis was performed using the two-tailed Student's *t*-test.

3. RESULTS AND DISCUSSION

Because SH-SY5Y cells contain at least five PKC isoforms (α, β_I, γ, δ and ε), and their expression is selectively down-regulated during neuritogenesis [24], we asked which of these isoforms might be specifically implicated in the acquisition of a morphologically differentiated phenotype in these cells. No PKC isoform-specific inhibitor is available at present. Therefore, we approached the problem of dissecting which PKC isoform was involved in the acquisition of a morphologically differentiated phenotype in this cell line by interfering with the individual isoforms using antibodies. For this purpose we transiently permeabilized SH-SY5Y cultures using lysolecithin [27] and introduced immunoprecipitating, isoform-specific antibodies against PKC-α, -β_I, -γ, -δ or -ε [11,25,26].

In order to ensure that the antibodies were penetrating the cells, we immunostained cultures with goat anti-rabbit secondary antibody coupled with horseradish peroxidase, which showed that the cells were effectively permeabilized and loaded with antibody (Fig. 1). In agreement with our previous data [24], anti-PKC-α antibodies were detected immunocytochemically only at the plasma membranes of treated cells (Fig. 1). No immunostaining in the cytoplasm was observed, as reported previously for SH-SY5Y cells differentiated by treatment with either staurosporine or TPA [24].

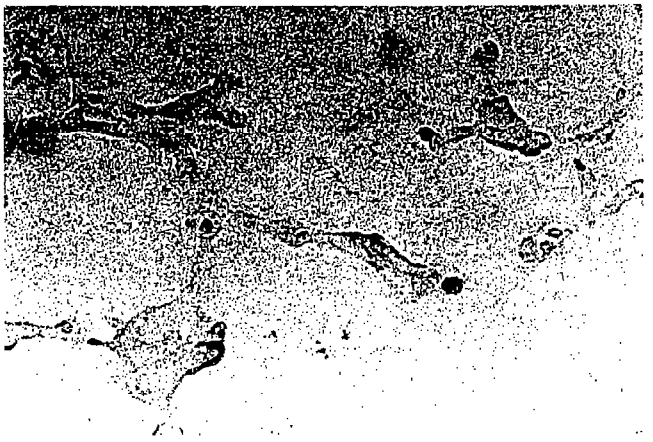

Fig. 1. Intracellular delivery of PKC-α-isoform-specific antibody. SH-SY5Y cells were permeabilized and loaded using lysolecithin and hyperosmolar shock [27], in the presence of 1:200 dilution of anti-PKC-α. The transmission light micrograph shows immunostaining with the antibody localized at the plasma membrane.

Anti-PKC-α or -ε, but not -β_I, -γ or -δ caused extension of processes longer than the cell body starting at one hour of exposure and continuing through the third hour (Table I and Fig. 2). Simultaneous treatment with anti-PKC-α and -ε produced an additive effect (Table I). Effective antibody dilution ranged from 1:200 (Table I and Fig. 2) to 1:2000, although they were less effective at these higher dilutions (not shown). In order to confirm the specificity of these effects we pre-adsorbed the antibodies with the peptide antigens used to raise them. Pre-treatment of both PKC-α and PKC-ε antibodies with an excess of their respective peptide antigens, decreased the stimulatory effects on neurite extension to values not statistically different from the control (Table I). Treatment with the antigens alone did not cause any change in the number of neurite-bearing cells (Table I).

With the protocol employed in these studies the effects of antigen binding on PKC-α and -ε activities cannot be established. However, it seems probable that in inducing neuritogenesis the antibodies have a negative effect on enzyme function, perhaps through increased breakdown of the antigen–antibody complex. This interpretation would be consistent with the observation that antisera to these C-terminal epitopes neither stimulate nor inhibit purified PKC isoforms in vitro (R. Marais and P.J.P., unpublished). This inferred loss of function would also be in agreement with published work showing selective decreases in the steady-state levels of mRNA for PKC-α and PKC-ε in the mouse neuroblastoma cell line neuro-2a during differentiation induced by retinoic acid [28,29], with a

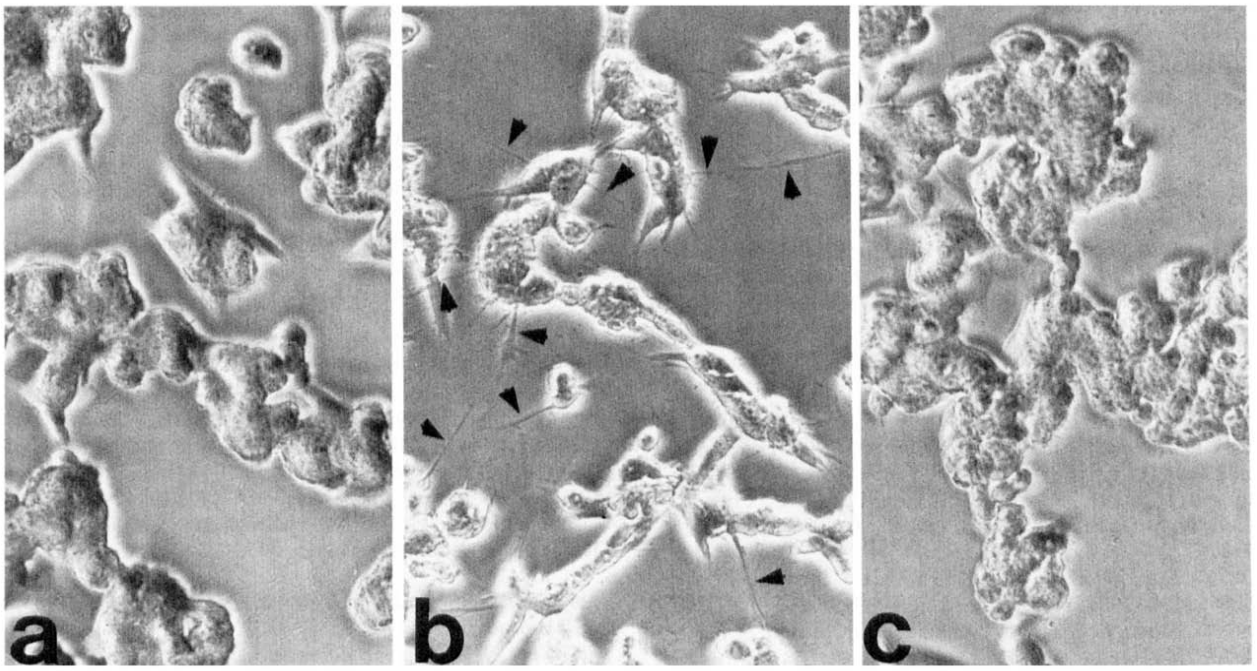

Fig. 2. Effects of PKC-isoform-specific antibody loading on the morphology of SH-SY5Y cells. SH-SY5Y cells were grown, permeabilized and loaded with a 1:200 dilution of anti-PKC-β (panel a) or -ε (panels b and c) as described in section 2. Phase-contrast micrographs were taken after 2 h. Neurite-like processes are indicated by arrowheads. Results similar to panel a were obtained using anti-PKC-β_{I}, -γ and -δ; and similar to panel b using anti-PKC-α, or anti-PKC-α + anti-PKC-ε (not shown). Panel c shows the effects of pre-adsorption of anti-PKC-ε with the ε-antigen peptide as described in Table I.

report demonstrating that decreased activity of PKC caused by actinomycin D induced extension of neurites [30], and with studies supporting that decreased phosphorylation is involved in neuritogenesis [31]. Further support to this view, that a decrease in the phosphorylation of some protein substrate of PKC may be involved in neuritogenesis also comes from studies showing that a correlation exists between phosphorylation of some intermediate filament proteins such as vimentin, and disassembly of the intermediate filament network during cell cycle [32,33].

The results presented here represent a direct demonstration that interference in the function of two specific PKC isoforms, PKC-α and PKC-ε, is sufficient to induce neurite extension in SH-SY5Y neuroblastoma cells. Differential roles of PKC-α and PKC-ε in the acquisition of a differentiated phenotype in SH-SY5Y cells are implied by the additive, rather than synergistic or mutually exclusive effects of anti-PKC-α and -ε (Table I). This suggests that down-regulation of PKC-α and PKC-ε may participate in separate steps of the process of neuroblastoma cell differentiation, in agreement with their different biochemical properties such as sensitivity to Ca^{2+}, preference for phospholipid co-factors and affinity to different protein substrates [10], and with their different subcellular localization [24]. Thus, PKC-α has been reported to associate with cytoskeletal components in several cell lines [34,35] and PKC-ε is

Table I

Effects of PKC-isoform-specific antibodies on extension of processes in SH-SY5Y cells

Additions	Cells with neurites	
	% of Control	$P<$ (n=10)
None	100.0 ± 21.3	
Anti-PKC-α	241.3 ± 38.7	0.001
Anti-PKC-β	141.3 ± 30.6	n.s.
Anti-PKC-γ	157.3 ± 43.7	n.s.
Anti-PKC-δ	104.0 ± 28.0	n.s.
Anti-PKC-ε	233.3 ± 41.3	0.003
Anti-PKC-α + α-antigen	162.7 ± 33.3	n.s.
α-antigen	84.0 ± 12.0	n.s.
Anti-PKC-ε + ε-antigen	137.3 ± 25.3	n.s.
ε-antigen	98.7 ± 8.0	n.s.
Anti-PKC-α + -ε	368.0 ± 40.0	0.0001
Anti-PKC-α + -ε + α- and ε-antigens	136.0 ± 29.3	n.s.

SH-SY5Y cells were permeabilized and loaded with 1:200 dilution of PKC-isoform-specific antibodies as described in section 2. PKC-α and PKC-ε antigen peptides were used to pre-absorb the sera. Data are means ± S.D. of data pooled from two separate experiments. Experiments were repeated more than 8 times with similar results, except for some variability in the basal number of cells bearing neurites. Data are expressed as percentages of controls to offset the variable baseline values. The percentage of neurite-bearing cells in untreated cultures (no antiserum in the permeabilization protocol) was 7.5 ± 1.6%.

nearly exclusively localized in the membranes and processes in SH-SY5Y cells [24].

In conclusion, the data presented here show that in neuroblastoma cells expressing multiple isoforms of PKC, it is the α and ε isoforms that are selectively involved in the control of the acquisition of a morphologically differentiated phenotype.

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