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Down-regulation of neuropathy target esterase by protein kinase C activation with PMA stimulation

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Abstract Neuropathy target esterase (NTE) was originally identified as the primary target site of those organophosphorus compounds that induce delayed neuropathy in human and some animals. Here we examined the role of protein kinase C (PKC) in the regulation of the NTE activity in mammalian cells. Six-hour exposure of human neuroblastoma SK-N-SH cell to a PKC activator phorbol 12-myristate 13-acetate (PMA) decreased the activity of NTE, and this effect was blocked by the PKC inhibitor staurosporine. These results suggest that PKC down-regulates the activity of NTE. NTE protein levels were down-regulated by PMA-stimulation as detected by Western blot analysis using the NTE-specific antibody, which resulted from down-regulation of NTE mRNA level as verified by real-time reverse transcription polymerase chain reaction (RT-PCR). However, there were no changes in the activity or protein levels of stable expression of NTE esterase activity domain (NEST) in SK-N-SH cells and transient expression of full-length NTE construct in COS7 cells driven by cytomegalovirus (CMV) promoter rather than by the cell's own one, despite the absence or presence of PMA stimulation. Together, these findings suggest that stimulation with PMA reduces the expression of NTE mRNA levels but does not affect the exogenous promoter-driven NTE expression in mammalian cells.

Keywords Neuropathy target esterase · Protein kinase C · Down-regulation · Mammalian cell

Abbreviation

cAMP	Cyclic AMP
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPC	Glycerophosphocholine
NEST	NTE esterase activity domain
NP40	Nonidet P-40
NTE	Neuropathy target esterase
OPIDN	OP-induced delayed neuropathy
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PtdCho	Phosphatidylcholine
PV	Phenyl valerate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Introduction

Neuropathy target esterase (NTE) was identified over 30 years ago as the primary target of organophosphorus compounds (OP) that cause a delayed paralyzing syndrome with degeneration of nerve axons [1]. Although the inhibition and subsequent aging of NTE has been proposed to be an initiating event in OP-induced delayed neuropathy (OPIDN), the events that occur between NTE inhibition and the appearance of clinical pathology are not completely understood [2]. As such, characterizing the molecular and cellular functions of NTE should elucidate the mechanism of OPIDN.

Neuropathy target esterase is a polypeptide of 1,327 amino acids and is anchored to the cytoplasmic face of endoplasmic reticulum (ER) through an amino-terminal transmembrane segment in cells [3, 4]. NTE displayed potent lysophospholipase activity in mouse brain and further characterized as a novel phospholipase B (PLB) responsible for converting phosphatidylcholine (PtdCho) to glycerophosphocholine (GPC) in mammalian cells and regulating PtdCho homeostasis in *Drosophila* [5–7]. Complete inactivation of mouse NTE gene resulted in embryonic lethality due to placental failure and impaired vasculogenesis [8, 9]. Additionally, mice with a brain-specific deletion of NTE exhibited neurodegeneration and loss of swiss cheese/NTE activity caused neuronal and glial death in adult *Drosophila* [4, 7], suggesting NTE is essential for embryonic and nervous development.

Although the character and function of NTE has been elucidated, little is known about the regulation of its activity. Potential regulation of NTE activity is suggested by the similarity of sequences within NTE's amino-terminal domain to the regulatory subunit of protein kinase A: the implication of this sequence homology is that NTE may be regulated directly by the binding of cyclic AMP (cAMP) [10]. By expressing the regulatory domain of NTE constructs in *E. coli*, binding of the radiolabeled cAMP to purified recombinant the regulatory domain of NTE was failed to be detected [11]. However, overexpression of all constructs of NTE containing the regulatory domain leads to aggregation of the recombinant polypeptide in mammalian cells so that its normal biologicallyactive conformation may not be attained [12]. NTE converts PtdCho to GPC in the CDP-choline pathway, so the most obvious regulator of NTE activity would be its substrate, PtdCho. More recently, Sec14p, an essential soluble protein possessing in vitro phosphatidylinositol (PI)/phosphatidylcholine (PC) transfer activity, has been shown to interact functionally with NTE1, the homologous protein of NTE in yeast, to increase the rate of PtdCho deacylation to GPC in yeast [13].

Protein kinase C (PKC) was first described as a calcium-activated, phospholipid-dependent serine/threonine protein kinase and the phorbol ester phorbol 12-myristate 13-acetate (PMA) is a potent activator of PKC, which plays an important role in signal transduction associated with a variety of cellular responses including cell growth and differentiation, gene expression, hormone secretion, and membrane function [14, 15]. Sequence analysis of NTE using the NetPhosK 1.0 server (http://www.cbs.dtu.dk/services/NetPhosK/) [16] and the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) [17] identified several putative phosphorylation sites by PKC, which suggested NTE activity may be regulated by PKC (our unpublished data). In addition, human lysophospholipase A (hLysoPLA I) was up regulated by PMA through a PKC-dependent pathway [18] and mouse brain NTE has potent lysophospholipase activity [5]. However, whether PKC regulates NTE activity with the stimulation of PMA still remains unknown. In order to know the possible regulation of NTE by PKC, we studied the effects of PMA-mediated PKC activation on the activity and expression of NTE in mammalian cells.

Materials and methods

Materials

The human neuroblastoma SK-N-SH cell line was obtained from Cell Center of Chinese Academy of Medical Sciences (Beijing, China). Monkey kidney COS7 cells were kindly provided by Prof. Y. -L. Wang (State Key Lab of Reproductive Biology, Beijing, China). SH/NEST, human neuroblastoma SK-N-SH cells with stable expression of NTE esterase activity domain (NEST), was generated in our laboratory [19]. Cell culture reagents were purchased from Gibco (Grand Island, NY, USA). NTE cDNA clone D16 plasmid was kindly provided by Dr. P. Glynn (MRC Toxicology Unit, Leicester, UK) and pCMV-Myc was obtained from Clontech (Clontech Palo Alto, CA, USA). The human NTE-specific antibody against peptides corresponding to amino acids 37–48/1316–1327 (VPKTPAPDGPRK/LPQEPPGSATDA) was generated by Shanghai Casarray Co. LTD (Shanghai, China). The anti-actin monoclonal antibody, anti-Myc monoclonal antibody, and anti-goat IgG and anti-IgY horseradish peroxidase conjugated antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Staurosporine was purchased from EMD Biosciences (Darmstadt, Germany). Enhanced chemiluminescence (ECL) reagents were obtained from Pierce (Rockford, IL, USA). Transfection regent lipofectamine 2000, Trizol, and Superscript™ First-strand Synthesis

System for RT-PCR reaction were purchased from Invitrogen Life Technologies (Groningen, The Netherlands). SYBR® RT-PCR Kit was purchased from Takara (Dalian, China). Paraoxon (90% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenyl valerate and mipafox were synthesized in our laboratory as described by Johnson [20] and USA patent (No. 2678334), respectively.

Cell culture, treatment, and transient transfection

SK-N-SH and COS7 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml each penicillin and streptomycin. Incubations were carried out at 37°C in a humidified atmosphere of 5% CO_2/95% (v/v) air. The cells were maintained in the logarithmic phase of growth and subcultured at 3–4 days intervals. To stimulate cells with PMA, cells were plated at 1×10^6 cells/well in 60 mm plates and grown at 37°C for 24 h. The cells were rinsed once and changed to the medium alone or medium containing the indicated amounts of PMA or proper controls. To antagonize PKC activation by PMA, cells were incubated with 100 nM staurosporine for 60 min, followed by PMA addition and incubation as described above. Myc-tagged full-length NTE plasmid (pCMV-Myc-NTE) was constructed by subcloning the NTE cDNA from D16 into pCMV-Myc. For pCMV-Myc-NTE transient transfection, COS7 cells were seeded in tissue culture plates for 18–24 h prior to transfection. At 90–95% confluency, transfections were performed using Lipofectamine 2000 reagent as recommended by the manufacturer and the cells were harvested at 48 h post-transfection for further analysis.

Neuropathy target esterase activity assay

The cells were harvested after rinsed with phosphate-buffered saline (PBS). Aliquots of the pellets were resuspended in TE buffer (50 mM Tris-HCl, 0.2 mM EDTA, pH 8.0), homogenized and centrifuged at $100 \times g$ at 4°C for 2 min. NTE activity in the supernatant fraction was determined from colorimetric assay of the phenol formed by the absorbance difference for PV hydrolysis between samples exposed to 40 μM paraoxon and those with both 40 μM paraoxon plus 50 μM mipafox [20, 21] according to the modified method for reduced volume microassay [22, 23]. Data shown are averages of at least three duplicate reactions from three independent experiments.

Western blotting analyses

Cells were rinsed three times with ice-cold PBS and lysed in lysis buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 5 mM EGTA, 1 mM EDTA, 0.5% (v/v) Triton X-100, 0.5% (v/v) NP40, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml final concentration of each of aprotinin, leupeptin, and pepstatin). The lysates were clarified by centrifugation at $10{,}000 \times g$ for 10 min at 4°C and the supernatant were collected for further treatment. Protein concentration was determined by Coomassie brilliant blue staining using bovine serum albumin (BSA) as a standard [24]. The protein samples were fractionated by SDS-PAGE with a 4% (w/v) stacking gel and 10% (w/v) separating gel and transferred onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The membranes were first blocked by incubation in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20) containing 5% (w/v) non-fat milk at room temperature at least 1 h, then incubated sequentially with primary antibody and HRP-conjugated secondary antibody, and then detected using ChemiDoc XRS system (Bio-Rad, Munich, Germany) by standard ECL reagents. To measure actin levels, blots were stripped and reprobed with anti-actin antibody.

Real-time Reverse Transcription (RT)-PCR analysis

Real-time RT-PCR was performed using Takara SYBR® RT-PCR Kit. Primers for amplification of human NTE and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were NTE-forward primer (5′-CCAAGAGTTCCGGCTG TCA-3′) and NTE-reverse primer (5′-CACAATGAGGAT GCAGTCGG-3′), GAPDH- forward primer (5′-ATGCTG GCGCTGAGTACGTC-3′) and GAPH- reverse primer (5′-GGTCATGAGTCCTTCCACGATA-3′), respectively. Total RNA was isolated with Trizol reagent according to the manufacturer's instructions and was quantified photometrically. cDNA templates were generated by reverse transcription using Superscirpt ™ First-strand Synthesis System. For fluorescence signal detection, a MX3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) was programed with an initial denaturation step of 10 s at 94°C, followed by 40 temperature cycles for 5 s at 94°C, 20 s at 55°C, and 20 s at 72°C, fluorescence signal was detected at 55°C, 1 cycle for 30 s at 94°C, 30 s at 55°C, 30 s at 94°C to collect fluorescence signal at all points for dissociation curve analysis which can respond to the specificity of amplification product. All measurements were produced in duplicate, and for real-time PCR primer set, reaction efficiency estimates were derived from standard curves that were generated using serial dilutions of the corresponding calibrator sample. These were then used to transform the cycle threshold (Ct) values for analysis. In terms of the standard curve and Cts, the ratios of initial cDNA quantity NTE/GAPDH were calculated as relative NTE mRNA level.

Statistical analysis

Data were generally expressed as Mean ± standard error (SE) values. Groups of data were compared by one-way ANOVA and by post-hoc analysis using Student-Keuls method. A difference between means was considered significant at $P < 0.05$.

Results

PKC-dependent down-regulation of the activity of NTE in SK-N-SH cells

Previously, we determined that human neuroblastoma SK-N-SH cells display high levels of NTE activity [19]. Therefore, we first evaluated the effects of PKC activation using different concentrations of PMA on the activity of endogenous NTE in SK-N-SH cells. As shown in Fig. 1, NTE activity was significantly decreased in SK-N-SH cells stimulated with 25 or 100 ng/ml PMA for 6 h. In 100 ng/ml PMA-treated cells, NTE activity was about one-third of that in control cells. Higher concentrations of PMA, such as 200 ng/ml PMA, did not further reduce NTE activity and shorter incubation times had less effect on NTE levels (data not shown). In order to determine whether the decreased NTE level is PKC-dependent, we inhibited PKC activation with staurosporine. Pretreatment of SK-N-SH cells with 100 nM of the PKC inhibitor staurosporine for 60 min completely blocked PMA-induced down-regulation of NTE while staurosporine alone did not affect NTE activity (Fig. 1). These results suggest that PKC down-regulates the activity of NTE.

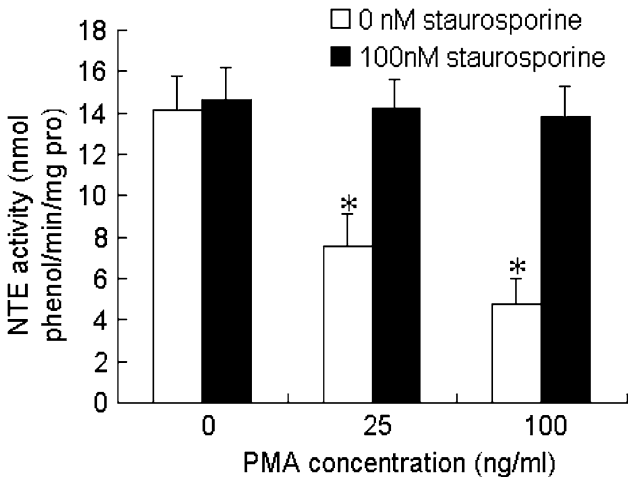

Fig. 1 Decreased NTE activity in SK-N-SH cells by PMA stimulation. Cells were incubated without or with 100 nM staurosporine for 60 min, following which PMA (0, 25, or 100 ng/ml) was added and incubated for 6 h, NTE activity was then assayed as described in the section of Materials and methods. Data are Mean ± SE (bars) from three independent experiments. Statistical analysis of control cells versus PMA-treated cells was carried out by ANOVA. *$P < 0.01$, compared with controls ($n = 3$)

PKC-dependent down-regulation of the mRNA level of NTE in SK-N-SH cells

The decrease in activity of NTE by PMA mediated PKC activation in SK-N-SH cells may result from the reduction of NTE content. As such, we generated an NTE-specific antibody to detect the changes of NTE protein levels after stimulation with PMA treatment in SK-N-SH cells. As indicated in Fig. 2, NTE content was decreased while the levels of actin were unaffected. So the reduction in NTE activity is due to reductions in the levels of NTE protein. Furthermore, the effect of PMA mediated PKC activation or staurosporine mediated PKC inhibition on the transcription of NTE was investigated by real-time RT-PCR. As shown in Table 1, the mRNA levels of NTE in PMA-treated cells were down-regulated compared with that in control cells. After 100 ng/ml PMA stimulation for 6 h, NTE mRNA level was significantly decreased; the level was only about one-third of that in control cells, which is consistent with the NTE activity results. In contrast, no changes of NTE mRNA levels were found between PMA-treated cells that were pretreated by staurosporine and its corresponding control cells. Therefore, PKC-dependent down-regulation of the mRNA level of NTE in SK-N-SH cells leads to the decrease of NTE protein levels and reduction of NTE activity.

PKC activation with stimulation of PMA has no effect on NTE expression activity driven by the exogenous promoter in mammalian cells

In order to investigate the effect of PKC activation with stimulation of PMA on the exogenous promoter-driven

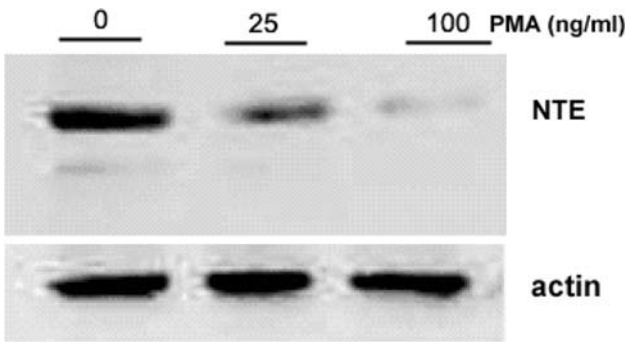

Fig. 2 Effect of PMA on NTE protein level in SK-N-SH cells. SK-N-SH cells were treated with PMA and then lysed in lysis buffer. Equal protein amounts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. NTE protein levels were detected with anti-NTE antibody and actin levels were analyzed by anti-actin antibody

Table 1 Effects of PKC activation/inhibition on the level of NTE mRNA in SK-N-SH cells

Group	Relative copies of NTE/GAPDH
Control (DMSO)	0.032 ± 0.006
PMA (25 ng/ml)	0.015 ± 0.008
PMA (100 ng/ml)	0.011 ± 0.006*
Control (staurosporine + DMSO)	0.034 ± 0.004
Staurosporine + PMA (25 ng/ml)	0.039 ± 0.007
Staurosporine + PMA (100 ng/ml)	0.038 ± 0.011

SK-N-SH cells were incubated with 100 nM staurosporine for 60 min, followed by PMA addition and incubation stimulated with PMA (0, 25, or 100 ng/ml) for 6 h, then the level of NTE mRNA was assayed by real-time PCR. The level of NTE mRNA is expressed as relative copies of NTE/GAPDH. The mRNA level of NTE in treated-cells was compared with that of corresponding control cells, *$P < 0.01$, $n = 3$

NTE expression in mammalian cells, we transiently over-expressed Myc-tagged NTE driven by cytomegalovirus (CMV) promoter in COS7 cells to measure the changes in NTE activity after PMA treatment. There was a very low basal NTE activity of 4.2 ± 0.3 nmol/min/mg protein in COS7 cells. At 48 h post-transfection, the activity of NTE was significantly increased to 185.0 ± 5.4 nmol/min/mg protein. As shown in Fig. 3A, PMA did not significantly reduce the activity of NTE in the COS7 cells that exhibited high levels of NTE activity due to Myc-tagged NTE expression. The levels of Myc-tagged NTE protein in PMA-treated cells were not reduced compared with that in control cells, as detected by the anti-Myc antibody in Western blot analysis (Fig. 3B). Moreover, the effect of PMA treatment on the NTE activity of NEST was investigated using SH/NEST, human neuroblastoma SK-N-SH cells with stable expression of NEST, as the cell model. As shown in Fig. 4, there was about a 1.7-fold increase in NTE activity in SH/NEST cells compared with SK-N-SH cells without PMA stimulation. After stimulation with 100 ng/ml PMA for 6 h, the NTE activity in both SK-N-SH and SH/NEST cells was decreased dramatically compared to that in the corresponding control cells without PMA stimulation (Fig. 4). However, the amplitude of decrease in NTE activity by the PMA stimulation was equal in the two cell lines. Therefore, PMA mediated activation of PKC has no effect on the exogenous promoter-driven NTE expression in mammalian cells.

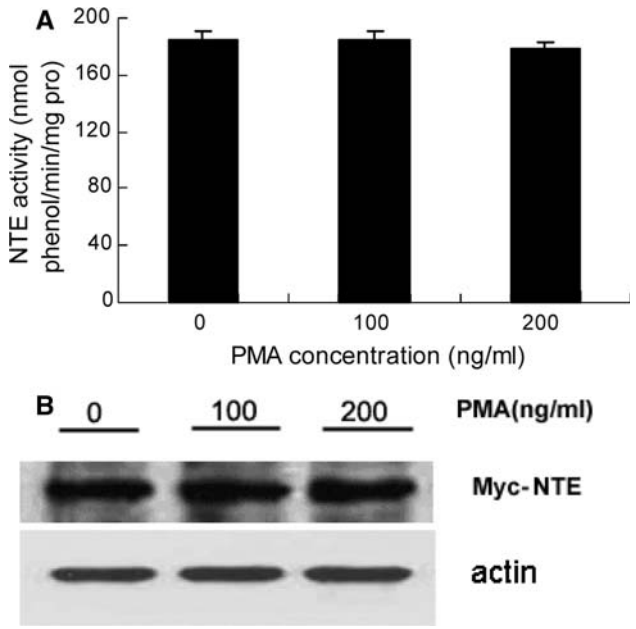

Fig. 3 Effect of PMA on NTE activity and protein content in COS7 cells. COS7 Cells were transfected with pCMV-Myc-NTE construct. 48 h post-transfection, cells were incubated in 100 or 200 ng/ml PMA for 6 h, then NTE activity (**A**) and Myc-tagged NTE protein (**B**) were assayed as described in the Materials and methods section. Results of NTE activity are means ± SE (bars) for duplicate dishes from three independent experiments for each panel ($n = 3$). Results of the expression of Myc-tagged NTE are representative of three independent experiments

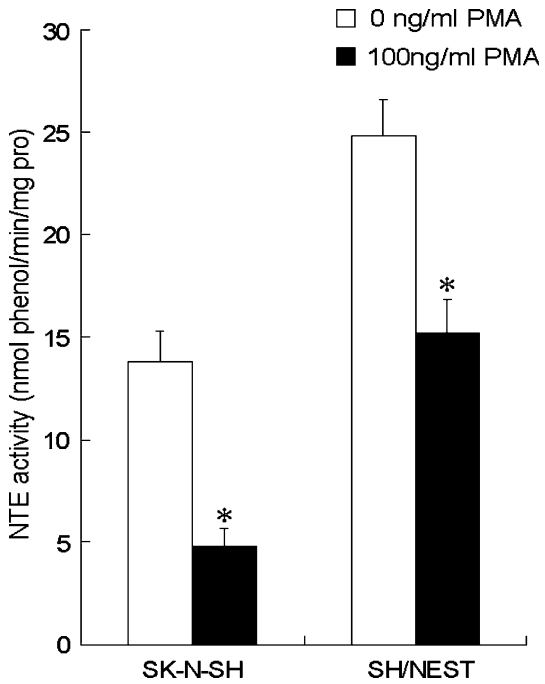

Fig. 4 Effect of PMA on NTE activity in SH/NEST cells. SH/NEST was maintained in DMEM medium containing 200 μg/ml G418. Control cells and SH/NEST cells were treated with 0 or 100 ng/ml PMA for 6 h. NTE activity was determined as described in the section of Materials and methods. Results are means ± SE (bars) from three independent experiments. Statistical analysis of control cells versus PMA-treated cells was carried out by ANOVA. *$P < 0.01$, compared with controls, $n = 3$

Discussion

In the present study, we show for the first time that a 6 h treatment of PMA induced down-regulated NTE activity, which coincides with a reduction of NTE protein levels in SK-N-SH cells. The real-time PCR experiment further revealed that the reduction of NTE protein level resulted from the decrease of its mRNA levels. The PMA-induced decrease in *NTE* mRNA levels could result from either decreased transcription or enhanced degradation of the mRNA. However, PMA stimulation has no effect on the expression of recombinant Myc-NTE and NEST driven by the CMV promoter, which is different from that of the expression of the cell's NTE driven by its own promoter. Moreover, it was reported that the half-life of *NTE* mRNA in mIMCD3 cells is about 24 h and it was unaffected by high NaCl, which can increase NTE mRNA and induce GPC synthesis [25]. These results suggest that the decrease of *NTE* mRNA levels after 6 h treatment of PMA result from decreased transcription, rather than enhanced degradation of the mRNA. Furthermore, the rapidity of the PMA-induced mRNA expression inhibition is striking and has implications for the slow rate of turnover of NTE. Previous results showed that it is a slow *de novo* synthesis of NTE in vivo after OP treatment; the half-life of reappearance of active NTE needed several days, such as 5 days in bovine chromaffin cell cultures [26], 4–6 days in hen nervous system [27, 28], and 2–3 days in chick nervous system [29, 30].

Although there are several predicted potential phosphorylation sites by PKC in the regulatory domain and NEST of human NTE, PKC activation by PMA treatment has no effect on the protein content and activity of the CMV promoter-driven expression of NTE or NEST. These results suggest that PKC may not directly phosphorylate NTE or regulate its activity after phosphorylation.

The general homeostasis between synthesis and turnover of the membrane phospholipid PtdCho is critical for cellular function. A very complex mechanism is associated with the membrane PtdCho homeostasis, which includes many important enzymes, such as phospholipase B (PLB) and phospholipase D (PLD). PtdCho can be degraded by PLB and PLD. PLD catalyzes the scission of a phosphoester bond to produce phosphatidic acid and choline. PLB deacylates PtdCho producing GPC and two free fatty acids and NTE is a devoted PLB against only cytidylyldiphosphocholine pathway derived PtdCho [6]. However, in human neuroblastoma SK-N-MC cells, activation of PLD by PMA was dependent on PMA concentration without change of PtdCho synthesis [31, 32]. As such, in order to keep PtdCho homeostasis, PMA stimulation may activate PtdCho-specific PLD and down-regulate NTE without the change of PtdCho synthesis in human neuroblastoma SK-N-SH.

In summary, in mammalian cells sustained PKC activation by PMA stimulation causes inhibition of NTE mRNA transcription, leading to down-regulation of the activity and protein levels of NTE, and has no effect on exogenous (but not cell's own promoter-driven) NTE expression. It will be of interest to further study the mechanism of the transcription of *NTE* mRNA by PMA.

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