

Enhanced Expression and Activation of CTP:Phosphocholine Cytidylyltransferase β 2 during Neurite Outgrowth*

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During differentiation neurons increase phospholipid biosynthesis to provide new membrane for neurite growth. We studied the regulation of phosphatidylcholine (PC) biosynthesis during differentiation of two neuronal cell lines: PC12 cells and Neuro2a cells. We hypothesized that in PC12 cells nerve growth factor (NGF) would up-regulate the activity and expression of the rate-limiting enzyme in PC biosynthesis, CTP:phosphocholine cytidylyltransferase (CT). During neurite outgrowth, NGF doubled the amount of cellular PC and CT activity. CT β 2 mRNA increased within 1 day of NGF application, prior to the formation of visible neurites, and continued to increase during neurite growth. When neurites retracted in response to NGF withdrawal, CT β 2 mRNA, protein, and CT activity decreased. NGF specifically activated CT β 2 by promoting its translocation from cytosol to membranes. In contrast, NGF did not alter CT α expression or translocation. The increase in both CT β 2 mRNA and CT activity was inhibited by U0126, an inhibitor of mitogen-activated kinase/extracellular signal-regulated kinase 1/2 (MEK1/2). In Neuro2a cells, retinoic acid significantly increased CT activity (by 54%) and increased CT β 2 protein, coincident with neurite outgrowth but did not change CT α expression. Together, these data suggest that the CT β 2 isoform of CT is specifically up-regulated and activated during neuronal differentiation to increase PC biosynthesis for growing neurites.

The differentiation of neuronal cell lines is characterized by a halt in proliferation, and the production of neurites. The rapid growth of neurites places a heavy demand on the differentiating cell for new membrane components. It is therefore not surprising that studies in rat pheochromocytoma (PC12)¹ cells

have shown that phosphatidylcholine (PC) biosynthesis increases during neurite outgrowth (1). Likewise, sympathetic neurons also increase phospholipid biosynthesis during axonal growth (2). PC, the predominant phospholipid in mammalian membranes, is synthesized primarily by the Kennedy pathway in which choline is phosphorylated by choline kinase to phosphocholine, which is converted to CDP-choline and subsequently to PC (3). In this pathway, the conversion of phosphocholine to CDP-choline, catalyzed by CTP:phosphocholine cytidylyltransferase (CT), is the rate-limiting step. To date, two murine/human genes encoding CT isoforms have been identified: *Pcyt1a* and *Pcyt1b*. CT α , the product of *Pcyt1a*, is expressed in all tissues. CT β 2 and CT β 3, which are splice variants of murine *Pcyt1b*, are both enriched in brain compared with other tissues (4, 5). However, whereas CT β 2 is expressed in both embryonic and adult mouse brain, CT β 3 is found only in adult brain (5). Orthologs of CT α and CT β 2 have been cloned from rat and have similar tissue distributions to those in mouse (6). Interestingly, in rat hippocampus, CT β 2 mRNA is up-regulated by arginine vasopressin_{4–8}, a metabolite of arginine vasopressin known to enhance memory and promote neurite growth in hippocampal neurons (6).

PC12 cells are widely used for studies on neurite growth that is induced by nerve growth factor (NGF) (7). NGF elicits neurite outgrowth via activation of mitogen-activated protein kinase (MAP kinase) (8). MAP kinase signaling has also been implicated in ganglioside-dependent neurite outgrowth in murine Neuro2a cells (9), which rapidly differentiate in response to retinoic acid.

We hypothesized that in differentiating neuronal cells PC biosynthesis is stimulated by an increase in CT activity and/or expression. To test this hypothesis, we examined PC biosynthesis, CT activity and CT isoform expression in PC12 cells and in Neuro2a cells. The results demonstrate a role for CT in neuritogenesis. Specifically, we observed a striking induction in expression of the CT β 2 isoform during neurite outgrowth.

EXPERIMENTAL PROCEDURES

Materials—The MAP kinase/ERK kinase1/2 inhibitor, U0126, was obtained from Promega. All *trans*-retinoic acid was from Sigma. All cell culture media and reagents were from Invitrogen.

Cell Culture—PC12 cells were obtained from the American Type Cell Culture Collection. Cells were maintained in F12-K medium supplemented with 15% heat-inactivated horse serum and 2.5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO₂. For differentiation experiments, cells were seeded on collagen-coated 35-mm dishes at a density of 1×10^5 cells/dish. Cells were incubated overnight and then 50 ng/ml 2.5 S NGF (Alomone Labs) was added. For NGF-withdrawn conditions, the cells were grown in NGF-containing medium for 5 days after which the medium was removed and replaced with control medium lacking NGF. All cells were harvested on day 9. The MAP kinase inhibitor, U0126, was dissolved in dimethyl sulfoxide and added to the medium to give a final concentration of inhibitor of 50 μ M. The control medium contained the same amount of vehicle.

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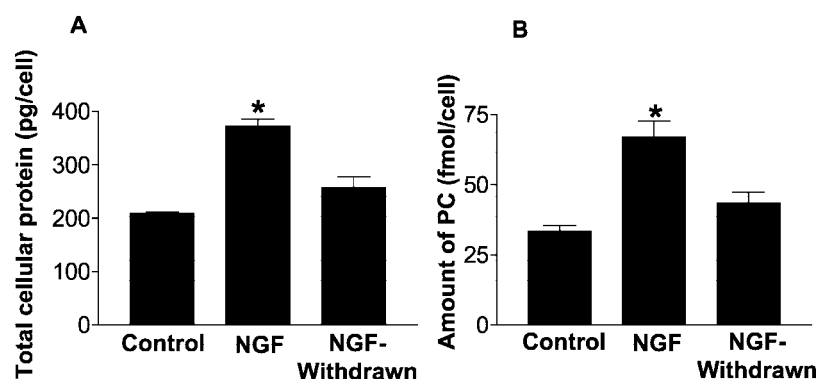
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¹ The abbreviations used are: PC12, rat pheochromocytoma; CT, CTP:phosphocholine cytidylyltransferase; DMEM, Dulbecco's modified Eagle's medium; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated kinase/extracellular signal-regulated kinase kinase; NGF, nerve growth factor; PC, phosphatidylcholine.

FIG. 1. Cellular protein and phosphatidylcholine are increased in NGF-treated PC12 cells. PC12 cells were incubated with 50 ng/ml NGF for 9 days (NGF), incubated for 5 days with NGF after which medium lacking NGF was present for 4 days (NGF-withdrawn), or incubated without NGF for 9 days (Control). Cells were harvested and lipids were extracted from cell lysates. The amount of cellular protein (A) and cellular phosphatidylcholine (PC) (B) were measured. Data represent means \pm S.E. ($n = 6$) of three independent experiments with duplicate dishes. *, control versus NGF, $p < 0.001$.



The murine neuroblastoma cell line, Neuro2a, was a generous gift from Dr. David Williams (State University of New York, Stonybrook). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1 ratio) containing 10% heat-inactivated fetal bovine serum. For differentiation experiments, cells were plated on 35-mm dishes at a density of 1.5×10^5 cells per dish in DMEM containing 2% fetal bovine serum. Following an overnight incubation, cell medium was replaced with DMEM with 2% fetal bovine serum containing 20 μ M retinoic acid (Sigma). After incubation for 48 h, cells were harvested in homogenization buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 100 μ g/ml each of leupeptin and aprotinin) and treated as described for PC12 cells.

PC Measurements and CT Assays—Cells were collected in 1 ml of homogenization buffer A. The cells were counted and then sonicated for 20 s at 4 $^{\circ}$ C. Cell lysates were centrifuged at $7,000 \times g$ for 5 min to pellet nuclei and unbroken cells. Aliquots of supernatant were used for lipid extractions, CT activity assays, and immunoblotting. For fractionation of membranes and cytosol, an aliquot of the cell lysate was centrifuged at $470,000 \times g$ for 30 min. The supernatant corresponded to the cytosol fraction; the pellet (microsomal fraction) was re-suspended in homogenization buffer A. All protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin used as a standard. CT activities in the total homogenate, soluble and membrane fractions were determined in the presence of PC:oleate vesicles by monitoring the conversion of phospho[3 H]choline to CDP-[3 H]choline as previously described (10).

RNA Preparation and Reverse Transcriptase-mediated PCR—Total poly(A) RNA was isolated using the TRIZOL (Invitrogen) reagent as described by the manufacturer. 1 μ g of total RNA was reverse-transcribed using a first strand cDNA synthesis kit (Superscript II, Invitrogen) according to the manufacturer's protocol. Aliquots of the reverse transcribed product were used for PCR amplification with the following sequence-specific primers for CT α : AGTGGAGGAGAAGAGCATCG (sense) and GGAAGTCTTGCCAGAGAAGG (antisense); choline kinase α , GTGAATGGATGGCTCCACTC (sense) and ACCTTCAAAGCCTGTGTCATG (antisense); cyclophilin TCCAAAGACAGCAGAAAACCTTTCG (sense) and TCTTCTTGCTGGTCTTGCCATTTC (antisense); CT β 3: AAATGCAGCATGGACAAGG (sense) and TTTGCTTGCTAAGTGCC (antisense). Two separate primer pairs were used for PCR amplification of CT β 2: pair 1, CCAAGGAGCTGAATGTCAGC (sense) and GCACTTGTTAACCAGGCACC (antisense); pair 2, GCCATGCCAGTAGTTACCACT (sense) and TTTGCTGCATAAGTGCC (antisense). All PCR amplifications used the following program: 94 $^{\circ}$ C for 1 min, 60 $^{\circ}$ C for 1.5 min and 72 $^{\circ}$ C for 1.5 min for a total of 32 thermocycles (40 cycles for CT β 3), which was within the linear response range for each primer pair. The primers generated amplicons of the following sizes: CT α , 232 bp; CT β 2, 452 bp (pair 1) and 302 bp (pair 2); CT β 3, 218 bp; choline kinase α (200 bp); and cyclophilin, 380 bp. All amplified products were resolved on 1.5% agarose gels and stained with ethidium bromide. The amounts of the products generated with the CT α , choline kinase α , CT β 2 and CT β 3 primer pairs were normalized to the amount of cyclophilin product.

RNase Protection Assays—Ribonuclease protection assays were performed using a RPA IIITM kit (Ambion) according to the manufacturer's protocol. Briefly, 5 μ g of total RNA was hybridized to 32 P-labeled antisense riboprobes corresponding to CT α , CT β 2, or cyclophilin. Following an overnight hybridization, the RNA was digested with a single-strand-specific RNase (Ambion) and then separated on a 5% denaturing polyacrylamide gel and detected by autoradiography. Riboprobe-protected

fragments were predicted to be 200bp (CT α), 200bp (CT β 2), and 250bp (cyclophilin).

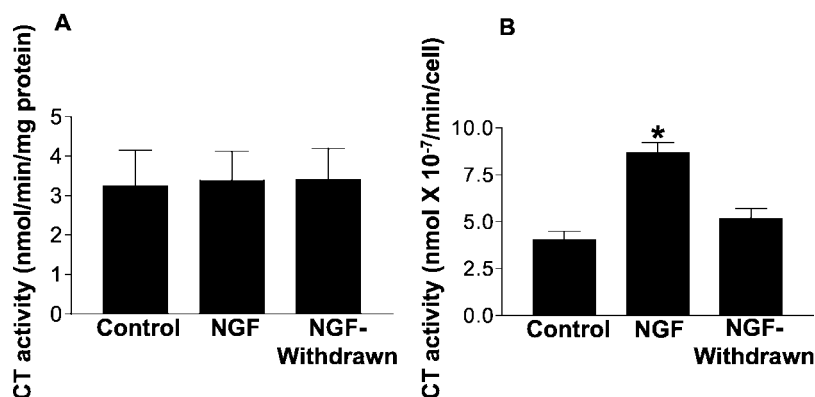
Immunoblots—Anti-human CT β 2 and anti-human CT α rabbit polyclonal antibodies were generous gifts from Dr. S. Jackowski (St. Jude Children's Research Hospital, Memphis, TN). The CT β 2 antibody was raised against a peptide corresponding to amino acids 347–365 of CT β 2 (4). The CT α antibody was raised against a peptide corresponding to amino acids 1–17 of CT α (4). Equal amounts of protein in cell lysates were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS and transferred onto Immobilon-P transfer membranes (Millipore). Ponceau S stain was used to compare protein loading in all lanes of the gel. The membranes were blocked for 2 h with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% Tween 20 (TBS-T) and then incubated overnight with either anti-CT α (dilution 1:1,000) or anti-CT β 2 (dilution 1:1,000) antibodies. The blots were washed with TBS-T for 1 h and incubated with secondary antibody, horseradish peroxidase linked to anti-rabbit IgG (1/2500 dilution) for 1 h. After incubation the blot was washed with TBS-T and visualized with Amersham Biosciences ECL reagents.

RESULTS

NGF Increases Cellular Protein and Phosphatidylcholine Levels in PC12 Cells—PC12 cells require NGF for differentiation to a neuronal phenotype; however, this differentiation is reversible. Once NGF is removed from the culture medium, the cells retract their neurites and resume proliferation. In our experiments, PC12 cells were incubated with medium containing 50 ng/ml NGF for 9 days (NGF), incubated with 50 ng/ml NGF for 5 days then cultured in medium lacking NGF for 4 more days (NGF-Withdrawn) or incubated without NGF throughout the experiment (Control). As expected, untreated cells continued to proliferate while NGF-treated cells stopped proliferating and started to extend neurites by day 3 of NGF treatment. NGF-withdrawn cells extended neurites during NGF treatment but the neurites retracted when NGF was removed from the culture medium.

Previous studies have shown that incubation of PC12 cells with NGF significantly increases cellular protein content as well as PC content (1). We used these two parameters, along with the presence of neurites, to confirm that the PC12 cells were responding appropriately to NGF treatment. After 9 days of NGF treatment, the amount of protein per cell increased by 78% compared with that of control cells (373 pg/cell versus 209 pg/cell) (Fig. 1A). Prior to NGF withdrawal on day 5, NGF-withdrawn cells had significantly higher cellular protein compared with that in control cells; however, at day 9, when the cells had been deprived of NGF for 4 days, cellular protein was no longer significantly different from that of control cells (258 pg/cell). Fig. 1B demonstrates that treatment with NGF doubled the PC content per cell ($p < 0.001$). In contrast, the level of PC in the NGF-withdrawn cells was not significantly different from that in control cells. These results confirm that the increase in cellular protein and PC content is dependent on NGF and coincident with neurite outgrowth.

FIG. 2. CT activity is stimulated by NGF in PC12 cells. Cells were incubated with 50 ng/ml NGF for 9 days (NGF), incubated for 5 days with NGF, then cultured for 4 more days in medium lacking NGF (NGF-withdrawn) or incubated without NGF for 9 days (Control). CT activity was measured in cell lysates and normalized to protein (A) or cell number (B). Data represent means \pm S.E. of four independent experiments with triplicate dishes. *, control versus NGF, $p < 0.001$.



NGF Stimulates CT Activity in PC12 Cells—Consistent with previous studies (1), NGF did not significantly increase CT activity *in vitro* when the activity was normalized to mg of protein (Fig. 2A, 3.24 nmol/min/mg protein and 3.39 nmol/min/mg protein in control and NGF-treated cells, respectively). Nor did NGF withdrawal alter CT activity. However, since NGF-dependent differentiation substantially increased the amount of protein per cell (Fig. 1A), presumably due to neurite outgrowth, we also normalized CT activity to cell number. Fig. 2B shows that NGF significantly increased CT activity per cell by greater than 100% ($p < 0.001$, Fig. 2B). Furthermore, in cells that were treated with NGF and in which NGF was subsequently withdrawn, CT activity returned to a level no longer significantly different from that in control cells. These results suggest that the increase in CT activity is dependent on NGF and coincides with neurite outgrowth.

CT, an amphitropic enzyme, is activated by translocation from a soluble form to a membrane-associated form (11, 12). We assayed soluble and membrane fractions of PC12 cells to determine whether or not NGF increased CT activity by promoting CT translocation to membranes. As shown in Fig. 3A, NGF significantly increased CT activity in the membrane fraction compared with that of control cells ($p < 0.01$). CT activity in membranes from NGF-withdrawn cells was not significantly different from that of control membranes. NGF did not significantly alter CT activity in the soluble fraction (Fig. 3B).

Together, these results suggest that NGF activates CT by increasing its activity on cellular membranes. However, since we did not observe a corresponding decrease in soluble CT activity (Fig. 3B), we hypothesized that NGF might also up-regulate CT expression.

NGF Induces CT β 2 mRNA Expression—To determine whether or not NGF increased expression of the different CT isoforms, we assessed the mRNA abundance of the three known rodent isoforms of CT (CT α , CT β 2, and CT β 3) by semi-quantitative RT-PCR. As shown in Fig. 4A, when PC12 cells were treated with NGF for 9 days, CT β 2 expression dramatically increased compared with that in cells not treated with NGF. In NGF-withdrawn cells CT β 2 mRNA was barely detectable. Furthermore, NGF increased CT β 2 expression as early as 1 day after application, and CT β 2 mRNA abundance increased with prolonged NGF exposure (Fig. 4B). In contrast, NGF did not alter CT α mRNA expression or choline kinase α mRNA expression. (Fig. 4A). Since neither the murine nor the rat cDNA encoding CDP-choline:diacylglycerol cholinephosphotransferase has been cloned, we could not examine if NGF affected its expression.

We also used a ribonuclease protection assay to obtain a more quantitative assessment of whether or not NGF affected CT α or CT β 2 expression. As shown in Fig. 5A, CT β 2 mRNA was barely detectable in PC12 cells incubated without NGF

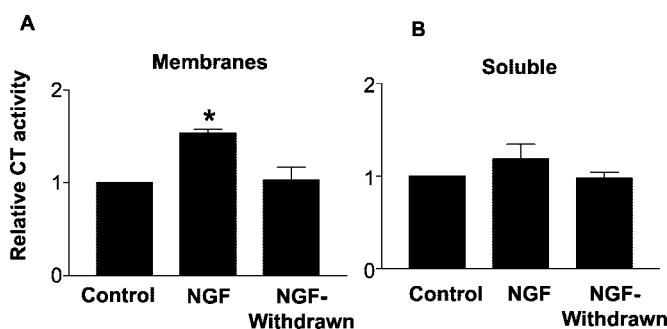


FIG. 3. NGF increases membrane-associated CT activity but not soluble CT activity in PC12 cells. Cells were incubated as in the legend to Fig. 2. Cell lysates were centrifuged at $470,000 \times g$ for 30 min. CT activity was measured in the pellet (membranes, panel A) and the supernatant (soluble fraction, panel B). CT activity was 0.309 fmol/min/cell in membranes of control cells and 1.27 fmol/min/cell in the soluble fraction of control cells. Data represent means \pm S.E. of three independent experiments with triplicate dishes. *, control versus NGF, $p < 0.01$.

(lane 2). However, CT β 2 mRNA was dramatically increased in cells treated with NGF for 7 days (lane 3). In contrast, and consistent with the RT-PCR results (Fig. 4A), CT α mRNA abundance was unaffected by NGF treatment. The approximately equal abundance of the 100-bp ribonuclease-protected cyclophilin mRNA fragment in all samples confirmed that equivalent amounts of RNA were used in the hybridization reactions.

Recently, a novel isoform of CT β has been identified, CT β 3, which is predicted to be a splice variant of CT β 2 (5). Like CT β 2, CT β 3 mRNA is found in mouse brain (5). We used RT-PCR to determine whether PC12 or Neuro2a cells expressed CT β 3 mRNA. As shown in Fig. 5B, CT β 3 mRNA was undetectable in PC12 cells after 40 PCR cycles. For this reason, we conclude that CT β 3 plays little or no role in the NGF-dependent increase in CT activity in PC12 cells.

NGF Increases the Amount of CT β 2 Protein and Promotes Translocation to Membranes—Since NGF treatment dramatically increased CT β 2 mRNA abundance, we hypothesized that NGF also increases the amount of CT β 2 protein. Fig. 6A demonstrates that NGF markedly increased the amount of CT β 2 protein compared with that in control cells. The anti-CT β 2 antibody produces a characteristic doublet which corresponds to the predicted 43-kDa CT β 2 protein and a larger, phosphorylated form of CT β 2 (4). Moreover, when NGF was removed from the culture medium, and the PC12 cells retracted their neurites, the amount of CT β 2 protein decreased to virtually undetectable levels. In contrast, NGF treatment did not alter the amount of CT α protein (Fig. 6A). These results suggest that the increase in the amount of CT β 2 protein also depends on NGF and coincides with neurite outgrowth, consistent with the

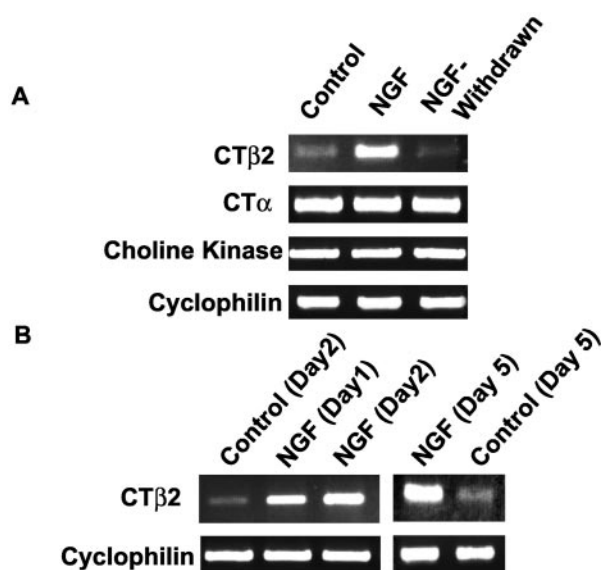


FIG. 4. CT α , CT β 2, and choline kinase mRNA expression in NGF-treated PC12 cells. A, cells were incubated as in Fig. 2. On day 9, total RNA was isolated. 1 μ g of RNA was reverse transcribed, then sequence-specific primers for CT α , CT β 2, CT β 3, choline kinase α , and cyclophilin were used in PCR amplifications to detect their relative abundance. B, PC12 cells were incubated without NGF for 9 days (Control) or incubated with 50 ng/ml NGF for 1, 2 or 5 days and total RNA was isolated. 1 μ g of total RNA was reverse-transcribed and CT β 2- and cyclophilin-specific primers were used in PCR amplifications to detect their relative abundance. Results were similar in two independent experiments.

observed increases in PC content and CT activity.

Since CT activity increased in the membrane fraction of NGF-treated PC12 cells, we hypothesized that NGF not only up-regulates the expression of CT β 2 specifically, but also activates CT β 2 by promoting its translocation from the soluble reservoir to membranes. Immunoblot analysis (Fig. 6B) demonstrated that membranes of NGF-treated PC12 cells contained more CT β 2 than did control or NGF-withdrawn cells. In contrast, membranes of NGF-treated cells did not show an appreciable increase in CT α immunoreactivity. The anti-CT α antibody revealed a doublet of immunoreactive bands in membranes from untreated, NGF-treated, and NGF-withdrawn PC12 cells, as well as in the positive control (10 μ g of protein from a lysate of McArdle 7777 rat hepatoma cells, which over-express CT α , data not shown). The lower band corresponds to the predicted size of CT α (41kDa) but the origin of the second, larger immunoreactive band in all of the samples is not clear.

We conclude that NGF increases CT activity in two specific ways. First, NGF up-regulates CT β 2, but not CT α , expression. Second, NGF specifically activates CT β 2, but not CT α , by promoting the association of CT β 2 with membranes.

U0126 Inhibits the NGF-dependent Increase in CT Activity and CT β 2 Expression—NGF signals through multiple intracellular cascades to promote neuronal survival and differentiation. Among these signaling cascades, MAP kinase is essential for neurite outgrowth (8, 13). In this pathway, NGF activates Ras, which activates mitogen-activated kinase/extracellular signal-regulated kinase kinase (MEK), which subsequently activates MAP kinase. We, therefore, hypothesized that NGF signals through the MAP kinase pathway to induce CT β 2 expression and activation. We treated PC12 cells with U0126, an inhibitor of MEK1/2, which inhibits the MEK-dependent activation of MAP kinase, and examined its effect on CT activity and CT β 2 expression in NGF-differentiated PC12 cells. Previous studies have shown that when NGF-treated PC12 cells are incubated with U0126 neurite extension is impaired compared

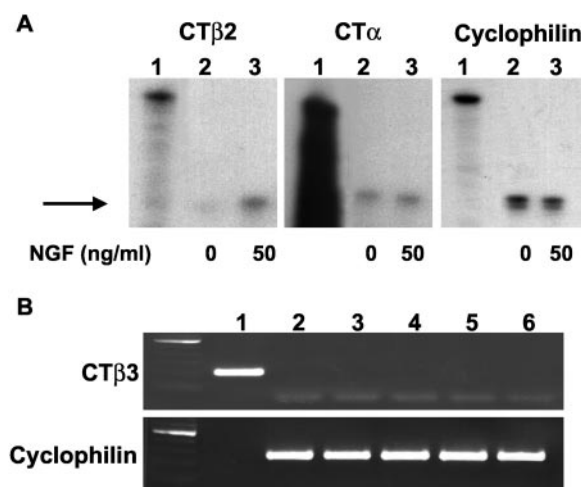


FIG. 5. CT isoform expression in NGF-treated PC12 cells. A, ribonuclease protection assay in NGF-treated PC12 cells. PC12 cells were cultured for 7 days in the absence (lane 2) or presence (lane 3) of 50 ng/ml NGF. Total RNA (5 μ g) was incubated with CT α -, CT β 2-, and cyclophilin-specific ribonucleotide antisense probes. Hybridization of the antisense probes to the target RNA gave rise to protected mRNA fragments of the following sizes: CT β 2, 193 nucleotides; CT α , 200 nucleotides; cyclophilin, 103 nucleotides. Lane 1 shows undigested ribonucleotide antisense probe specific CT β 2, CT α or cyclophilin as indicated. B, PC12 cells were incubated as described in the legend to Fig. 2. Neuro2a cells were plated and incubated for 48 h in the presence or absence of 20 μ M retinoic acid. Total RNA was isolated as described for Fig. 4. Primers for CT β 3 and cyclophilin were used in PCR amplifications to detect their relative abundance. cDNA templates used in the PCR reactions were as follows: lane 1, plasmid containing full-length CT β 3 cDNA; lane 2, PC12 cells incubated without NGF; lane 3, NGF-treated PC12 cells; lane 4, NGF-withdrawn PC12 cells; lane 5, Neuro2a cells incubated without retinoic acid; lane 6, retinoic acid-treated Neuro2a cells. Results were similar in two independent experiments.

with that in cells treated with NGF alone (14). We, therefore, incubated PC12 cells for 5 days with 50 ng/ml NGF (NGF), with 50 ng/ml NGF and 50 μ M U0126 (NGF/U0126), with 50 μ M U0126 alone (U0126) or without both NGF and U0126 (control). Control and U0126-treated cells proliferated and exhibited no discernible morphological differences. Both NGF- and NGF/U0126-treated cells differentiated. However, fewer NGF/U0126-treated cells extended neurites and the average neurite length at 5 days was shorter than in NGF-treated cells. Consistent with the 9-day differentiation experiments (Fig. 2B), cells incubated with NGF for 5 days contained a significantly higher CT activity per cell than did control cells ($p < 0.01$, Fig. 7A). However, when PC12 cells were incubated with NGF in the presence of U0126, CT activity was not significantly different from that of control cells.

We used semi-quantitative RT-PCR to examine whether or not U0126 attenuated CT β 2 mRNA and CT α mRNA expression. In cells treated with NGF and U0126 together, CT β 2 mRNA abundance was appreciably less than in NGF-treated cells (Fig. 7B). CT α mRNA abundance was the same under all treatment conditions (data not shown). These results support our hypothesis that MAP kinase signaling mediates the NGF-dependent increase in CT β 2 expression and the subsequent increase in CT activity.

Retinoic Acid Stimulates CT Activity in Neuro2a Cells—Neurite outgrowth is characteristic of all differentiating neuronal cells. We, therefore, hypothesized that CT β 2 expression and CT activity would be increased in any cells that were actively growing neurites, rather than only in cells, which differentiate upon NGF treatment. To test this hypothesis, we measured CT activity in Neuro2a cells, which do not require NGF for differentiation but instead rapidly differentiate in

FIG. 6. NGF increases the amount of CT β 2, but not CT α , protein. Cells were incubated as described in the legend to Fig. 2. Proteins (25 μ g) from cell lysates (A) or membranes (B) were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. Amounts of CT α and CT β 2 protein were assessed by immunoblotting with antibodies against CT α or CT β 2. Results were similar in two independent experiments.

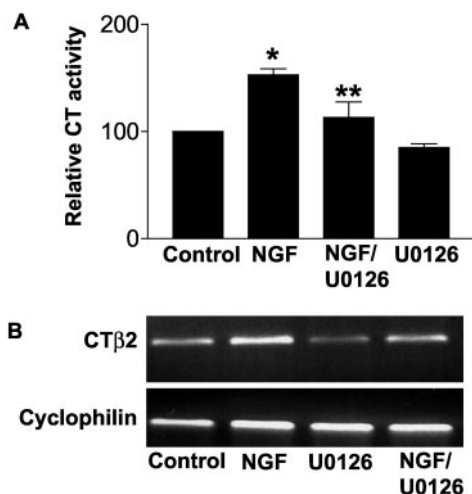
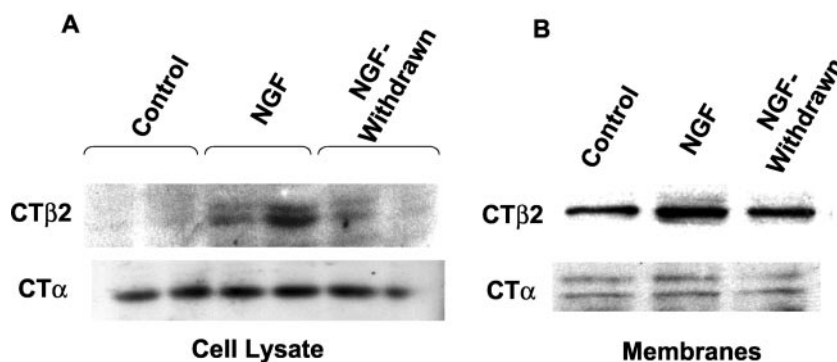


FIG. 7. The MEK inhibitor, U0126, prevents the NGF-induced increase in CT activity and CT β 2 mRNA. PC12 cells were incubated without NGF (Control) or incubated with 50 ng/ml NGF (NGF), 50 ng/ml NGF in the presence of 50 μ M U0126 (NGF/U0126) or 50 μ M U0126 (U0126) for 5 days. A, CT activity was measured in cell lysates. CT activity in control cell lysates was 2.67 fmol/min/cell. Data represent means \pm S.E. of three independent experiments. B, total RNA was isolated, reverse-transcribed, and CT β 2 and cyclophilin mRNA expression were detected by RT-PCR. Results were similar in two independent experiments. *, control versus NGF, $p < 0.01$; **, NGF versus NGF/U0126, $p < 0.05$.

response to treatment with retinoic acid (15). We plated Neuro2a cells on 35 mm dishes and treated them with 20 μ M retinoic acid for 48 h. Within 12 h after retinoic acid application, virtually all cells had stopped proliferating and had extended neurites longer than the diameter of a cell body.

Retinoic acid-dependent differentiation increased cellular protein in Neuro2a cells ($p < 0.01$, Fig. 8A), similar to the situation in NGF-dependent differentiation of PC12 cells (Fig. 1A). Also similar to PC12 cells, CT activity per mg of protein did not increase (not shown) whereas the activity per cell increased by 54% ($p < 0.0025$) from 3.9 fmol/min/cell in cells incubated without retinoic acid to 6.0 fmol/min/cell in cells incubated with retinoic acid (Fig. 8B). From these data, we conclude that in both Neuro2a and PC12 cells, which differentiate in response to retinoic acid and NGF, respectively, CT activity is significantly increased during neurite outgrowth.

Retinoic Acid Increases the Amount of CT β 2 Protein in Neuro2a Cells—Since retinoic acid treatment significantly increased CT activity in Neuro2a cells, we immunoblotted Neuro2a cell lysates to determine whether or not retinoic acid increased the amount of CT protein. As shown in Fig. 9, the amount of CT β 2 protein was substantially higher in retinoic acid-treated cells than in control cells. The amount of CT α protein in Neuro2a cells, however, was unaffected by retinoic acid treatment. Together these results support our hypothesis

that CT β 2 expression and CT activity are increased during neurite outgrowth irrespective of the method of induction of differentiation.

DISCUSSION

Neurite outgrowth places a high demand on neurons for membrane biosynthesis. This is especially true for PC biosynthesis since PC is the most abundant phospholipid in mammalian membranes. We have shown that during neurite growth of PC12 cells, NGF stimulates PC biosynthesis by specifically up-regulating the expression and activity of CT β 2. Furthermore, in Neuro2a cells, retinoic acid-induced differentiation also stimulated CT β 2 expression and increased CT activity. We conclude that in both of these neuronal cell lines, the demand for PC during neurite growth is accommodated by an elevation of CT β 2 expression, with a concomitant elevation in CT activity.

CT Activity and Isoform Expression in NGF-treated PC12 Cells—CT is an amphitrophic enzyme (3), existing in a soluble form with low activity, and an active, membrane-associated form. Translocation of CT from a soluble form to membranes is a major mechanism of regulation of CT activity (11, 12). Accordingly, we found that in PC12 cells NGF regulated CT activity by promoting translocation of CT to membranes. Furthermore, CT β 2, but not CT α , protein increased in the membranes of NGF-treated cells (Fig. 6B). These observations provide the first evidence that one isoform of CT can be differentially activated over another isoform. The increase in membrane-associated CT β 2 in response to NGF was not simply due to an increase in the amount of CT β 2 protein, because the cytosolic CT activity was not increased in parallel. Previous reports have shown that the incorporation of labeled choline into PC increases dramatically when PC12 cells are exposed to NGF and that this increase in PC biosynthesis coincides with neurite outgrowth (1). Similarly, in our experiments, PC mass per cell doubled in cells treated for 9 days with NGF. Consistent with a previous report (1), NGF treatment did not increase CT activity when measured *in vitro* per mg of protein. Nevertheless, as expected, the dramatic morphological changes of neuronal differentiation were associated with significant increases in cellular protein content (Fig. 1B); thus, we normalized our CT activity measurements to cell number as well as mg of protein. CT activity per cell increased by 50% after 5 days of NGF treatment and by 100% after 9 days of NGF treatment (compare Fig. 7B to Fig. 2B). Although Araki and Wurtman (1) did not report CT activity per cell, they did measure the rate of the CT-catalyzed reaction in intact cells. They reported that the CT-catalyzed reaction was stimulated by ~ 2 -fold in PC12 cells after 2 and 4 days of incubation with NGF (1). Our data suggest that this increase in CT activity corresponds to the differential activation and expression of the CT β 2 isoform. Araki and Wurtman (1) also reported that cholinephosphotransferase activity, which catalyzes the final step in PC biosynthesis, in-

FIG. 8. Retinoic acid increases both CT protein and activity in Neuro2a cells. Murine Neuro2a cells were incubated without retinoic acid (*Control*) or treated with 20 μ M retinoic acid for 48 h (*Retinoic acid*), and then harvested as described in the legend to Fig. 2. *A*, cellular protein was measured. *B*, CT activity was measured in cell lysates. Data represent means \pm S.E. of three independent experiments. *, $p < 0.05$.

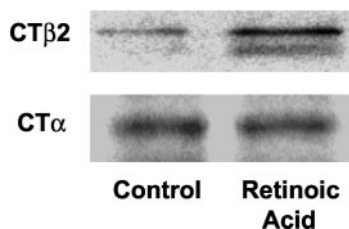
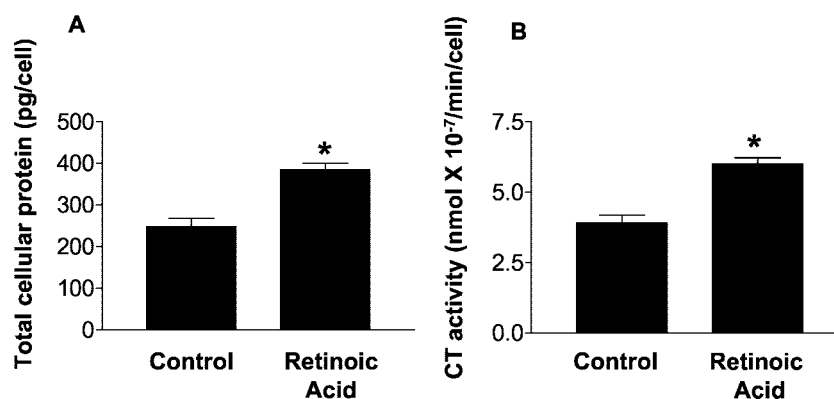


FIG. 9. Retinoic acid increases the amount of CT β 2, but not CT α , protein in Neuro2A cells. Neuro2A cells were cultured as described in the legend to Fig. 8. Proteins (25 μ g) from cell lysates were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. Amounts of CT α and CT β 2 protein were assessed by immunoblotting with antibodies against CT α (*A*) or CT β 2 (*B*). Results were similar in two independent experiments.

creased by $\sim 50\%$ after 4 days of NGF treatment. Since the amount of diacylglycerol (a substrate for this enzyme) increased 4-fold after 4 days of NGF treatment, the authors speculated that the increase in cholinephosphotransferase activity was likely due to the increase in the amount of this substrate. Thus, CPT activity may also increase to accommodate the demand for PC biosynthesis.

Diacylglycerol is also a lipid activator of CT in many types of cells including human neuroblastoma (16) cells and HeLa cells (17). In HeLa cells, diacylglycerol is increased by phorbol ester treatment and activates CT by promoting CT translocation to membranes (17). Given that diacylglycerol levels increase 4-fold after 4 days of NGF treatment (1), it is possible that NGF activates CT β 2 by increasing diacylglycerol levels and thereby promoting the translocation of CT to membranes.

CT can also be regulated by phosphorylation (3) and NGF might regulate CT β 2 by altering its phosphorylation status. To date, the exact role of phosphorylation remains unclear in the regulation of CT α and has not been studied with CT β 2. It is known that CT α does not have to be dephosphorylated for membrane binding, but CT α activity is reduced by phosphorylation (18, 19). Like CT α , CT β 2 protein has many potential phosphorylation sites within its carboxyl terminus (4). However, the amino acid sequence does differ between the two isoforms. In HeLa cells, that are non-neuronal cells abundantly expressing CT β 2 mRNA in addition to CT β 1 and CT α mRNAs (4), both insulin and epidermal growth factor can stimulate CT phosphorylation without altering its distribution between cytosol and membranes (20). It should be noted that the insulin/epidermal growth factor experiments were done prior to the identification of CT β 1/2, thus it is unclear which CT isoforms were phosphorylated. In our experiments with PC12 cells, immunoblotting showed no appreciable doublet for CT β 2 in membranes. However, immunoblots of proteins from cell lysates revealed the characteristic doublet (Fig. 6A). Perhaps the membrane-associated form of CT β 2 requires a certain phosphorylation status for membrane binding. Also, when lysates from

NGF-treated PC12 cells were immunoblotted, the lower molecular weight band of CT β 2 predominated. In contrast, in retinoic acid-treated Neuro2a cells, the higher molecular weight band, which likely corresponds to the more-highly phosphorylated form of CT β 2 (4), was predominant. Based on these initial observations, we speculate that phosphorylation may play a role in the regulation of CT β 2 activity during neurite outgrowth.

In addition, we found that NGF increased CT β 2 mRNA levels prior to neurite sprouting: while 5 days of NGF treatment were necessary for 50% of PC12 cells to bear neurites (21), CT β 2 mRNA was up-regulated within 1 day of NGF treatment. Furthermore, CT β 2 expression was clearly dependent on NGF because when NGF was withdrawn, CT β 2 mRNA and protein levels were the same as in cells incubated without NGF. Our results are consistent with some differential display PCR studies in rat hippocampal neurons in which CT β 2 mRNA was the most highly up-regulated transcript in response to arginine vasopressin $_{4-8}$, a peptide that facilitates memory acquisition and stimulates neurite outgrowth (5). Arginine vasopressin $_{4-8}$ can also up-regulate NGF expression (22). Thus, the increase in CT β 2 mRNA induced by arginine vasopressin might result from an elevation in NGF expression.

To date, there is no information about the transcriptional regulation of *Pcyl1b*, the gene encoding the CT β isoforms. Several Sp-related transcription factors regulate transcription of *Pcyl1a*, the gene encoding CT α (23). Moreover, when Sp3 is overexpressed in murine fibroblasts, both CT α and CT β 2 mRNAs are up-regulated, suggesting that Sp3 might also regulate transcription of *Pcyl1b* (23). The present study demonstrates that differentiating PC12 cells specifically up-regulate CT β 2 mRNA but not CT α mRNA. Thus, we speculate that a neuron-specific, or differentiation-specific, transcription factor regulates expression of the *Pcyl1b* gene during neurite outgrowth. In PC12 cells, the expression of several immediate early genes, predominantly transcription factors (e.g. MafK), is increased within 1 h of NGF exposure (24). Unlike several other identified NGF-responsive immediate early genes, MafK, a basic region/leucine zipper transcription factor, is specifically up-regulated upon NGF exposure and is essential for neurite outgrowth in both PC12 cells and immature telencephalic neurons (21).

CT β 2 and the MAP Kinase Cascade—NGF signals through myriad signaling cascades to ensure neuronal survival and differentiation. In PC12 cells, both NGF and pituitary adenylate cyclase-activating polypeptide converge upon, and require, MAP kinase signaling for neurite outgrowth (8, 13). Since NGF treatment up-regulated CT β 2 expression coincident with neurite outgrowth, we examined whether or not the MEK1/2-specific inhibitor, U0126, impaired CT β 2 expression. Consistent with previous studies, when PC12 cells were incubated with both NGF and U0126, fewer cells had visible neurites. More-

over, CT β 2 mRNA levels, but not CT α mRNA, decreased in the U0126/NGF-treated cells as compared with NGF treatment alone. Because CT activity was also dramatically reduced in U0126/NGF-treated cells ($p < 0.05$) so that it was no longer any different from control cells, elevations in CT β 2 expression may completely account for the elevation in CT activity during neurite outgrowth.

Our laboratory recently published data that further implicate MAP kinase signaling in CT β 2 expression in oncogenic Ha-Ras-overexpressing fibroblasts (25). Other studies, in HeLa cells, have shown that purified CT (isoform unknown) can be phosphorylated *in vitro* by p44 MAP kinase (20); however, it is unclear which CT isoform is phosphorylated because the studies were done prior to the identification of CT β 1/2. It is possible that MAP kinase signaling governs both the elevation in CT β 2 expression via transcriptional regulation as well as phosphorylation of CT β 2 to regulate its activity.

CT Expression and Activity in Neuro2A Cells—We hypothesize that enhanced PC biosynthesis and CT β 2 expression are necessary in all neurons during neurite growth. We used Neuro2a cells as a second model of neuronal differentiation because, unlike PC12 cells, Neuro2a cells produce neurites in response to retinoic acid (15). Similar to the differentiated PC12 cells, CT activity/cell and the amount of CT β 2 protein were significantly increased in retinoic acid-treated Neuro2a cells. Furthermore, immunoblot analyses showed that retinoic acid substantially increased the amount of CT β 2 protein.

Where do retinoic acid and NGF signaling converge to upregulate CT β 2 protein and CT activity? While little is known about how retinoic acid stimulates neurite outgrowth in Neuro2a cells, Neuro2a cell differentiation is stimulated by the ganglioside GM₂ via a c-Src-dependent sustained activation of MAP kinase (9). In PC12 cells, NGF also induces a sustained (60 min) activation of MAP kinase, which is essential for differentiation and neurite outgrowth (8). Based on these observations, we speculate that MAP kinase signaling is likely important in regulating CT β 2 expression and CT activity in both Neuro2a cells and PC12 cells.

Despite the appreciable increase in CT β 2 protein and CT activity in retinoic acid-treated Neuro2a cells, we did not detect an increase in CT β 2 mRNA. This may be explained by an increase in the stability of CT β 2 protein. Indeed, enhanced CT activity associated with neurite growth has been observed with no apparent effect on CT α or CT β 2 expression (26). In Gaucher disease, an inherited metabolic disorder with defective lysosomal glucocerebrosidase, the accumulation of glucosylceramide accelerated axon growth with a concomitant increase in PC biosynthesis (26). In a mouse model of Gaucher disease (*Gba*^{-/-}), the elevation in PC biosynthesis is directly related to activation of CT. However, neither CT α nor CT β 1/2 expression (mRNA and protein) were altered in *Gba*^{-/-} mice. Consequently, the authors concluded that CT was activated by post-transcriptional mechanisms (26). Studies with cholesterol-loaded macrophages have demonstrated directly that an increase in CT activity can be elicited exclusively by post-translational mechanisms for example solely by a partial dephosphorylation of the membrane-bound pool of CT (27).

CT β 2 Expression and Neurite Outgrowth—As shown with *in vitro* CT activity assays of membrane and soluble fractions, PC12 cells, like all other cells, have a large reserve pool of soluble CT. An important question is: why do PC12 cells and hippocampal neurons specifically elevate CT β 2 expression during neurite outgrowth rather than mobilizing the pre-existing soluble CT pool? We speculate that CT β 2 is targeted to neurites so that PC can be synthesized *in situ* for neurite outgrowth. PC biosynthetic enzymes have been detected in rat brain synap-

tosomes (28), squid axoplasm (29) and distal axons of sympathetic neurons (2, 30). CT α is unlikely to be targeted to the neurite because of the nuclear localization signal within its amino terminus. Moreover, immunofluorescence studies have localized CT α predominantly to the nucleus of cells (31). In contrast, CT β 2 does not contain a nuclear localization signal and immunofluorescence studies in murine macrophages found that CT β 2 localized to the endoplasmic reticulum (4). For these reasons, we predict that CT β 2 might be targeted to neurites.

In conclusion, we have provided evidence that enhanced CT β 2 expression and CT activity coincide with neurite outgrowth irrespective of the molecular signal for neuritogenesis. Moreover, in PC12 cells, CT β 2 expression, like neurite outgrowth, is dependent on MAP kinase signaling. We propose that CT β 2 activation accounts for the increase in CT activity that occurs during neurite outgrowth. Together, the data strongly support a role for CT activity, and specifically CT β 2 expression, in neurite outgrowth.

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Enhanced Expression and Activation of CTP:Phosphocholine Cytidylyltransferase β 2 during Neurite Outgrowth

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