Phosphatidylcholine Biosynthesis via CTP:Phosphocholine Cytidyllyltransferase β2 Facilitates Neurite Outgrowth and Branching*

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Hallmarks of neuronal differentiation are neurite sprouting, extension, and branching. We previously showed that increased expression of CTP:phosphocholine cytidyllyltransferase β2 (CTβ2), an isoform of a key phosphatidylcholine (PC) biosynthetic enzyme, accompanies neurite outgrowth (Carter, J. M., Waite, K. A., Campenot, R. B., Vance, J. E., and Vance, D. E. (2003) J. Biol. Chem. 278, 44988–44994). CTβ2 mRNA is highly expressed in the brain. We show that CTβ2 is abundant in axons of rat sympathetic neurons and retinal ganglion cells. We used RNA silencing to decrease CTβ2 expression in PC12 cells differentiated by nerve growth factor. In CTβ2-silenced cells, numbers of primary and secondary neurites were markedly reduced, suggesting that CTβ2 facilitates neurite outgrowth and branching. However, the length of individual neurites was significantly increased, and the total amount of neuronal membrane was unchanged. Neurite branching of PC12 cells is known to be inhibited by activation of Akt and promoted by the Akt inhibitor LY294002. Our experiments showed that LY294002 increases neurite sprouting and branching in control PC12 cells but not in CTβ2-deficient cells. CTβ2 was not phosphorylated in vitro by Akt. However, inhibition of Cdk5 by roscovitin blocked CTβ2 phosphorylation and reduced neurite outgrowth and branching. These results highlight the importance of CTβ2 in neurons for promoting neurite outgrowth and branching and represent the first identification of a lipid biosynthetic enzyme that facilitates these functions.

In response to nerve growth factor (NGF), rat pheochromocytoma (PC12) cells stop proliferating and differentiate into sympathetic neuron-like cells (1). The morphological hallmark of neuronal differentiation is neurite sprouting and elongation and subsequent maturation of neurites into axons and dendrites. These processes increase the demand for membrane components. Accordingly, the biosynthesis of the predominant membrane phospholipid, phosphatidylcholine (PC), is accelerated during neurite outgrowth in response to NGF (2, 3).

PC is synthesized in neurons by the CDP-choline pathway (4), in which the rate-limiting reaction is catalyzed by CTP:phosphocholine cytidyllyltransferase (CT) (5). Three CT isoforms, encoded by two genes, have been identified in rodents. CTα is encoded by the Pcyt1a gene, whereas CTβ2 and CTβ3 are derived from the Pcyt1b gene (6, 7). CTα and CTβ2 share considerable sequence identity, but CTα contains a nuclear localization signal (8), whereas CTβ2 does not (7). Immunofluorescence studies have found that CTα is primarily located in the nucleus and that CTβ2 resides in the cytosol and on the endoplasmic reticulum (9). The subcellular localization of CTβ3 has not yet been reported. Although CTα is expressed in all tissues, CTβ2 and CTβ3 mRNAs are predominantly expressed in the brain (6). In neurons, PC is synthesized not only in cell bodies but also in distal axons (4, 10–12). During neurite outgrowth of PC12 cells and Neuro2a cells, CTβ2 expression and CT activity are increased, whereas CTα expression is unchanged, indicating a link between CTβ2 expression and neurite outgrowth (3).

Neurites are categorized by their location relative to the cell body. Primary neurites project directly from the cell body. Secondary neurites (i.e. branches) project from primary neurites. The sprouting of primary neurites and branch formation are distinct processes that are independently regulated (13). During neuronal differentiation, NGF activates phosphatidylinositol-3-kinase, which activates Akt (14). Neurite extension and branching are promoted in some populations of neurons by activation of Akt, whereas in other neurons activation of Akt impairs neurite elongation and branching (15). In PC12 cells, Akt activation inhibits neurite branching (16).

On the basis of these findings, we hypothesized that neurons require CTβ2 in axons to provide a localized source of PC for neurite outgrowth, extension, and/or branching. We show that CTβ2 is abundant in PC12 cells and in distal axons of primary cultures of neurons. RNA silencing of CTβ2 in PC12 cells reduced the number of primary neurites and markedly reduced the number of branches but increased the length of individual neurites. Moreover, stimulation of neurite branching in PC12...
cells, a process that is normally induced by inhibition of Akt, was abrogated in CTβ2-deficient cells. These data provide important new insights into the role of CTβ2 and PC biosynthesis in neurite sprouting and branch formation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Rat pheochromocytoma cells (PC12) 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 at 37 °C in a humidified atmosphere containing 5% CO₂. For differentiation and transfection experiments, cells were seeded on collagen-coated 35-mm dishes at a density of 2 × 10⁵ cells/dish. The cells were incubated overnight and then treated with medium containing 0.5% horse serum and 50 ng/ml 2.5 S NGF (Alomone Laboratories, Jerusalem, Israel), and cells were grown for up to 5 days.

**Compartmented Cultures of Rat Sympathetic Neurons and Retinal Ganglion Cells**—Sympathetic neurons from the superior cervical ganglia of 1-day-old rats were isolated as previously described (17). Briefly, following dissection, the ganglia were mechanically and enzymatically dissociated and plated into the center compartment of compartmented culture dishes. Initially, all compartments contained 2.5 S NGF (100 ng/ml). NGF was withdrawn from the center compartment (containing cell bodies and proximal axons) after 7 days. The side compartments (containing distal axons alone) contained 100 ng/ml NGF throughout the experiments. Retinal ganglion cells were isolated from 1-day-old rats and cultured in compartmented culture dishes as previously described (18). Proximal axons/cell bodies, distal axons of sympathetic neurons, and distal axons of retinal ganglion cells were harvested from 2-week-old cultures (18, 19).

**Antibodies**—Anti-M rabbit polyclonal antibodies that recognize CTβ2 were generated against the conserved membrane domain of rat liver CT (amino acids 256–288) and were a generous gift from Dr. R. Cornell (Simon Fraser University, Vancouver, Canada). Anti-human CTβ2 rabbit polyclonal antibodies were a generous gift from Dr. S. Jackowski (St. Jude Children’s Research Hospital, Memphis, TN). The CTβ2-specific antibody was raised against a peptide corresponding to amino acids 347–365 of CTβ2 (7). The mouse anti-tubulin monoclonal antibodies were from Sigma, and the anti-phospho-Akt (Ser-473) and anti-Akt antibodies were from Cell Signaling Technology (Beverly, MA). The Cdk5 (cycdin-dependent kinase 5) antibody was purchased from Chemicon International, Inc. (Temecula, CA).

**Transfection of PC12 Cells and Measurement of Neurite Length and Branching**—PC12 cells were plated at a density of 2 × 10⁵ cells/35-mm dish for 12 h, after which the medium was changed to F12-K medium without serum. Cells were transfected with 2.5 µg of DNA/35-mm dish. For RNA silencing, cells were co-transfected with a 3.7-kb mammalian expression vector encoding recombinant green fluorescent protein (GFP) (phrGFP) (Stratagene, Cedar Creek, TX) and pSILENCER 4.0 encoding rat CTβ2. PC12 cells were transfected with equimolar amounts of phrGFP and either the silencing construct (pSiCTβ2) or pSILENCER 4.0 containing a scrambled, noncoding insert. For CTβ2 overexpression studies, cells were transfected with a cDNA encoding a hemagglutinin (HA)-tagged CTβ2 (pCI[CTβ2-HA]) and phrGFP. The cells were transfected in the presence of Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Briefly, pSiCTβ2, pSILENCER 4.0 containing the scrambled insert, or pCI[CTβ2-HA] and phrGFP were added in a 3:1 ratio to Lipofectamine 2000 for 20 min to allow formation of DNA complexes, which were then applied to PC12 cells. After 12 h, the cells were given serum-free F12-K medium containing 50 ng/ml NGF to promote differentiation and neurite outgrowth. After 2, 3, and 4 days, cells were viewed by fluorescence microscopy. Cells that had been co-transfected with phr-GFP and pSiCTβ2 were identified by their fluorescence at 520 nm.

Cells bearing at least one neurite longer than 20 pixels were considered to be differentiated, and all neurites and branches were counted and/or measured. At each time point, at least 20 random fields of view were sampled.

**Immunoprecipitation and Immunoblotting**—Two dishes of PC12 cells or six dishes of sympathetic neurons and retinal ganglion cells were rinsed with ice-cold phosphate-buffered saline and harvested into 1 ml of immunoprecipitation lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, and a protease inhibitor mixture (Sigma). Cell lysates were centrifuged at 10,000 × g for 15 min at 4 °C, and then supernatants were incubated for 1 h with 3 µl of anti-M antibodies on a rotting shaker at 4 °C. A 50% slurry of Protein A-Sepharose beads (40 µl) was added, and the mixtures were incubated for 1 h at 4 °C. Protein A-Sepharose bead conjugates containing immunoprecipitated CTβ2 were washed three times with buffer containing 50 mM Tris-HCl (pH 7.4) and 0.1% Triton X-100. Proteins were eluted from the Protein A-Sepharose beads using SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 8.3), 10% (v/v) glycerol, 1% SDS, and 0.04% bromphenol blue) and heated at 70 °C for 3 min. Immunoprecipitated proteins were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS and then transferred to Immobilon-P transfer membranes (Millipore, Cambridge, Canada). Ponceau S staining was used to compare protein loading in lanes of the gel. The membranes were blocked for 2 h with 5% skimmed milk in Tris-buffered saline (20 mM Tris-HCl (pH 7.5) containing 500 mM NaCl and 0.1% Tween 20) and then incubated overnight with anti-M (membrane domain) (dilution 1:1,000), anti-CTβ2 (dilution 1:1,000), or mouse anti-tubulin (dilution 1:1,000) antibodies. All blots were washed for 1 h and subsequently incubated with anti-rabbit, or anti-mouse, IgG conjugated to horseradish peroxidase (1:2,500 dilution) for 1 h. Immunoreactivity was detected using Westdura or Westfemto ECL reagent (Amersham Biosciences).

**In Vitro Measurement of CT Activity**—PC12 cells from two 35-mm dishes were collected in 1 ml of homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 100 µg/ml each of leupeptin and aprotinin. The cells were sonicated for 20 s at 4 °C. Cell lysates were centrifuged at 7,000 × g for 5 min to remove nuclei and unbroken cells. Aliquots of the supernatant were used for measurement of CT activity and immunoblot analysis. CT activity was determined using Westdura or Westfemto ECL reagent (Amersham Biosciences).
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in the presence of PC/oleate vesicles by monitoring the conversion of phospho-[3H]choline to CDP-[3H]choline (20).

Incubation of PC12 Cells with [3H]Choline—PC12 cells were transfected with control plasmid or phrGFP + pSiCTβ2. Six h later, the medium was changed to serum-free medium that contained 50 ng/ml NGF. After 48 h, cells were incubated with [3H]choline (5 μCi/35-mm dish) for 3 h. Cells were harvested, the amount of cellular protein was determined, and radioactivity in PC was measured.

In Vivo Phosphorylation of CTβ2—PC12 cells were incubated with 50 ng/ml NGF for 4 days, after which the cells were incubated for 48 h with LY294002 or for 1 h with 25 μM roscovitine or an equivalent volume of vehicle (dimethyl sulfoxide) in the presence of 100 μM [32P]orthophosphate (Amersham Biosciences). Cells were rinsed with ice-cold phosphate-buffered saline and harvested into immunoprecipitation lysis buffer. The cell lysates were then centrifuged at 10,000 × g for 15 min at 4 °C, after which supernatants were incubated with 3 μl of anti-M antibody on a rotating shaker at 4 °C. After 1 h, 40 μl of a 50% slurry of protein A-Sepharose beads were added, and the mixture was incubated for an additional 1 h at 4 °C. Protein A-Sepharose bead conjugates containing immunoprecipitated CTβ2 were washed three times. CTβ2 was eluted from the beads by heating for 3 min in SDS-PAGE sample buffer at 70 °C. Proteins were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. The gel was dried overnight and then exposed to a PhosphorImager screen (Eastman Kodak Co.) for up to 5 days. Quantification of [32P]orthophosphate-labeled CT protein was performed with a Bio-Rad PhosphorImager.

In Vitro Phosphorylation Assays—PC12 cells were harvested into 1 ml of immunoprecipitation buffer. CTβ2 was immunoprecipitated using anti-M antibody as described above. Tubulin was immunoprecipitated using anti-tubulin antibodies. Cdk5 was immunoprecipitated using 10 μl of anti-Cdk5 antibodies. Following immunoprecipitation, Cdk5-bound protein A-Sepharose beads were warmed at 30 °C for 10 min. CTβ2 protein was eluted from the beads with 40 μl of buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 2 mM dithiothreitol and boiled for 5 min. The eluted CTβ2 protein was added to Cdk5-protein A-Sepharose beads in the presence of 1 μCi of [32P]ATP and incubated for 15 min at 30 °C. The kinase reaction was terminated by heating at 100 °C for 3 min in the presence of 5× SDS-sample buffer. Proteins were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. The gels were dried overnight and then exposed to a PhosphorImager screen (Eastman Kodak Co.) for up to 3 days.

Phospholipid Extraction and Quantification—Cell lysates were centrifuged at 7,000 × g for 5 min to remove nuclei and unbroken cells. Phospholipids were extracted with chloroform/methanol (2:1, v/v) and separated by thin layer chromatography on silica gel G60 plates with chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:1) as developing solvent. Phospholipids were visualized by exposure of the plate to iodine vapor and identified by comparison with phospholipid standards. The relevant spots were scraped from the plate, and amounts of phospholipids were determined by phosphorus analysis (21).

CTβ2-silencing RNA Oligonucleotides—An oligonucleotide specific for CTβ2 mRNA (ACA GGT ATC CCA AAA TCCC) was designed using a sequence finder algorithm (Ambion, Austin, TX) and synthesized at the core facility (Department of Biochemistry, University of Alberta). The sequence showed no homology to other sequences in the NCBI data base; nor was the siCTβ2 oligonucleotide homologous to any other mRNA listed in GenBank™. The siCTβ2 oligonucleotide was inserted into pSILENCER 4.1CMV (Ambion, Austin, TX), and the plasmid was named pSiCTβ2. Insertion of the cDNA was confirmed by sequencing.

Cloning and Hemagglutinin Modification of CTβ2 cDNA—A cDNA encoding the entire open reading frame of CTβ2 was cloned from a cDNA population generated from PC12 cell mRNA using the following primers: 5’-GCCATGCCAGTAGTTACCCT-3’ (forward) and 3’-GCTAAGGTGGTGTTG-GTTGTCC-5’ (reverse). The amplicon was inserted into TOPO 2.1 vector (Invitrogen) and used as a template for the addition of an HA tag to the 3’-end of the open reading frame. The following sequence encoded the HA tag: 3’-TCAAGCATATACTG-GAACATCATATGGATCTCCTCCTATCCCCCTCACCTCAT-5’. The amplicon was cloned into the mammalian expression vector pcI (Invitrogen) and named pcI(CTβ2-HA).

Expression of CTβ2-HA protein was confirmed by immunoblotting of proteins from lysates of cells using anti-HA and anti-CTβ2 antibodies.

Other Methods—Protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin as a standard. LY294002 and roscovitine were purchased from Sigma and dissolved in dimethyl sulfoxide.

RESULTS

CTβ2 Is Present in Axons of Sympathetic Neurons and Retinal Ganglion Cells—In light of our previous observation that NGF increases the expression of CTβ2 in PC12 cells (3), we reasoned that the subcellular localization of CT isoforms within neurons might provide insight into their contributions to PC biosynthesis and neurite growth. Our laboratory has previously demonstrated that PC biosynthetic enzyme activities, including CT, are present in distal axons of rat sympathetic neurons (4). The finding that in most cell types CTα is predominantly nuclear, whereas CTβ is cytoplasmic, suggested that CTβ2, rather than CTα, is a functionally important CT isoform in axons. We therefore immunoprecipitated CT from PC12 cells, rat sympathetic neurons, and retinal ganglion cells using an antibody (anti-M) raised against the membrane domain of rat liver CT. We confirmed that CTβ2 was immunoprecipitated by the anti-M antibody by immunoblotting the immunoprecipitated proteins from retinal ganglion cells with anti-CTβ2 antibodies (Fig. 1). Based on these immunoblots, and consistent with the molecular mass of CTβ2 (43 kDa), we identified the upper band in Fig. 1 as CTβ2. Immunoblotting of the immunoprecipitated proteins in the absence of primary antibody identified the major band in Fig. 1 as the IgG heavy chain of the anti-M antibody. CTβ2 was also detected in PC12 cells and rat sympathetic neurons (Fig. 1, right-hand panels).

To determine whether or not axons contain CTβ2, we immunoprecipitated CT from distal axons of compartmented
cultures of sympathetic neurons and retinal ganglion cells. In this culture system, cell bodies and proximal axons are physically separated from distal axons by a silicone grease barrier and a Teflon divider (17). Thus, distal axons can be harvested independently from cell bodies/proximal axons. As shown in Fig. 1 (left), immunoblotting of anti-M-immunoprecipitated proteins with anti-M or anti-CTβ2 antibodies revealed that CTβ2 is abundant in both distal axons and cell bodies/proximal axons of sympathetic neurons and also in distal axons of retinal ganglion cells. These immunoblotting experiments demonstrate that CTβ2 is present in PC12 cells as well as in distal axons of rat sympathetic neurons and rat retinal ganglion cells.

**CTβ2 Facilitates Sprouting of Neurites and Branch Formation**—Since CTβ2 is present in axons (Fig. 1), and since the amount of CTβ2, but not CTα, is increased during neurite outgrowth and/or extension (3), we considered that CTβ2 might regulate neurite growth. We therefore hypothesized that suppression of CTβ2 expression in PC12 cells would impair neurite growth. To test this hypothesis, we used a RNA silencing strategy to “knock down” expression of CTβ2 in PC12 cells. PC12 cells were co-transfected with a GFP-encoding mammalian expression vector (phrGFP) and either a vector encoding small interfering RNA targeted to CTβ2 mRNA (pSiCTβ2) or a vector containing a scrambled insert (designated “control”). The transfected cells were incubated with NGF to induce differentiation and neurite outgrowth. We estimated that transfection efficiency was ~30% according to the proportion of cells that expressed GFP in several random fields of view. In pSiCTβ2-transfected cultures, the amount of CTβ2 protein, as detected by immunoblotting, was reduced compared with that in control cells (Fig. 2A). Immunoblotting of tubulin demonstrated that protein synthesis in general was not suppressed (Fig. 2A). We also measured the in vitro activity of CT in cellular homogenates. This activity represents the combined activities of CTα and CTβ. In pSiCTβ2-transfected cells, CT activity was 2.9 nmol/min/mg protein, whereas CT activity of control cells was 4.3 nmol/min/mg protein (Fig. 2B). A trend (p < 0.09) toward reduced CT activity (~30%) in CTβ2-silenced cells was observed. However, since CT activity reflects the activities of both CTα (the predicted major isoform of CT) and CTβ, and since RNA interference-silencing of CTβ2 was operative in only ~30% of the cells, it is likely that CTβ2 activity was reduced in the SiCTβ2-transfected cells.

Consistent with the lack of reduction of total CT activity in SiCTβ2-transfected cells, the incorporation of [3H]choline into PC in PC12 cells over a 3-h time period was not significantly reduced (4.69 × 10⁶ ± 0.37 × 10⁶ dpm/mg of protein for control and 4.41 × 10⁶ ± 0.19 × 10⁶ dpm/mg of protein for SiCTβ2-transfected cells).

To determine if suppression of CTβ2 expression altered the morphology of NGF-treated PC12 cells, the cells were co-transfected with phrGFP and pSiCTβ2, and the morphologies of cells transfected with pSiCTβ2 and those transfected with the empty vector were compared (Fig. 3). Transfected cells were identified by the presence of GFP fluorescence. Within 2 days of NGF treatment, the number of neurites (primary neurites plus branches) per cell was markedly less in CTβ2-deficient cells than in cells transfected with pSILENCER containing a scrambled insert or in control cells transfected with empty vector. After 4 days of NGF treatment, differences in the number of neurites per cell, the degree of neurite branching, and neurite length between SiCTβ2-transfected cells and control cells were more pronounced (Fig. 3, A and B versus C and D). Moreover, the neurites of siCTβ2-expressing cells grew in a more linear fashion, with fewer points of attachment to the collagen substrate and fewer directional changes.
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**FIGURE 3.** Decreased neurite sprouting and branching in pSiCT\(\beta2\)-transfected PC12 cells. PC12 cells were co-transfected with an expression vector encoding GFP (phrGFP) and the pSILENCER 4.0 vector containing a scrambled siRNA insert (control) (A and B) or an RNA-silencing oligonucleotide directed against CT\(\beta2\) (C and D). Cells were differentiated for 4 days and then viewed by fluorescence microscopy (A, C, and D) or phase-contrast microscopy (B). Results were similar in four independent experiments.

**FIGURE 4.** Knockdown of CT\(\beta2\) reduces neurite sprouting and branching. A, primary neurites and branches are classified according to their positions relative to the cell body. Primary neurites project from the cell body, whereas branches project from primary neurites. Distal to the branch site, the continuance of primary neurites and branches is regulated by separate signaling pathways (22), we determined if a decrease in the amount of CT\(\beta2\) affected each of these neurite populations. Within 2 days of NGF treatment, the extent of neurite sprouting was significantly less in CT\(\beta2\)-deficient cells than in control cells (2.52 ± 0.09 versus 1.83 ± 0.01 neurites/cell (\(p < 0.01\))) (Fig. 4C). The difference in the number of primary neurites was still apparent after 4 days of NGF-induced differentiation (control versus siCT\(\beta2\)-expressing cells: 3.48 ± 0.12 versus 2.46 ± 0.18 neurites/cell (\(p < 0.01\))) (Fig. 4C). Furthermore, CT\(\beta2\)-deficient cells had far fewer branches than did control cells after 2 and 4 days of NGF treatment (Fig. 4D). Thus, CT\(\beta2\) deficiency impairs neurite sprouting and branching.

**CT\(\beta2\) Is Not Required for Neurite Extension**—When a primary neurite has sprouted from the cell body, either the neurite continues to elongate or neurite extension pauses for branch formation. Since CT\(\beta2\)-deficient PC12 cells contained fewer neurites (both primary neurites and branches) than did control cells (Figs. 3 and 4), we measured the lengths of primary neurites and branches to determine whether or not CT\(\beta2\) is required for neurite extension. In control cells, mean neurite length (including both primary neurites and branches) did not increase between days 2 and 5: 84 ± 1.5 pixels at day 2 and 100 ± 37 pixels at day 5 (Fig. 5A). Typically, once a neurite attained a length of ∼80 pixels, it had produced at least one branch, and further elongation of the primary neurite was limited. However, extension of primary neurites of CT\(\beta2\)-deficient cells continued throughout the period of NGF treatment. Consequently, by day 5, neurites of CT\(\beta2\)-deficient cells were 2.5 times longer than those of control cells (245 ± 1.4 versus 100 ± 37 pixels (\(p < 0.05\))). In addition to the striking increase in length and lack of branches, neurites of pSiCT\(\beta2\)-transfected cells also differed morphologically from neurites of control cells. Compared with neurites of control cells, neurites of CT\(\beta2\)-deficient cells extended in straight lines with far fewer points of attachment to the collagen-coated dishes or regions of localized thickening within the neurite (Fig. 3).

A potential explanation for why silencing of CT\(\beta2\) expression in PC12 cells decreased the number of neurites is that the synthesis and availability of PC were reduced. To investigate this possibility, we calculated the total length of all neurites (i.e. the sum of the length of all primary neurites and branches) in control cells and CT\(\beta2\)-deficient cells. After 2 and 4 days of NGF treatment, the total length of neurites was not different between control and CT\(\beta2\)-deficient cells (day 2, 257 ± 5 pixels/cell versus 315 ± 41 pixels/cell, respectively; day 4, 580 ± 89 pixels/cell and 713 ± 86 pixels/cell, respectively) (Fig. 5B). Thus, the total length of all neurites per cell and, presumably,
the total amount of membrane were the same in control and siCTβ2-expressing cells, implying that reduction in the amount of CTβ2 does not impair neurite elongation.

**Overexpression of CTβ2 in PC12 Cells Does Not Stimulate Neurite Sprouting or Branch Formation**—Since siRNA-mediated reduction of CTβ2 expression in PC12 cells significantly decreased total neurite number, we investigated whether or not overexpression of CTβ2 increased neurite sprouting and branch formation. We therefore generated a cDNA encoding HA-tagged CTβ2 (CTβ2-HA) and cloned it into a mammalian expression vector. Expression of CTβ2-HA protein in PC12 cells was verified by immunoblotting of lysates of CTβ2-HA-transfected cells with anti-HA antibodies. Fig. 6A shows that a 43-kDa, HA-containing protein corresponding to the predicted size of CTβ2-HA was expressed in CTβ2-HA-transfected cells. Accordingly, CT activity in lysates of CTβ2-HA-transfected cells was significantly (30%) higher than in control cells (1.30 ± 0.09 nmol/min/mg protein versus 1.00 ± 0.01 nmol/min/mg protein, respectively (p < 0.05)) (Fig. 6B). To determine if CTβ2 overexpression altered neurite morphology, we co-transfected PC12 cells with a GFP-encoding vector to select and visualize cells that expressed CTβ2-HA. After 2 days of NGF treatment, the cells were visualized by fluorescence microscopy, and numbers of primary neurites and branches were determined. CTβ2-HA-expressing cells contained 4.9 ± 0.6 neurites/cell, whereas control cells contained 3.7 ± 0.4 neurites/cell (Fig. 6C).

Although this difference is not statistically significant (p < 0.15), there is a trend toward an increased number of neurites in CTβ2-HA-expressing cells. Furthermore, enhanced expression of CTβ2 did not significantly increase the number of primary neurites (3.05 ± 0.25 neurites/cell in control cells versus 3.57 ± 0.16 neurites/cell (p < 0.15) in CTβ2-HA-transfected cells). Despite a strong trend toward increased number of branches, overexpression of CTβ2 did not significantly increase the number of branches (0.66 ± 0.31/cell in control cells versus 1.38 ± 0.30/cell in CTβ2-HA-transfected cells (p < 0.17)). Thus, whereas suppression of CTβ2 expression dramatically reduced the number of primary neurites and branches (Fig. 4), overexpression of CTβ2 did not significantly increase the number of primary neurites or branches (Fig. 6).
brane phospholipids in PC12 cells would be unaffected by alteration (Fig. 5A) of CTβ2 expression. We investigated the mechanism by which CTβ2 plays a role in regulating neurite outgrowth. previous report (16), PC12 cells increased the number of primary neurites/cell by 81% (from 0.57 ± 0.03 to 0.12 ± 0.02 primary neurites/cell). Whereas CTβ2-deficient cells contained 0.10 ± 0.005 for control versus LY294002; **, p < 0.01 for control versus LY294002; ***. p < 0.005 for control versus LY294002; $, p < 0.05 for SiCTβ2 versus SICl[CTβ2-HA].

Since silencing of CTβ2 expression did not reduce total neurite length (Fig. 5B), we hypothesized that the amounts of membrane phospholipids in PC12 cells would be unaffected by alterations in CTβ2 expression. Indeed, the mass (nmol of phospholipid/mg of cell protein) of PC, phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, and phosphatidylserine was not significantly altered by overexpression of CTβ2 (CTβ2-HA-transfected cells) or in cells in which CTβ2 expression was reduced (i.e. pSiCTβ2-transfected cells (Fig. 7). Thus, the total amount of phospholipids in PC12 cells was not altered by either a decrease or an increase in the level of CTβ2. Taken together, these data demonstrate that CTβ2 is required for normal neurite branching and sprouting but not for neurite extension.

CTβ2 is Required for LY294002-dependent Stimulation of Neurite Outgrowth—We investigated the mechanism by which reduction of CTβ2 impairs neurite sprouting and branching. Activation of Akt has been proposed to inhibit neurite branching in PC12 cells, since treatment of the cells with LY294002, an inhibitor of phosphatidylinositol 3-kinase, abolished Akt activation and promoted neurite branching (16). Because our data suggested that CTβ2 plays a role in regulating neurite outgrowth and branch formation, we hypothesized that CTβ2 might be a downstream target of the phosphatidylinositol-3-kinase/Akt signaling cascade. To test this hypothesis, we compared the effect of LY294002 on neurite outgrowth and branch formation in control PC12 cells and in PC12 cells in which CTβ2 expression had been reduced. Immunoblotting of cellular proteins with anti-phospho-Akt antibodies confirmed that LY294002 (10 μM) reduced the phosphorylation of Akt (Fig. 8A). Consistent with a previous report (16), PC12 cells incubated with LY294002 contained more neurites than did untreated cells (5.4 ± 0.2 neurites/cell for LY294002-treated cells versus 3.1 ± 0.06 neurites/cell for control cells (p < 0.01)). In sharp contrast, however, decreased expression of CTβ2 in pSiCTβ2-transfected cells blocked the increase in the total number of neurites that was observed in control cells treated with LY294002 (Fig. 8B); CTβ2-deficient cells in the absence of LY294002 contained 2.05 ± 0.16 neurites/cell, and LY294002-treated siCTβ2-transfected cells contained 1.95 ± 0.02 neurites/cell.

FIGURE 7. Modulation of CTβ2 expression does not alter phospholipid mass. PC12 cells were transfected with either a pCI vector containing no insert (control), pCI encoding HA-tagged CTβ2 (pCI[CTβ2-HA]), or pSILENCER 4.0 containing siRNA directed against CTβ2 (siCTβ2). Cells were differentiated for 4 days. Phospholipids were extracted, and amounts of PC, phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), sphingomyelin (SM), and phosphatidylserine (PS) were quantified. Data are means ± S.E. from triplicate analyses of three independent experiments.

FIGURE 8. CTβ2 is required for LY294002-dependent neurite sprouting. Cells were differentiated for 2 days in the presence of either 10 μM LY294002 or an equivalent volume of vehicle (dimethyl sulfoxide). A, cells were harvested, and equal amounts of protein (20 μg) from homogenates were separated by polyacrylamide gel electrophoresis. The phosphorylation of Akt (Ser-473) and the total amount of Akt were assessed by immunoblotting with anti-phospho-Akt and anti-Akt antibodies, respectively. Shown (top of A) are representative data from three independent experiments. Data from densitometric scanning of the bands for phospho-Akt and total Akt were quantiﬁed for LY294002-treated cells relative to untreated control cells (bottom of A); data are averages ± S.D. of four samples from two independent experiments. B, C, and D, cells were co-transfected with a vector encoding GFP and pSILENCER 4.0 vector containing either siRNA directed against CTβ2 (SiCTβ2) or a scrambled siRNA insert (Control). Cells were differentiated for 2 days in the presence of either LY294002 (10 μM) or an equivalent volume of vehicle and then viewed by fluorescence microscopy. Numbers of all primary neurites and branches (greater than 20 pixels) were determined. B, total number of neurites/cell. C, number of primary neurites/cell. D, number of branches/cell. All data are means ± S.E. of number of neurites on GFP-positive cells. A minimum of 50 cells were scored for each time point. *, p < 0.05 for control versus LY294002; **, p < 0.01 for control versus LY294002; ***. p < 0.005 for control versus LY294002; $, p < 0.05 for SiCTβ2 versus SICl[CTβ2-HA].
these data strengthen the conclusion that CT2 is important for neurite branching.

**Phosphorylation of CTβ2**—More than one CT isoform contributes to CT activity in PC12 cells. We therefore assessed whether or not treatment with LY294002 directly affected the phosphorylation status of CTβ2. Since the phosphatidylinositol 3-kinase inhibitor, LY294002, did not stimulate neurite sprouting and only slightly increased branching in CTβ2-deficient cells, we speculated that CTβ2 might be a downstream target of the phosphatidylinositol 3-kinase/Akt signaling pathway. CTβ2 is heavily phosphorylated in vivo (7). However, studies on the kinases and phosphatases that act on CTβ2 have not been reported. In HeLa cells, both insulin and epidermal growth factor can stimulate CT phosphorylation (23), and HeLa cells express both CTα and CTβ mRNAs (7). Thus, it is likely that CTβ2 is phosphorylated in response to growth factors, including NGF.

From the amino acid sequence of CTβ2, we identified a putative Akt phosphorylation motif (RSRSPS) within the carboxyl terminus of CTβ2. To determine if CTβ2 was a substrate for phosphorylation by Akt, we incubated differentiated PC12 cells with LY294002 or an equivalent volume of vehicle for 48 h. The cells were then incubated with [32P]orthophosphate for 1 h, and CTβ2 was immunoprecipitated and separated by SDS-PAGE. As shown in Fig. 8A, treatment of PC12 cells with LY294002 reduced the phosphorylation of Akt but did not significantly alter the 32P labeling of CTβ2 (Fig. 9). Thus, in NGF-differentiated PC12 cells, CTβ2 is unlikely to be directly regulated by Akt phosphorylation.

Another candidate kinase for phosphorylation of CTβ2 is Cdk5 (24). Cdk5s comprise a family of proline-directed threonine and serine kinases that orchestrate transitions through the cell cycle. Cdk5 has 60% sequence identity to other Cdk5s and is the sole isoform that is highly enriched in brain (25). Cdk5 is required for axon growth (26) and is activated during regeneration of facial nerve axons after injury (27). CTβ2 has a putative Cdk5 phosphorylation consensus motif (SPSR) within its carboxyl terminus. We therefore investigated whether or not CTβ2 was directly phosphorylated by Cdk5. CTβ2 was immunoprecipitated from homogenates of PC12 cells and used as a substrate for Cdk5 phosphorylation in an in vitro kinase assay. We used tubulin as a negative control, because it lacks a Cdk5 phosphorylation motif and is not known to be phosphorylated by Cdk5. As shown in Fig. 10A, CTβ2 was phosphorylated in vitro by Cdk5, whereas tubulin was not.

Since Cdk5 phosphorylates CTβ2 in vitro, we next determined if Cdk5 also phosphorylated CTβ2 in intact cells. If CTβ2 were phosphorylated by Cdk5, incubation of PC12 cells with roscovitine, a Cdk inhibitor, would be expected to diminish Cdk5-dependent 32P labeling of CTβ2. To test this hypothesis, PC12 cells were incubated with and without roscovitine for 1 h prior to labeling of the cells with [32P]orthophosphate. CTβ2 was immunoprecipitated from cell lysates with anti-M antibodies, and the incorporation of [32P] into CTβ2 was assessed. Fig. 10B shows that less 32P was incorporated into CTβ2 in PC12 cells treated with roscovitine than in untreated cells. These observations suggest that CTβ2 is a substrate for Cdk5. In addition, roscovitine treatment of PC12 cells markedly reduced neurite branching (Fig. 10C), suggesting a link between reduced phosphorylation and activity of CTβ2 and inhibition of neurite branching. At the concentration of roscovitine used for these experiments (25 μM for 48 h), PC12 cell viability was unaffected. For example, for six fields counted for each condition, the number of cells per field was the same for control cells (39.0 ± 12.6) as for roscovitine-treated cells (37.4 ± 9.3).

**DISCUSSION**

We investigated the role of CTβ2, an isoform of CT (the rate-limiting enzyme in PC biosynthesis) in neurite sprouting, extension, and branching of PC12 cells. We show that CTβ2 is abundant in PC12 cells, as well as in axons of rat sympathetic neurons and rat retinal ganglion cells. When the amount of CTβ2 protein was reduced in PC12 cells by RNA silencing, neurite branching, the total number of neurites per cell, and the number of primary neurites per cell were markedly diminished, whereas the total length of neurites was unchanged. These data demonstrate that CTβ2 plays a role in neurite sprouting and branching but not in neurite elongation. Our studies also indicate that CTβ2 is required for the stimulation of neurite outgrowth that occurs when the phosphatidylinositol-3-kinase/
Akt signaling pathway is inactivated. We show that CTβ2 is phosphorylated by Cdk5 and that inhibition of Cdk5 by roscovitine reduces neurite branching, consistent with a link between phosphorylation and activity of CTβ2 and neurite branching.  

CTβ2 Is Abundant in Axons and Facilitates Neurite Sprouting and Branch Formation—Phospholipids are not synthesized exclusively in cell bodies of neurons (12), and enzymes of the Kennedy pathway for PC biosynthesis have been detected in distal axons of rat sympathetic neurons (4) and in rat brain synaptosomes (10). Indeed, at least 50% of PC in axonal membranes is biosynthesized within axons (28). Moreover, when distal axons, but not cell bodies, of sympathetic neurons are treated with inhibitors of PC biosynthesis, axonal growth is significantly impaired (28). The biosynthesis of phosphatidylethanolamine, phosphatidylserine, and sphingomyelin has also been detected in axons of rat sympathetic neurons (4). Furthermore, the presence of GM3 synthase, a ganglioside-specific sialyltransferase, has been reported in axons of hippocampal neurons (29). Interestingly, however, not all lipid classes are made in distal axons, since cholesterol biosynthesis appears to be restricted to cell bodies/proximal axons (4).

The most pronounced expression of CTβ2 mRNA in the mouse is in the brain (30). However, since ~90% of the cells in the brain are glial cells, not neurons (reviewed in Ref. 31), we do not know the relative level of expression of CTβ2 in neurons compared with other types of cells in the brain. We now show that CTβ2 is abundant in PC12 cells and in distal axons of sympathetic neurons (peripheral neurons), and retinal ganglion cells (central nervous system neurons). Based on these findings, we proposed that axonal synthesis of PC via CTβ2 would be important for some aspects of neurite growth, such as sprouting, branching, and/or extension. We, therefore, suppressed CTβ2 expression in differentiating PC12 cells. Silencing of CTβ2 expression strikingly altered neurite morphology and reduced neurite sprouting and branch formation. In contrast, the total combined length of all neurites was not decreased. As an additional test of whether or not CTβ2 regulates neurite sprouting and branching, we expressed HA-tagged CTβ2 in differentiating PC12 cells. The amount of CTβ2 protein and CT activity were increased, but the number of neurites was not significantly increased. Thus, either the residual amount of CTβ2 and CTα in PC12 cells can together produce sufficient PC for neurite sprouting and branching or, alternatively, CTβ2 is not the sole requirement for these processes. Several reports have identified proteins in PC12 cells, including the microtubule-binding protein, raspolin, and the signaling molecule, Raf, that are required for proper branching of neurites (32). Like CTβ2, raspolin promotes branch formation, and in its absence PC12 cells produce fewer branches.  

CTβ2 Is Required for LY294002-stimulated Neurite Outgrowth—Successful neurotogenesis requires a delicate balance between neurite extension and branch formation (33). In CTβ2-deficient PC12 cells, we observed a dramatic decrease in neurite branching but an increase in the length of individual primary neurites. Time lapse recording of axon extension in sensory neurons has shown that axon growth pauses for up to several hours at the site of future branch points (34). During this pause, microtubules become splayed and disorganized at the future branch points. Eventually, the microtubular network reorganizes and short microtubules project into the developing neurite (reviewed in Ref. 33). When an axon or neurite is unable to recognize signals for pausing and reorganization for branch formation, uninterrupted growth of the neurites results in production of abnormally long neurites. This phenomenon has also been observed in PC12 cells that lack Akt, a serine/threonine kinase known to promote neurite growth and inhibit branch formation. PC12 cells lacking Akt contain significantly fewer neurites than do control cells that contain Akt, but the neurites extend greater distances than in control cells that contain Akt (16). Thus, inactivation of Akt appears to facilitate neurite formation and/or branching.

LY294002 is a phosphatidylinositol 3-kinase inhibitor that prevents the activation of Akt (16). Incubation of control PC12 cells with LY294002 increased the number of neurites per cell, both primary neurites and branches. However, in CTβ2-deficient PC12 cells, LY294002 failed to increase the number of primary neurites (i.e. sprouting). Thus, CTβ2 is required for the
promotion of sprouting induced by inhibition of the phosphatidylinositol 3-kinase/Akt signaling pathway.

Within its carboxyl terminus, CTβ2 contains a putative Akt phosphorylation motif, RSRSPS. We therefore determined whether or not CTβ2 is phosphorylated by Akt in intact cells. LY294002 treatment of PC12 cells did not alter the 32P labeling of CTβ2 (Fig. 9B), suggesting that CTβ2 is not directly regulated by phosphorylation via Akt.

NGF is known to activate many signaling pathways in neurons (35). Cdk5 and its co-activator, p35, are abundant in the brain and are among the proteins activated by NGF (36); these proteins have been strongly implicated in axon growth and recovery after injury (26, 27), and Cdk5 appears to be involved in efficient cytoskeletal remodeling. Since CTβ2 contains a Cdk5 phosphorylation consensus motif (SPSR) within its carboxyl terminus, we hypothesized that Cdk5 directly phosphorylates CTβ2. Indeed, we demonstrated that Cdk5 can phosphorylate CTβ2 in vitro and in intact cells (Fig. 10). In addition, short term treatment of PC12 cells with roscovitine, a Cdk inhibitor, markedly decreased CTβ2 phosphorylation and neurite branching in intact cells. CTβ2 and Cdk5 (38) are both present within axons. Several lines of evidence suggest that Cdk5 promotes axon extension and impairs axon branching. In Cdk5-null mice, motor axons exhibit extensive anomalous branching patterns (37). Furthermore, inhibition of Cdk5 with roscovitine attenuates axon elongation (38). Thus, we propose that when Cdk5 is activated, CTβ2 is phosphorylated and activated, and consequently, neurite outgrowth and branching are promoted.

Phosphatidylcholine Synthesis and Neurite Formation—To our knowledge, we have identified the first lipid biosynthetic enzyme that facilitates neurite sprouting and branch formation. Marszalek et al. (39) reported that overexpression of a fatty acid-metabolizing enzyme, acyl-CoA synthetase-2, significantly increases neurite extension in PC12 cells. However, the numbers of neurites and branches were unchanged. It is possible that CTβ2 provides a locally synthesized pool of PC in axons at future branch points. Consistent with this idea, when axons of Aplysia sensory neurons were injured, the axonal membrane, rather than the cell body, provided lipid for rescaling the membrane and for the rapid growth following axotomy (40).

In addition to providing new membrane material for neurite sprouting and branching, CTβ2 might replace PC that has been degraded in axons. Phospholipase D hydrolyzes PC (41) and is present in PC12 cells (42). Neurite outgrowth in PC12 cells requires activation of phospholipase D via the MAP kinase signaling cascade (43). In our previous study (2), we demonstrated that inhibition of the mitogen-activated protein kinase pathway by U0126 decreased CT activity, consistent with a role for mitogen-activated protein kinase in activation of CTβ2. Thus, CTβ2 might replenish the pool of PC that has been hydrolyzed by phospholipase D. Alternatively, or in addition, CTβ2 might synthesize PC that provides phosphocholine for sphingomyelin synthesis. Sphingomyelin can be made in distal axons of sympathetic neurons (4) and is a component of detergent-insoluble glycolipid complexes in axonal membranes (44). Last, we cannot rule out a role for CTβ2 in neurite outgrowth and/or branching that is independent of its catalytic role in PC synthesis.

The CDP-choline pathway for PC biosynthesis is, under most metabolic conditions, including in NGF-stimulated rat sympathetic neurons (5), regulated by CT activity. In PC12 cells, PC biosynthesis has also been reported to be regulated by the availability of diacylglycerol and by the activity of cholinephosphotransferase, the enzyme that catalyzes the final step of the CDP-choline pathway (2). Furthermore, cytidine and uridine (the product of cytidine deamination) can enhance PC biosynthesis in PC12 cells (45) and rat brain slices (46). Interestingly, treatment of PC12 cells with uridine increased CDP-choline levels and enhanced neurite outgrowth and branch formation in a dose-dependent manner (47). This enhancement of neurite outgrowth was attributed, at least in part, to an increased amount of CDP-choline (47). Thus, it appears that both the amount of CTβ2 protein and substrate availability can regulate PC biosynthesis during PC12 cell differentiation.

In summary, we provide evidence that CTβ2 facilitates neurite outgrowth and branching. Since CTβ2 appears to be involved in neurite branch formation and is abundant in the brain, we predict that a complete deficiency of CTβ2 might result in subtle neurological defects, perhaps in learning and memory or motor coordination, rather than gross neuroanatomical deficits.

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