

Axonal Synthesis of Phosphatidylcholine is Required for Normal Axonal Growth in Rat Sympathetic Neurons

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Abstract. The goal of this study was to assess the relative importance of the axonal synthesis of phosphatidylcholine for neurite growth using rat sympathetic neurons maintained in compartmented culture dishes. In a double-labeling experiment [¹⁴C]choline was added to compartments that contained only distal axons and [³H]choline was added to compartments that contained cell bodies and proximal axons. The specific radioactivity of labeled choline was equalized in all compartments. The results show that ~50% of phosphatidylcholine in distal axons is locally synthesized by axons.

The requirement of axonal phosphatidylcholine synthesis for neurite growth was investigated. The neurons were supplied with medium lacking choline, an essential substrate for phosphatidylcholine synthesis.

In the cells grown in choline-deficient medium for 5 d, the incorporation of [³H]palmitate into phosphatidylcholine was reduced by 54% compared to that in cells cultured in choline-containing medium. When phosphatidylcholine synthesis was reduced in this manner in distal axons alone, growth of distal neurites was inhibited by ~50%. In contrast, when phosphatidylcholine synthesis was inhibited only in the compartment containing cell bodies with proximal axons, growth of distal neurites continued normally. These experiments imply that the synthesis of phosphatidylcholine in cell bodies is neither necessary nor sufficient for growth of distal neurites. Rather, the local synthesis of phosphatidylcholine in distal axons is required for normal growth.

NERVE fiber elongation involves the expansion of surface membranes. It has been well documented that neuronal proteins are made in cell bodies, and possibly in dendrites (Torre and Steward, 1992), but no protein synthesis has been detected in axons. Until recently, axonal lipids also were presumed to be synthesized exclusively in cell bodies (Pfenninger and Johnson, 1983; Ledeen, 1985; Toews and Morell, 1985; Alberts et al., 1989) and co-transported with proteins in membranous transport vesicles into the axons by an anterograde transport mechanism. Available data suggest that it is unlikely that lateral diffusion of a lipid in the plane of the plasma membrane contributes significantly to the mass of axonal lipids derived from the cell body because a lipid would require 6 d to move 1 mm along the axon (Futerman et al., 1993).

Contrary to the dogma, however, in the last few years some evidence has emerged suggesting that axons themselves can synthesize membrane lipids. For example, the activities of some enzymes of glycolipid biosynthesis were detected in rat axolemma (De Vries et al., 1983), and the synthesis of small amounts of several phospholipids was

reported in extruded squid axoplasm (Tanaka et al., 1987) and in mouse sciatic nerves (Gould, 1976; Gould et al., 1987; Kumari-Siri and Gould, 1980; Padilla and Pope, 1991). Moreover, phosphatidylinositol synthesis was observed in mouse sciatic nerves (Gould, 1976) and squid axons (Gould et al., 1983). In addition, enzymes catalyzing the final steps in the biosynthesis of phosphatidylcholine (PtdCho)¹ and phosphatidylethanolamine (PtdEtn) were detected in lysed rat brain synaptosomes (Strosznajder et al., 1979).

In our laboratories, using radiolabeled precursors of lipids, we have shown that the biosynthesis of the major phospholipids—PtdCho, PtdEtn, phosphatidylserine (PtdSer) and phosphatidylinositol—as well as fatty acids, but not cholesterol, occurs in pure axons of rat sympathetic neurons (Vance et al., 1991, 1994). For these studies rat sympathetic neurons were cultured in dishes divided into three compartments (Campenot, 1982, 1992). A major advantage of this culture system of neurons compared to others is that metabolic events occurring in distal axons can be studied independently of those occurring in cell bodies. Cell bodies are plated in the center compartment and axons extend at a

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1. *Abbreviations used in this paper:* PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

rate of ~ 1 mm/day under Teflon-silicone grease barriers into the two side compartments. The center compartment contains proximal axons as well as cell bodies, whereas the left and right compartments contain distal axons alone. Small molecules that are added to the culture medium of one compartment are unable to diffuse into adjacent compartments (Campenot, 1982; Campenot and Draker, 1989). The axonal synthesis of PtdCho was confirmed by experiments in which the specific activities of three enzymes involved in the biosynthesis of PtdCho via the CDP-choline pathway were found to be of similar magnitude in the distal axons and in the cell body-containing compartment and of similar magnitude also to the activities in rat hepatocytes (Vance et al., 1994). Our data did not, however, imply that all of the PtdCho in axons was synthesized in axons. Indeed, in the compartmentalized model for neuron culture, synthesis of PtdCho in the cell body-containing compartment was observed with a concomitant transfer of the PtdCho into distal axons (Vance et al., 1991). These studies support the observations of Pfenninger and Johnson (1983) who examined PtdCho synthesis and anterograde transport in explant cultures of rat sympathetic neurons. These authors concluded, however, that PtdCho synthesis occurred exclusively in cell bodies, although in their study the synthesis of PtdCho in pure axons was not examined.

Our data raised the possibility that axonal membrane lipids may be synthesized largely in situ in axons rather than solely in cell bodies. Therefore, in the present study we have asked: what fraction of axonal PtdCho is synthesized in axons and how important is the axonal synthesis of PtdCho for neurite extension? We report that local production of PtdCho in axons contributes $\sim 50\%$ of the axonal PtdCho. Moreover, we provide evidence that when PtdCho biosynthesis is inhibited in distal axons, but not in cell bodies and proximal axons, the growth of distal axons is substantially decreased. The data imply that synthesis of PtdCho in axons is required for normal axonal extension.

Materials and Methods

Materials

The radiolabeled chemicals [*methyl*- ^3H]choline (specific activity 15 Ci/mmol) [*methyl*- ^{14}C]choline (specific activity 55 mCi/mmol), and [^3H]palmitate (specific activity 54 Ci/mmol) were purchased from Amersham Canada (Oakville, ON). Thin-layer chromatography plates (silica gel G, 0.25-mm thickness) were obtained from BDH Chemicals (Edmonton, Canada). Standard phospholipids were either isolated from rat liver or were purchased from Avanti Polar Lipids (Birmingham, AL). Choline, phosphocholine, CDP-choline, palmitate, and phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were from Sigma or Fisher Scientific (Edmonton, Canada).

Neuronal Cultures

The general culture procedures for the neurons were as previously reported (Campenot et al., 1991). Briefly, superior cervical ganglia were dissected from 1-d-old Sprague-Dawley rats supplied by the University of Alberta farm. Ganglia were enzymatically and mechanically dissociated and the cells were plated either at a density of 2 ganglia/well in a 24-well plate coated with collagen or in the center compartment of the compartmented dishes (0.5 ganglion/dish). Compartmented culture dishes were constructed essentially as previously described (Campenot, 1992). Collagen was dried onto 35-mm Falcon tissue culture dishes to provide a culture substratum.

20 parallel tracks were then formed on the dish surface by scraping the collagen from the dish with a pin rake. A Teflon divider was seated on the surface of the dish with silicone grease. Neurons, plated in the center compartment, settled on the substratum of the collagen tracks, and axons elongated along these tracks crossing under the silicone grease barriers and entering left and right compartments. L15 medium without antibiotics (GIBCO BRL, Gaithersburg, MD), but supplemented with the additives prescribed by Hawrot and Patterson (1979) including bicarbonate and methylcellulose, was used for culture of the neurons. Rat serum (2.5%, provided by the University of Alberta Laboratory Animal Services) and ascorbic acid (1 mg/ml) were supplied only in medium given to the cell body-containing compartments. Culture medium was routinely changed every 3–6 d. Non-neuronal cells were eliminated by exposure of the cultures for the first 6 d to medium containing 10 μM cytosine arabinoside. Initially, all three compartments were supplied with mouse 2.5 S nerve growth factor (200 ng/ml) (Cedarlane Laboratories Ltd., Hornby, ON, Canada) to allow cell survival and to permit neurite growth in all compartments. The use of nerve growth factor was discontinued in the center compartment after 6 d, so that subsequent neurite growth was largely confined to distal neurites in the side compartments which contained nerve growth factor (Campenot, 1982). In some experiments, delipidated rat serum, prepared as previously described (Yao and Vance, 1988), was used. Choline-deficient medium consisted of L15 medium containing all the ingredients listed by GIBCO BRL, but with choline omitted. The concentration of choline in the normal, choline-containing medium was 11 μM .

In some experiments, cultures were neuritotomized and regeneration of neurites was examined. Neuritotomy consisted of a mechanical removal of distal neurites from left and right compartments with a jet of sterile distilled water delivered with a syringe through a 22-gauge needle. The water was aspirated, the washing step was repeated twice, then fresh culture medium was added. This procedure reliably removes virtually all visible traces of neurites from the side compartments (Campenot, 1992). In one set of experiments, the cellular material was similarly removed from the center compartment, leaving the distal axons intact.

Measurements of neurite growth were made as previously described (Campenot, 1992) using a Nikon Diaphot inverted microscope with phase contrast optics outfitted with a MD2 microscope digitizer (Minnesota Datametrics Corp., Minneapolis, MN) which tracks stage movements to an accuracy of ± 5 μm . An online personal computer using custom software (Minnesota Datametrics Corp.) calculated the distance from the edge of the silicone grease to the farthest extending neurite on each track and combined these measurements to obtain means and standard errors. In each culture neurites in 16 tracks were measured in left and right compartments.

Incorporation of Radiolabeled Precursors into PtdCho and Intermediates of PtdCho Synthesis

Neurons, either in 24-well dishes or in compartmented dishes, were incubated with medium containing [*methyl*- ^3H]choline or [*methyl*- ^{14}C]choline. For the double-labeling experiments, unlabeled choline was added to the [*methyl*- ^3H]choline-containing medium to give the same choline concentration as in the [*methyl*- ^{14}C]choline medium (i.e., 92 μM). After the desired incubation times, the radiolabeled medium was aspirated and the cells were washed twice with ice-cold phosphate-buffered saline. Cellular material from each compartment or well was collected in methanol:water (1:1, vol/vol). Chloroform was added to give a final ratio of chloroform:methanol:water of 2:1:1 (vol/vol). The lipids were extracted according to the procedure of Folch et al. (1959) and separated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, vol/vol). The band corresponding to authentic PtdCho was scraped from the plates and radioactivity was measured. Incorporation of radiolabeled choline into the water-soluble precursors of PtdCho was measured as follows, in some experiments. The upper aqueous phase from the Folch extraction, which contained choline, phosphocholine and CDP-choline, was evaporated to dryness and the residue was dissolved in methanol:water, 1:1, (vol/vol). The sample was applied to a thin-layer chromatography plate and the components were separated in a solvent system consisting of ammonia/methanol/0.6% NaCl, 5:50:50 (vol/vol). The bands corresponding to authentic standards of choline, phosphocholine and CDP-choline were scraped from the plate and radioactive incorporation was measured. In the experiments in which lipids were radiolabeled with [^3H]palmitate, the cells in 24-well dishes were incubated with 10 $\mu\text{Ci/ml}$ [^3H]palmitate. PtdCho and PtdEtn were isolated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/formic acid/water, 70:30:12:4:2 (vol/vol) and radioactive incorporation was measured.

Other Methods

The phospholipid content of the cells was measured by lipid phosphorus determination (Chalvardjian and Rudnicki, 1970). The protein content of cells was measured with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

Results

Axonal Synthesis of Phosphatidylcholine

We have previously demonstrated by radiolabeling experiments with compartmented neuron cultures and from direct assay of phospholipid biosynthetic enzyme activities that axons have the capacity to synthesize the most abundant membrane phospholipids PtdCho, PtdEtn, PtdSer, phosphatidylinositol, and sphingomyelin (Vance et al., 1991, 1994). To confirm that the synthesis of PtdCho in axons was not an artifact of rapid retrograde diffusion of radiolabeled choline from distal axons into cell bodies and synthesis in the cell bodies, followed by rapid diffusion of products into the axons, we examined the ability of severed axons to synthesize PtdCho. The cell bodies and proximal axons in the center compartment of 3-compartmented dishes containing rat sympathetic neurons (Campenot, 1982, 1992) were removed by washing, and the severed distal axons that remained in the side compartments were incubated with [*methyl*-³H]choline (10 μ Ci/ml) for 5 h. We compared the incorporation of radioactivity into PtdCho in severed distal neurites to that in distal axons of intact neuron cultures. The incorporation of [³H]choline into PtdCho was $131,760 \pm 11,360$ dpm/nmol phospholipid for distal axons of intact cells and $120,481 \pm 8,324$ dpm/nmol phospholipid for distal axons severed from their cell bodies. Thus, the attachment of cell bodies had no significant effect upon the ability of distal axons to convert [³H]choline into PtdCho.

While distal axons clearly synthesize PtdCho, our experiments did not indicate how much of the PtdCho of distal

axons is made locally compared to how much is made in cell bodies/proximal axons and transported into distal axons. We therefore attempted to evaluate the proportion of axonal PtdCho that is made in situ in axons in a double-labeling experiment. [*Methyl*-³H]choline was added to the compartment containing the cell bodies (center compartment) and [*methyl*-¹⁴C]choline was added to the compartments containing distal axons (left and right compartments). The specific radioactivity of choline was equalized (55 μ Ci/nmol) in the media of all three compartments (the final concentration of choline was 92 μ M) by addition of unlabeled choline to the cell body-containing compartment. The incorporation of radioactivity into PtdCho was measured after time intervals of 1 h to 9 d (Fig. 1). The amounts of [³H]choline and [¹⁴C]choline (dpm/dish) incorporated into PtdCho in the axon-containing side compartments were approximately equal (Fig. 1 B), showing that half of axonal PtdCho had been derived from [³H]choline and half from [¹⁴C]choline. These data indicated that over the entire 9 d of the experiment 50% of axonal PtdCho had been synthesized in distal axons.

These data do not imply that the exclusive site of PtdCho synthesis is the distal axons because the remaining 50% of PtdCho of distal axons was made from [³H]choline supplied to the cell body-containing compartment. The transport of [³H]choline, or its metabolites, from the cell body-containing compartment to distal axons was substantial: approximately one half of the [³H]PtdCho made by the neurons during the first day of incubation was present in distal neurites. Since some of the cellular material in the center cell body-containing compartment consists of proximal axons, it is likely that a significant quantity of PtdCho synthesized in the center compartment was not made by cell bodies, but by proximal axons. Furthermore, [³H]choline added to the cell body-containing compartment could have been transported into distal axons in the form of choline, phosphocholine or CDP-choline and subsequently converted into PtdCho in the distal axons. The incorporation of radioactivity into two precursors of PtdCho, phosphocholine (Fig. 2) and CDP-choline (data not shown), in axons and in the cell body-containing compartment followed the same general pattern

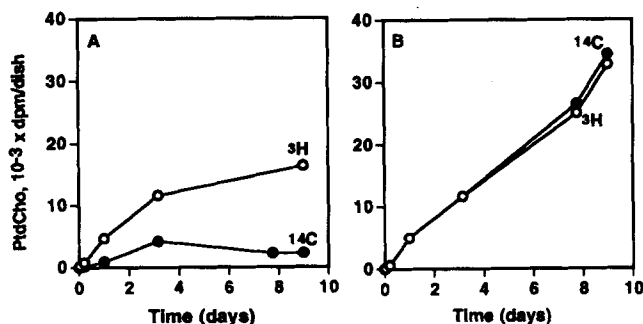


Figure 1. Axons produce ~50% of their own phosphatidylcholine. [*Methyl*-³H]choline (5 μ Ci/ml) (open circles) and [*methyl*-¹⁴C]choline (5 μ Ci/ml) (solid circles) were added to cell body-containing compartments and distal axon-containing compartments, respectively, of isolated rat sympathetic neurons that had been cultured for 11 d. The specific radioactivity of choline was equalized (55 μ Ci/nmol) in all compartments by adding unlabeled choline to the cell body-containing compartment to a final concentration of 92 μ M. At the indicated times, cellular material of the cell body-containing compartment (A), and the distal axon-containing compartments (B) was collected and lipids were extracted. PtdCho was isolated by thin-layer chromatography and radioactivity was determined. The experiment was repeated twice with similar results.

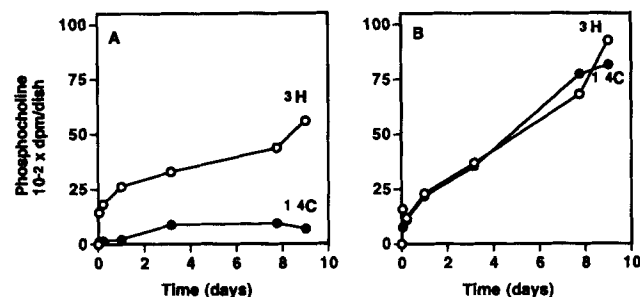


Figure 2. Incorporation of choline into phosphocholine. From the experiment described in Fig. 1, the water-soluble metabolites of choline were separated by thin-layer chromatography. The spot corresponding to authentic phosphocholine was scraped and radioactivity was measured. (A) Cell body-containing compartment; (B) distal axon-containing compartments. Incorporation of radioactivity from [³H]choline, open circles, and from [¹⁴C]choline, solid circles. Each point is the average of four individual cultures. The experiment was repeated twice with similar results.

as incorporation into PtdCho. Our results do not preclude a substantial production of PtdCho in cell bodies but do suggest that at least 50% of PtdCho in distal axons was derived from local synthesis in axons.

In contrast to the anterograde movement of [^3H]label into PtdCho in distal neurites relatively little retrograde movement of [^{14}C] label from distal axons into proximal axons/cell bodies occurred. During the first 24 h of the experiment only 15% of PtdCho made from [^{14}C] supplied to distal axons was found in the cell bodies/proximal axons (Fig. 1 A). Consequently, the retrograde movement of [^{14}C] label from distal neurites makes a relatively small contribution to PtdCho in the cell bodies/proximal axons. Over the time course of the experiment 80–90% of PtdCho in the center compartment had been made locally from [^3H]choline supplied to that compartment (Fig. 1 A).

We cannot exclude the possibility that some diffusion of PtdCho in the plane of the membrane occurs. However, the contribution of this process to the transfer of PtdCho between compartments would be expected to be small in light of the considerations of Futerman and coworkers (1993) who calculated that a lipid would take ~ 6 d to move 1 mm along an axon by lateral diffusion. Ledeen (1985) has also suggested that such diffusion could not provide a mechanism by which phospholipids would be distributed to the distal regions of longer processes.

Phosphatidylcholine Synthesis and Neurite Growth in the Absence of Exogenous Choline

Since the experiment described above demonstrates that axons synthesize $\sim 50\%$ of their own PtdCho in situ, we wished to determine if the absence of local PtdCho synthesis in axons would inhibit axonal growth. A highly specific way in which to inhibit PtdCho biosynthesis is to eliminate choline, an essential precursor of PtdCho, from the culture medium (Yao and Vance, 1988).

The effect of choline deficiency on PtdCho synthesis in neurons was measured. In a representative experiment, 9-d-old neurons cultured in 24-well plates were incubated with medium containing or lacking choline (11 μM). After 5 d,

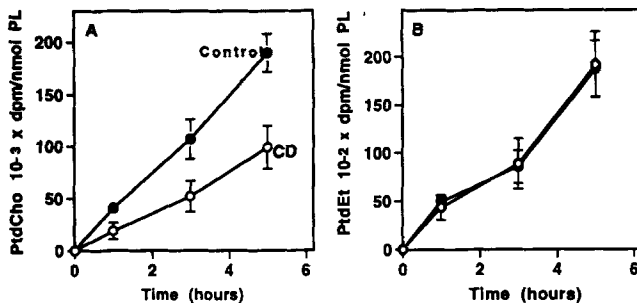


Figure 3. Choline deficiency inhibits PtdCho synthesis in sympathetic neurons. Neurons that had been cultured for 9 d in 24-well dishes were incubated with medium containing (control, *solid circles*) or lacking (CD, *open circles*) choline (11 μM). After 5 additional days [^3H]palmitate (10 $\mu\text{Ci/ml}$) was added. The cells were collected at the indicated times and lipids were extracted and separated by thin-layer chromatography. The data are given as dpm in PtdCho (A) or PtdEtn (B) per nmol of total phospholipid, and are averages \pm SD of four individual cultures. The experiment was repeated twice with similar results.

[^3H]palmitate (10 $\mu\text{Ci/ml}$) was added and the incorporation into PtdCho was measured (Fig. 3 A). In choline-deficient neurons the incorporation of [^3H]palmitate into PtdCho was reduced by $\sim 50\%$ compared to that in choline-supplemented neurons. The incorporation of [^3H]palmitate into PtdEtn was the same in choline-deficient and choline-supplemented cells (Fig. 3 B).

The effect of choline deficiency, and the corresponding reduction in PtdCho synthesis, on neurite growth was measured. Rat sympathetic neurons were plated in the center compartment of the dishes and allowed to grow for 11 d. At this time, neurites were washed from the left and right compartments. The cultures were incubated for the indicated times as follows: (a) without choline in the compartment containing cell bodies and proximal axons, but with choline in the distal axon-containing compartments; (b) without choline in the distal axon-containing compartments but with choline in the center compartment; (c) without choline in the center and side compartments; (d) with choline in all compartments. The extension of the neurites was measured after 2, 3, 4, 5, and 7 d (Fig. 4). In cultures that had been deprived of choline in the distal axon-containing compartments, neurite extension was 50–60% less than in cultures that had been grown in medium containing choline in these compartments. In contrast, when the cell body-containing compartment alone was deprived of choline for 7 d, neurite growth was equal to that of cultures which contained choline in all compartments (Fig. 4). The slopes of the curves which represent the growth rates (mm/d) are: for choline present in all compartments, 0.79; for choline in distal axon compartments but no choline in the center compartment, 0.79; for choline deficiency in the axon compartments but with choline present in the cell body-containing compartment, 0.42; for choline deficiency in all compartments, 0.48. The effect of choline deficiency on neurite growth appeared to be increasingly more severe as the duration of the choline deficiency in-

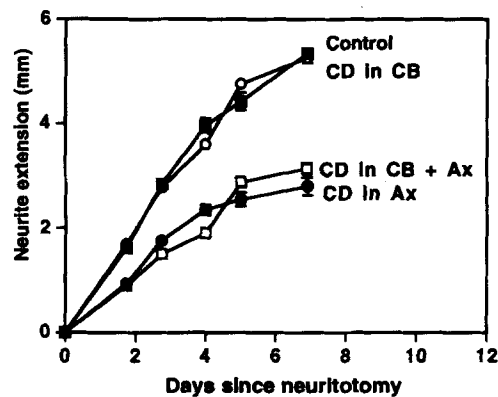


Figure 4. Choline deficiency (CD) in distal axons inhibits neurite growth. Neurons that had been cultured for 11 d were neuritotomized and incubated with the following media: without choline in the cell body-containing compartment (CD in CB, *open circles*), without choline in the distal axon-containing compartments (CD in Ax, *solid circles*), without choline in all compartments (CD in CB + Ax, *open squares*), or with choline in all compartments (Control, *solid squares*). Neurite extension was measured at the indicated times. Data are averages \pm SEM from measurement of 62–64 tracks. The experiment was repeated three times with similar results.

creased, as shown in the following experiment. Three groups of cultures were prepared. One group was preincubated for 5 d in choline-free medium, then axotomized and maintained in choline-free medium. A second group of cells was preincubated for 5 d with choline-supplemented medium, then axotomized and incubated with choline-free medium in all compartments. The third group of cells was given choline-supplemented medium throughout the experiment. Neurite extension 3 d after the neuritotomy was 2.77 ± 0.03 mm for cells incubated in choline-supplemented medium throughout the experiment, 0.88 ± 0.02 mm for choline-deficient cells preincubated in the presence of choline, and 0.55 ± 0.03 mm for choline-deficient cells preincubated without choline. This experiment suggests that the inhibitory effect of choline deficiency on axonal extension increases with time of incubation without choline.

In another experiment, we demonstrated that the detrimental effect of axonal choline deficiency on neurite extension was largely reversible upon supplementation with choline. When choline was added to the distal axon-containing compartments of cells that had been deprived of choline for three days, neurite growth resumed at almost the same rate as in cultures that had been incubated throughout the experiment with choline in the distal axon-containing compartments (data not shown).

One possible complicating factor in these experiments was that the culture medium added to the cell body-containing compartment contained 2.5% rat serum which may have been a source of some choline from choline-containing lipids such as PtdCho and lyso-PtdCho. Therefore, to ensure that the "choline-deficient" medium supplied to the cell body compartment was indeed free of choline in any form, the above experiments were repeated with delipidated rat serum and the results were the same (data not shown). In combination, these experiments suggested that inhibition of neurite growth in response to choline deficiency in the distal axons was largely due to the axons lacking choline, and presumably being unable to synthesize PtdCho at the normal rate. In contrast, when choline was eliminated from the medium supplied to the cell bodies, growth of distal neurites was not affected. Thus, although distal axons normally derive some of their PtdCho by anterograde transport from proximal axons/cell bodies, the PtdCho supplied from this source appears to be less important for normal axonal growth than is PtdCho made in the axons themselves.

Discussion

These studies demonstrate that for rat sympathetic neurons grown in the compartmented cultures, axonal synthesis of PtdCho, the major membrane lipid, is an important source of PtdCho required for axonal elongation. We have shown that axons are capable of the synthesis of $\sim 50\%$ of their own PtdCho. We can evaluate neither the amount of PtdCho made by proximal axons in the cell body-containing compartment nor the amount of PtdCho made in axons from [3 H]choline-labeled precursors imported from the cell bodies. Therefore, the actual magnitude of the contribution of axonal PtdCho synthesis to PtdCho in axons may be considerably greater than 50%.

Popov et al. (1993) have recently demonstrated that lipids are inserted into the growing neuron at the cell body and all

along the neurite, rather than exclusively at the growth cone. However, in their studies Popov et al. (1993) did not examine the site of synthesis of the membrane lipids. Since smooth endoplasmic reticulum is most likely distributed throughout axons we anticipate that PtdCho synthesis would occur all along the axons and not be restricted to the growth cones.

Because axonal PtdCho synthesis is a quantitatively important source of PtdCho in axons, we considered the possibility that PtdCho synthesis in axons may be required for axon elongation. We therefore tested the hypothesis that if PtdCho synthesis in axons were inhibited, neurite extension would decrease or cease. At the same time, we examined whether or not PtdCho synthesis in the cell bodies was required for axonal growth. PtdCho biosynthesis was specifically inhibited by removal of choline, a required precursor of PtdCho, from the culture medium. Previous studies in our laboratories have shown that when rats are fed a choline-deficient diet for 3 d, the rate of PtdCho synthesis in the liver is reduced by $\sim 70\%$ (Yao and Vance, 1988). As a result of the choline deficiency, translocation of the regulated and rate-limiting enzyme of PtdCho synthesis, CTP/phosphocholine cytidylyltransferase from its inactive pool in the cytosol to its active form on the endoplasmic reticulum membranes, is increased (Yao et al., 1990; Vance, 1990). However, under these conditions PtdCho synthesis does not proceed at the maximal rate because the substrate, choline, is lacking. This model is particularly useful for inhibition of PtdCho synthesis because the effect is highly specific. We have now shown that in rat sympathetic neurons cultured for 5 d in choline-free medium PtdCho biosynthesis was inhibited by 54% whereas choline deficiency did not affect the biosynthesis of PtdEtn.

The effect of choline deficiency on axonal elongation was examined. A lack of choline in the distal axon-containing compartments strongly impaired neurite growth, and the effect was the same during global choline deficiency. However, if choline were removed from the cell body-containing compartment alone, extension of distal neurites bathed in choline-containing medium continued normally. These results indicated that PtdCho made in the axons was required for axonal growth, and that synthesis of PtdCho in cell bodies alone was not sufficient for axon elongation. Whether the local synthesis of PtdCho in axons was required for providing the lipid for membrane expansion or for another role, such as supplying PtdCho as a precursor of lipid "signaling" molecules, is not known. It is noteworthy that even in the complete absence of choline from all compartments for several days PtdCho synthesis proceeded at a reduced rate and the neurites continued to elongate slowly, which may be due to the large pool of phosphocholine in cells of the nervous system (Millington and Wurtman, 1982), the reutilization of choline from PtdCho catabolism and/or a compensatory decrease in PtdCho catabolism.

The significance of phospholipid synthesis in axons for axonal elongation of sympathetic neurons *in vivo* is not clear. Although we have demonstrated that the axonal synthesis of fatty acids and phospholipids occurs, our data strongly indicate that cholesterol synthesis does not occur in the axons (Vance et al., 1994). Mahley and his coworkers (Mahley, 1988) have proposed a novel mechanism for delivery of cholesterol to axons. They have suggested that apolipoprotein E, which is produced by astrocytes, plays a major role in the

delivery of cholesterol to axons during regeneration after injury. However, the delivery of phospholipids to axons by this mechanism has not been reported. Therefore, one possible explanation for the finding that cholesterol synthesis is restricted to cell bodies, whereas phospholipid synthesis occurs in both cell bodies and axons, is that cholesterol can be delivered via apolipoprotein E to axons from the microenvironment of the axons, but phospholipids cannot. In addition, neurons might possess a highly efficient mechanism for delivery of cholesterol via anterograde axonal transport. The transfer of phospholipid precursors, and probably the phospholipids themselves, from axons to myelin has been described (Ledeen, 1985), but transport of phospholipids in the reverse direction (i.e., to axons) has not, to our knowledge, been demonstrated.

In conclusion, our data demonstrate that at least 50% of axonal PtdCho is made locally in axons. Moreover, inhibition of PtdCho synthesis in axons by choline deficiency inhibits neurite elongation. In contrast, synthesis of PtdCho in cell bodies and proximal axons does not appear to be essential for growth of axons.

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