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Compartmentalization of Choline and Acetylcholine Metabolism in Cultured Sympathetic Neurons[1]

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To determine the relative contribution of cell bodies and distal axons to the production of acetylcholine, we used retinoic acid to induce a cholinergic phenotype in compartmented cultures of rat sympathetic neurons. When [^{3}H]choline was given to cell bodies/proximal axons for 24 h, 98% of the radiolabel was recovered as choline, phosphocholine, CDP-choline and phosphatidylcholine, whereas only 1 to 2% of the radiolabel was incorporated into acetylcholine. Choline taken up by cell bodies and transported to axons is poorly utilized for acetylcholine biosynthesis. In contrast, when distal axons were supplied with [^{3}H]choline, 11% of the radiolabel was recovered in acetylcholine after 24 h, the remainder being incorporated into precursors/metabolites of phosphatidylcholine. The lack of acetylcholine synthesis in cell bodies/proximal axons could not be ascribed to an absence of choline acetyltransferase activity in this region of the neurons, since the specific activity of this enzyme was similar in cell bodies/proximal axons and distal axons. The rate of choline uptake by distal axons (15.3 ± 4.4 nmol/5 min/mg protein) was ~10-fold greater than by cell bodies/proximal axons (1.6 ± 0.8 nmol/5 min/mg protein). Moreover, choline uptake into distal axons was inhibited by 74.5% by hemicholinium-3, and by 80.1% by removal of Na^+ from the medium. In contrast, choline uptake by cell bodies/proximal axons was not significantly inhibited by hemicholinium-3 or Na^+ removal. These results suggest that the majority of axonal acetylcholine is synthesized in distal axons/axon terminals from choline taken up by a high-affinity choline transporter in distal axons.

Key words: acetylcholine, choline acetyltransferase, high affinity choline transport, phosphatidylcholine, retinoic acid.

When an action potential arrives at a cholinergic nerve terminal, synaptic vesicles fuse with the plasma membrane releasing acetylcholine (ACh) into the synaptic cleft where ACh binds to and activates ACh receptors on the post-synaptic cell. Receptor activation is rapidly terminated by hydrolysis of ACh to choline and acetate mediated by acetylcholinesterase. All of the released ACh can be hydrolyzed within a few hundred microseconds of its release. This rapid termination of the signal is required for the synapse to transmit signals at high frequency. Thus, cholinergic synaptic transmission requires a constant supply of ACh at the axon terminal. While it has been suggested that some of the ACh used for neurotransmission is synthesized in cell bodies and transported intracellularly to axon terminals (*1*), an alternative source is re-uptake of choline from the synaptic cleft and synthesis of ACh from choline and acetyl-CoA in axon terminals (*2, 3*). The ACh is subsequently transported into small synaptic vesicles by the vesicular ACh transporter (*4, 5*). In cholinergic neurons, a specialized high-affinity choline transport (HACT) system is expressed and is involved in the re-uptake of choline for ACh synthesis (*6–10*). The cholinergic HACT system is sodium-dependent, sensitive to inhibition by the choline analog hemicholinium-3 (HC-3), and its capacity is coupled to synaptic activity (*11–13*). HACT has been suggested to be the rate-limiting, and perhaps regulated, step of ACh synthesis (*11–15*). Denervation experiments (*16, 17*), and studies examining choline uptake in synaptosomal preparations (*6–8*), suggest that HACT is localized to axon terminals of cholinergic neurons.

Choline acetyltransferase (ChAT), the enzyme that catalyzes the synthesis of ACh, is, like most other neuronal proteins, synthesized in cell bodies and anterogradely transported along the axon to the axon terminal (*1*). In humans, four species of ChAT mRNA have been identified which produce proteins with a range of molecular masses between 66 and 70 kDa (*18*). Interestingly, the first intron of the ChAT gene contains the entire sequence of the vesicular ACh transporter gene (*19*). Histochemical and immunocytochemical studies have indicated that ChAT is distributed

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Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; HACT, high-affinity choline transport; HC-3, hemicholinium-3; PC, phosphatidylcholine; RA, retinoic acid.

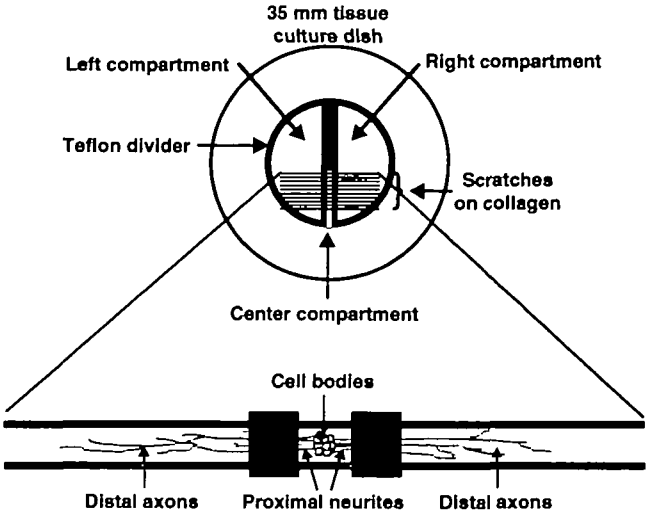

Fig. 1. **Schematic diagram of a compartmented culture of rat sympathetic neurons.** A compartmented culture and the enlargement of a single track are illustrated. The center compartment contains cell bodies and proximal axons, whereas the side compartments contain distal axons and axon terminals.

throughout all regions (cell body, dendrites, axon, and axon terminals) of cholinergic neurons (*20, 21*). In subcellular fractionation studies of brain homogenates, however, ChAT was found to be enriched in synaptosomal fractions, suggesting that the enzyme is concentrated in nerve terminals (*22, 23*). Moreover, Fonnum *et al.* (*24*) estimated that in cholinergic neurons of the hypoglossal nucleus, 2% of total ChAT activity is present in cell bodies, 42% in axons, and 56% in axon terminals. Some investigators have speculated that HACT and ChAT might be physically (*25*) or kinetically (*26*) coupled, since choline that is imported *via* HACT is rapidly acetylated to ACh. Other evidence, however, suggests that ACh can be synthesized from an intra-synaptosomal pool of choline (*27–29*), as well as from choline recently imported by HACT. Whether choline transported *via* HACT mixes with intracellular pools of choline, and whether ACh can be synthesized from intracellular pools of choline, have not been determined.

Our laboratories have used the compartmented culture system of sympathetic neurons (Fig. 1) as a model to study events occurring in distal axons/axon terminals independently of events occurring in cell bodies/proximal axons (*30, 31*). In some situations, sympathetic neurons undergo cholinergic differentiation upon innervation of their target (*32*). However, the contributions of choline in cell bodies and axons to acetylcholine biosynthesis have not been investigated. We now report utilization of the compartmented culture system to assess the relative contributions of cell bodies/proximal axons and distal axons to choline uptake and acetylcholine bioysynthesis. We have found that in sympathetic neurons in which a cholinergic phenotype was induced by retinoic acid treatment, the vast majority of axonal ACh is synthesized locally in distal axons from choline taken up by distal axons. Furthermore, although ChAT enzyme activity was distributed throughout all regions of the neurons (cell bodies/proximal neurites and distal axons), HACT was localized to distal axons. These results support the hypothesis that extracellular choline produced from the hydrolysis of ACh in the synaptic cleft is taken up into the axon terminals, where it is converted to ACh that is used for sustained synaptic transmission.

MATERIALS AND METHODS

[*methyl*-^{3}H]Choline chloride (81 Ci/mmol) was obtained from NEN Products (Boston, MA). Thin-layer chromatography plates (glass silica gel G plates, 0.25 mm thickness, and plastic cellulose plates, 0.1 mm thickness) were purchased from BDH (Edmonton, Canada). Eserine sulfate, HC-3, all-*trans*-retinoic acid, acetyl-CoA, ACh, choline, phosphocholine, CDP-choline and PC were purchased from Sigma (St. Louis, MO). All other reagents and chemicals were from Sigma or Fisher Scientific.

Preparation of Compartmented Cultures of Rat Sympathetic Neurons—Compartmented cultures of rat sympathetic neurons (Fig. 1) were prepared as described by Campenot (*30, 31*). Briefly, Falcon tissue culture dishes (35 mm) were coated with rat tail collagen and scratched so that 20 parallel tracks were formed on the dish surface. A Teflon divider (Tyler Research Instruments, Edmonton, AB) was sealed to the floor of the dish with silicone grease, thus partitioning the dish into three compartments. After trypsinization and mechanical dissociation of the superior cervical ganglia from newborn rats, the dissociated neurons were plated in the center compartments of compartmented dishes at a density of 0.6 to 0.8 ganglia/dish. Within 1 to 2 days axons elongated along the tracks, penetrated the silicone grease barrier beneath the dividers and entered the distal compartments. After 1 to 2 weeks of growth, the center compartments contained cell bodies and proximal axons, whereas the side compartments contained distal axons and axon terminals (Fig. 1). Each compartment maintains a separate fluid environment with virtually no flow between compartments (*33, 34*). L15 medium (GIBCO Laboratories, Grand Island, NY) without antibiotics, but supplemented with the additives prescribed by Hawrot and Patterson (*35*), including bicarbonate and methylcellulose, was used as the basal culture medium. For the first 6 days of growth, medium supplied to the center, cell body–containing compartments was supplemented with 2.5% rat serum (Lab Animal Services, University of Alberta), 1 mg/ml ascorbic acid, 10 mM cytosine arabinoside (to prevent growth of non-neuronal cells), and 10 ng/ml nerve growth factor (Alamone Labs, Jerusalem, Israel). Medium supplied to the side compartments contained 100 ng/ml nerve growth factor. After 6 days, the treatment with cytosine arabinoside and nerve growth factor was discontinued in the center compartments, and nerve growth factor was confined to the side compartments. Culture medium was routinely changed every 3 to 6 days.

Incorporation of [^{3}H]Choline into ACh, PC, and Intermediates of PC Biosynthesis—Neurons were cultured for 7 days in compartmented culture dishes, then treated with 5 μM retinoic acid in all three compartments for 9 days. At this time, the cultures were given 10 μCi/ml [*methyl*-^{3}H]-choline in either the center or side compartments, as indicated, and the incubation was continued for 5 or 24 h. The choline concentration of the medium was 10 μM. Cells were washed, and cellular material from the center and side compartments was separately harvested in Tris-buffered saline [20 mM Tris-HCl, 150 mM NaCl (pH 7.4)]. Both the

washing solution and the harvesting buffer contained 15 μg/ml eserine, an inhibitor of acetylcholinesterase. Aqueous and organic extracts of the cells were isolated according to the procedure of Folch *et al.* (*36*).

The water-soluble metabolites of choline were obtained by evaporation of the aqueous extract to dryness. The residue was dissolved in an aqueous solution containing 1 mg/ml choline and 1 mg/ml ACh as carriers. The samples were applied to cellulose thin-layer chromatography plates that had been spotted with CDP-choline (100 μg) and phosphocholine (100 μg) carriers. The metabolites were resolved in the solvent system *n*-butanol/95% ethanol/acetic acid/water 70:21:10:29 (v/v) according to Marchbanks and Israel (*37*). Bands corresponding to ACh, choline, phosphocholine. and CDP-choline were scraped, and radioactive incorporation was measured by liquid scintillation counting.

PC was isolated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/formic acid/water, 70:30:12:4:2 (v/v). The band corresponding to authentic PC was scraped, and radioactive incorporation measured.

Assay of Choline Transport into Neurons—Transport of choline into either 2.5-week-old compartmented cultures or non-compartmented cultures of neurons was measured essentially as described by Rylett *et al.* (*38*). Cultures were washed 3 times with Krebs-Ringer buffer [124 mM NaCl, 5.0 mM KCl, 1.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 20 mM NaOH, and 10 mM glucose (pH 7.4)]. Choline uptake was initiated by addition of Krebs-Ringer buffer containing 10 μCi/ml [*methyl*-^{3}H]choline (0.125 μM final choline concentration), and the incubation was continued for 5 min. Uptake was terminated by aspiration of the incubation buffer and rapid washing of the cells with ice-cold Krebs-Ringer buffer (completion of 3 washes required less than 20 s). Cellular material was lysed by addition of 0.1 M NaOH. Aliquots of the lysate were analyzed for protein content and radioactive incorporation. Parallel cultures were incubated with [^{3}H]-choline in the presence of 10 or 50 μM hemicholinium-3, or in sodium-free Krebs-Ringer buffer in which NaCl had been replaced iso-osmotically with LiCl, and HEPES-NaOH had been replaced with HEPES-Tris. The center and side compartments of compartmented cultures were assayed simultaneously. Choline transport was expressed as nmol of choline incorporated per mg protein per 5 min. In separate experiments, no transport of choline between compartments of culture dishes without cells was detected during 5 min of labeling with [^{3}H]choline added to either the center or side compartment.

Other Methods—The amount of protein was determined by a micro version of the bicinchoninic acid procedure (Pierce) using bovine serum albumin as the standard. Data expressed per mg of protein refer to total protein recovered from all compartments. ChAT activity was determined according to the method of Fonnum (*39*).

RESULTS

Choline Taken Up by Cell Bodies Is Poorly Incorporated into ACh—To determine whether choline taken up by cell bodies of cholinergic neurons is utilized for ACh synthesis, one-week-old compartmented cultures of sympathetic neurons were treated for 9 days in all compartments with 5 μM retinoic acid (RA). Previous work has shown that these conditions induce cholinergic differentiation of rat sympathetic neurons (*32, 40–42*). [^{3}H]Choline was then supplied for 24 h to either the center, cell body-containing compartments alone or to the side, distal axon-containing compartments alone. Cellular material from the center and side compartments was harvested separately, and the incorporation of [^{3}H]choline into metabolites of PC, as well as into ACh, was measured in each compartment. Other studies from our laboratories have demonstrated that in rat sympathetic neurons PC and sphingomyelin are synthesized from [^{3}H]choline in both cell bodies/proximal axons and distal axons (*43, 44*). Moreover, the axonal synthesis of PC is required for normal axonal elongation (*45, 46*). In cultures given [^{3}H]choline to the center compartments alone, 45.9% of the radiolabel taken up by cell bodies/proximal axons was anterogradely transported into the side compartments after 24 h. Very little incorporation of [^{3}H]choline into ACh was detected in either the center or side compartments (Fig. 2, top and bottom panels, respectively). In contrast, most of the radiolabel (~98%) was recovered in PC and its biosynthetic precursors/metabolites—choline, phosphocholine, and CDP-choline. Incorporation into sphingomyelin and lyso-PC was not measured in these studies. Thus, [^{3}H]-choline is readily taken up into cell bodies/proximal axons of RA-treated sympathetic neurons. Although a significant amount of the radiolabel transported into cell bodies/proximal axons was present in the cell body-containing compartment as [^{3}H]choline after 24 h, very little of this radiolabel was incorporated into ACh.

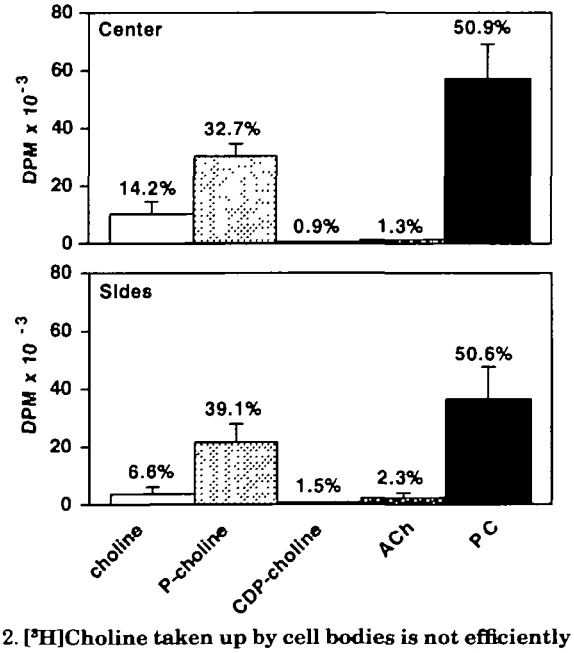

Fig. 2. **[^{3}H]Choline taken up by cell bodies is not efficiently incorporated into ACh.** Compartmented cultures of sympathetic neurons were treated with 5 μM RA for 9 days, after which the center compartments alone were incubated for 24 h with medium containing 10 μCi/ml [^{3}H]choline. The radiolabel incorporated into choline, phosphocholine (P-choline), CDP-choline, ACh and PC in the center (top panel) and side (bottom panel) compartments of 3 dishes combined was measured. Each value represents the mean ± SD of 3 separate groups of dishes. The value above each bar indicates the percentage of radioactivity in that product as a percentage of total radioactivity in that compartment.

In cultures in which [^{3}H]choline was supplied to the side compartments alone, 84.3% of the radiolabel taken up remained in the distal axons after 24 h, and 15.7% was retrogradely transported into the cell body-containing compartment. Of the radiolabel present in distal axons, 11% was in ACh, while the remainder was in choline (13.3%), phosphocholine (37.2%), CDP-choline (0.3%), and PC (38.2%) (Fig. 3, bottom panel). Essentially all of the radiolabel transported into the center compartment (98.3%) was present in PC and its biosynthetic intermediates (Fig. 3, top panel). Thus, distal axons of RA-treated sympathetic neurons utilize locally supplied extracellular choline for both ACh and PC synthesis. When RA-treated compartmented cultures of sympathetic neurons were labeled with [^{3}H]choline in either the center or side compartments for 5 h (not shown), the same profile of ^{3}H incorporation was observed, although less radiolabel was axonally transported in 5 h than in 24 h. Moreover, after 5 h compared to 24 h, proportionately more radiolabel was recovered in the water-soluble metabolites of PC than in PC itself. These experiments suggest that ACh is synthesized almost exclusively within distal axons of sympathetic neurons and that extracellular choline is utilized for ACh synthesis.

ChAT Activity Is Distributed throughout Rat Sympathetic Neurons—To determine the location of ChAT activity in RA-treated sympathetic neurons, one-week-old compartmented cultures were either left untreated or treated with 5 μM RA for 9 days. Cellular material from the center and side compartments was harvested separately and assayed for ChAT activity. RA treatment induced a 6.9-fold increase in the specific activity of ChAT in cellular material from the center compartment, and a 7.9-fold increase in distal axons (Fig. 4). The specific activity of ChAT in RA-treated distal axons was of the same order of magnitude as that in cell bodies/proximal axons. Thus, the finding that exogenous choline was incorporated into ACh only when the choline was supplied to distal axons (Figs. 2 and 3) cannot be explained by an absence of ChAT activity in cell bodies/proximal axons.

HACT Is Localized to Distal Axons of Sympathetic Neurons—A likely source of choline for ACh synthesis is recycled, extracellular choline that is transported into the neuron *via* HACT (*11–15*). Previous experiments have shown that HACT occurs in rat sympathetic neurons (*41*). Moreover, HACT is thought to be localized specifically to cholinergic axon terminals (*6–8, 16, 17, 47*). Consequently, one possible explanation for the finding that the synthesis of ACh is compartmentalized to distal axons is that HACT is restricted to distal axons. To test this hypothesis, we compared the uptake of choline into distal axons and cell bodies/proximal axons of compartmented cultures of sympathetic neurons. Distal axons and cell bodies/proximal axons were separately labeled for 5 min with [^{3}H]choline in the absence or presence of 10 μM HC-3, 50 μM HC-3, or in Na^+-free buffer. HACT is inhibited by HC-3, as well as by a deficiency of Na^+ (*11–15*). As shown in Fig. 5A, a small amount of choline was taken up by cell bodies/proximal axons of control neurons (1.6 ± 0.8 nmol/5 min/mg protein) and this uptake was unaffected by HC-3 or by a lack of Na^+. The rate of choline uptake (15.3 ± 4.4 nmol/5 min/mg protein) was ~10 times higher in distal axons than in cell bodies/proximal axons, and the transport of choline into distal axons was markedly inhibited by HC-3 and by the absence of Na^+ (Fig. 5A). These characteristics of choline uptake into distal axons were similar to those of the transport of choline into non-compartmented neuron cultures

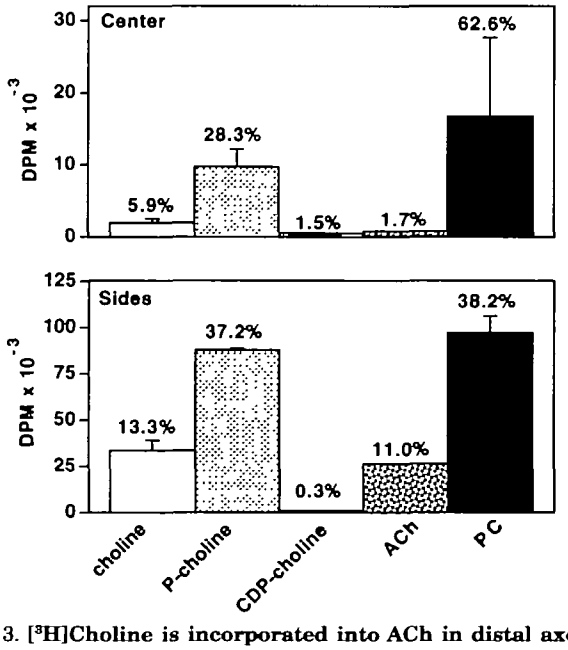

Fig. 3. **[^{3}H]Choline is incorporated into ACh in distal axons/axon terminals of rat sympathetic neurons.** Compartmented cultures of sympathetic neurons were incubated with 5 μM RA for 9 days, after which the side compartments alone were incubated for 24 h with medium containing 10 μCi/ml [*methyl*-^{3}H]choline. Radiolabel incorporated into choline, phosphocholine (P-choline), CDP-choline, ACh and PC in the center (top panel) and side (bottom panel) compartments of 3 dishes combined was measured. Each value represents the mean ± SD of 3 separate groups of dishes. The value above each bar indicates the percentage of radioactivity in that product as a percentage of total radioactivity in that compartment.

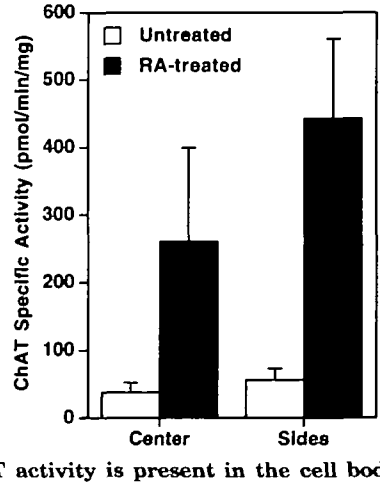

Fig. 4. **ChAT activity is present in the cell bodies/proximal neurites and distal axons of retinoic acid–treated sympathetic neurons.** Compartmented cultures of sympathetic neurons were either left untreated (open bars) or treated with 5 μM RA for 9 days (solid bars). Cellular material from the center and side compartments was harvested separately, and ChAT activity (pmoles ACh formed/min/mg protein) was measured. Values are means ± SD of 3 separate groups of dishes.

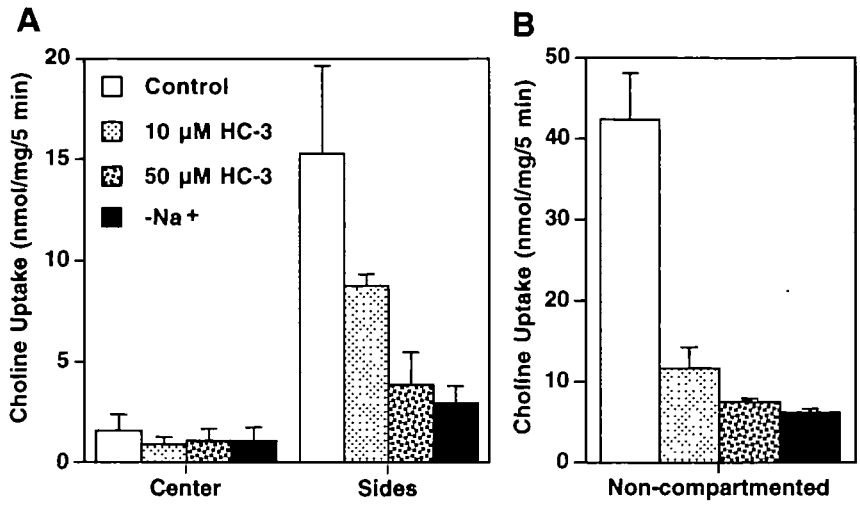

Fig. 5. **HACT is localized to distal axons/axon terminals of rat sympathetic neurons.** Compartmented and non-compartmented cultures of sympathetic neurons were incubated for 5 min with 10 μCi/ml [*methyl*-^{3}H]choline. Total radioactivity incorporated into distal axons and cell bodies/proximal axons of compartmented cultures (panel A) or non-compartmented cultures (panel B) was determined. Choline uptake in the presence of 10 μM HC-3, 50 μM HC-3, or in Na^+-free buffer was determined in parallel. Values represent means ± SD of 4 individual cultures.

(Fig. 5B). These observations demonstrate that HACT is highly enriched in distal axons/axon terminals compared to cell bodies/proximal axons.

DISCUSSION

The results presented herein suggest that choline that is transported into cell bodies/proximal axons of RA-treated sympathetic neurons is poorly utilized for ACh synthesis either locally or after anterograde transport from cell bodies into distal axons/axon terminals. Despite ChAT having a similar specific activity in cell bodies/proximal axons and distal axons, significant ACh synthesis was observed only in distal axons, and only when the distal axons were supplied with exogenous choline. These observations imply that the bulk of choline used for ACh synthesis does not originate from choline transported intracellularly from cell bodies/proximal axons, but instead is derived directly from the uptake of extracellular choline into distal axons. Similar conclusions were reached by Suidan and Tolkovsky (*48*), who reported that ACh labeled from exogenously-added choline was found mainly in neurites. The interpretation of the results of these investigators is, however, less certain than the present study for several reasons. First, they could not distinguish between the uptake of choline by axons and cell bodies. Second, the site of synthesis of the labeled choline metabolites (*i.e.*, cell bodies or distal axons) was not established. Third, the purity of the cell body and axon preparations was not discussed. Finally, the explant cultures used were not pure.

Our observations raise the question of whether the choline moiety is transported along axons as choline *per se* or as a metabolite of choline. This question is important because the lack of incorporation of [^{3}H]choline given to cell bodies into ACh in distal axons could, perhaps, be explained if a metabolite of choline, rather than choline *per se*, were anterogradely transported. For example, choline transported into the cell bodies/proximal axons might have been rapidly phosphorylated to phosphocholine (*43, 44, 49*) and transported in this form. Consequently, this pool of (phospho)choline might not be available for ACh synthesis in distal axons. However, our data (Fig. 2, bottom panel) show that significant amounts of [^{3}H]choline are present in distal axons when [^{3}H]choline is given to the center compartment. For example, 6.6% of the radiolabel from [^{3}H]-choline added to the center compartment was recovered in choline in the side compartments after a 24-h incubation; after a 5-h incubation of the center compartment with [^{3}H]-choline, 33.3% of the label in distal axons was recovered as choline, but only 2.7% as ACh. Thus, although [^{3}H]choline accumulates in distal axons of compartmented cultures supplied with [^{3}H]choline in the cell body/proximal axon-containing compartments, this pool of choline is poorly incorporated into ACh in either cell bodies/proximal axons or distal axons.

Another possible explanation for the lack of significant incorporation of [^{3}H]choline supplied to cell bodies/proximal axons into ACh in distal axons is that when [^{3}H]choline is taken up by cell bodies/proximal axons and transported into distal axons, its radioactivity might be diluted by a large pool of unlabeled choline in distal axons. This situation would result in a pool of choline with a low specific radioactivity in distal axons. Therefore, even though ACh synthesis might have occurred, little radioactivity would have been detected in ACh. A corollary of this scenario, however, is that the amount of [^{3}H]choline incorporated into PC in distal axons would also be low. As shown in Fig. 2, however, the [^{3}H]choline that was taken up by cell bodies was robustly incorporated into PC and intermediates of PC synthesis in distal axons.

Localization of ChAT Activity and HACT in Neurons—Our data show that the specific activity of ChAT is of a similar magnitude in cell bodies/proximal axons and distal axons (Fig. 4). However, extracellular [^{3}H]choline was incorporated into ACh to a much greater extent when added to distal axons (Fig. 3) than when added to cell bodies/proximal axons (Fig. 2). A likely explanation for these observations is that ACh is synthesized primarily from choline that is taken up *via* HACT (*11–15*). Our data show that HACT is restricted to distal axons and is not detected in cell bodies (Fig. 5). Consequently, even though ChAT activity is present in all regions of the neurons, the substrate, choline, is transported via HACT only in distal axons. Thus, ACh is synthesized almost entirely in distal axons.

Previous experiments have suggested that HACT is localized to cholinergic nerve terminals. For example,

HACT activity was enriched in synaptosomal preparations (*6–8, 47*), and a loss of HACT was observed in denervation experiments (*16, 17*). That HACT is an important component of ACh synthesis is also indicated by studies in which inhibition of HACT [for example, by removal of extracellular Na^+ or by treatment with HC-3 or choline mustard aziridinium ion, an irreversible inhibitor of HACT (*50*)], also inhibited ACh synthesis (*7, 32, 47, 50, 51*). Moreover, HACT is modulated by neuronal activity, since the rate of choline transport is increased during periods of synaptic activity (*52–55*). Thus, our data are consistent with the hypothesis that HACT plays a key role in the synthesis and maintenance of ACh stores (*12, 13*). Previous results from our laboratory have also suggested that choline transported into sympathetic neurons *via* HACT is utilized both for ACh and PC synthesis (*32*).

Our data do not exclude the possibility that some ACh is made in cell bodies/proximal axons but is rapidly hydrolyzed by acetylcholinesterase. Even if this were the case, however, the conclusion that cell bodies do not make significant amounts of the ACh that is available for release at axon terminals, remains valid.

Compartmentalization of Choline Pools within Sympathetic Neurons—Our data support the idea that (at least) two distinct intracellular pools of choline exist in sympathetic neurons: one pool that is restricted to axons and is supplied by HACT for utilization in ACh and PC synthesis in distal axons/axon terminals, and the other pool that is used exclusively for PC, but not ACh, synthesis, throughout all parts of the neuron. The idea that metabolically distinct pools of choline exist in neurons is also suggested by Spence and coworkers (*56*), who proposed that substrate channeling of the intermediates of PC biosynthesis occurs in rat glioma cells. Their studies provided evidence that the intermediates of the PC biosynthetic pathway are not freely diffusible within the cell, but that the uptake of exogenously-added choline is channeled towards PC synthesis. The integrity of the channeling was found to be dependent on intracellular Ca^{2+} levels (*57, 58*). Since a change in the intracellular Ca^{2+} level in nerve terminals is an integral aspect of cholinergic synaptic transmission, and since choline taken up by HACT is utilized for both ACh and PC synthesis (*32*), it would be interesting to determine if substrate channeling of choline specifically for the biosynthesis of PC or ACh occurs in cholinergic neurons.

Our results do not exclude the possibility that under certain conditions (*e.g.*, when the extracellular supply of choline is limited), intracellular choline is utilized for ACh synthesis (*59*). Evidence has been provided that ACh synthesis from choline derived from PC degradation occurs in neuronal cell lines (*60, 61*). Choline, liberated from PC by the action of phospholipases might, therefore, be released intracellularly into the cytosol and used for ACh synthesis (*61*). Repeated depolarization of striatal slices (*59, 62*) decreases the amount of membrane PC, an effect that can be reversed by choline administration (*62*). It has been hypothesized that depletion of membrane PC as a source of choline for ACh synthesis might contribute to the selective vulnerability of cholinergic neurons in Alzheimer's disease and other cholinergic neurodegenerative disorders (*63*).

In conclusion, our results indicate that in RA-treated rat sympathetic neurons, choline that is used for ACh synthesis originates primarily from extracellular choline taken up by distal axons/axon terminals via HACT. In contrast, choline taken up by cell bodies is poorly incorporated into ACh either within the cell bodies *per se* or after anterograde transport into distal axons/axon terminals. In agreement with this concept, we have found that HACT, which is thought to supply the choline utilized for ACh synthesis, is localized to distal axons/axon terminals of RA-treated rat sympathetic neurons. These results suggest that recycling of choline in the synapse is a major source of choline for ACh synthesis.

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