Electron Microscope Localization of Acetylcholinesterase and Butyrylcholinesterase in the Superior Cervical Ganglion of the Cat

II. Preganglionically Denervated Ganglion

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ABSTRACT

Cat superior cervical ganglia (SCG), denervated preganglionically 6–8 d previously, were stained for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) by the bis-(thioacetoxy)aurate (I), or Au(TA)2, method and compared by electron microscopy with normal SCG described previously (Davis, R., and G. B. Koelle. 1978. J. Cell Biol. 78:785–809). In confirmation of earlier light microscopic findings by the highly specific copper thiocholine method, there was nearly a total disappearance of AChE from the ganglion; no myelinated or unmyelinated axons with AChE-stained axolemmas were found, and only occasional traces of AChE staining were noted at dendritic and perikaryonal plasma membranes. Considerable staining for BuChE persisted at the latter sites, however. As in the normal SCG, physostigmine-resistant staining, caused by noncholinesterase enzymes plus the possible presence of very low concentrations of AChE or BuChE, was noted at external mitochondrial membranes, elements of the endoplasmic reticulum of neurites and Schwann cells, and also in lysosomes. These findings confirm the previous identification of AChE-stained myelinated fibers in the normal SCG as preganglionic and of the unstained myelinated fibers as postganglionic. It is proposed that the maintenance of AChE at postsynaptic sites in normal ganglia is caused by the release of a trophic factor(s) from presynaptic terminals. The source of the postsynaptic BuChE, which is apparently completely absent from the endoplasmic reticulum of the ganglion cells, remains unexplained.

In a preceding paper (1), we have described the electron microscopic (EM) localization of acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7; AChE) and butyrylcholinesterase (acylcholine acyl-hydrolase; pseudocholinesterase; EC 3.1.1.8; BuChE) in the normal cat superior cervical ganglion (SCG) by the bis-(thioacetoxy)aurate (I), or Au(TA)2, method combined with the use of selective cholinesterase inhibitors in vivo and in vitro to achieve specificity. It was concluded that AChE is located along the entire lengths of the axolemmas of the preganglionic cholinergic axons down to and including their terminal varicosities at the synaptic junctions. The enzyme was found also in occasional synaptic vesicles within the terminals. In addition, AChE was localized extensively at the postsynaptic ganglion cell perikaryonal and dendritic plasma membranes. BuChE was absent from the preganglionic axons, but present at the ganglion cell plasmalemmas at sites identical to those of AChE. Neither AChE nor BuChE appeared to be present in appreciable concentrations on the plasmalemmas or in the cytoplasm of Schwann cells. Intracellular staining of neurons and their processes and Schwann cells was noted at mitochondria, membranes of the endoplasmic reticulum (ER), and lysosomes; staining at these sites was not eliminated by physostigmine and is, therefore, predominantly nonspecific; however, it could not be stated conclusively whether small amounts of AChE and/or BuChE were present there in addition to other esterase(s). It was also observed that in identifiable
preganglionic axonal processes the inner folded as well as the outer membranes of mitochondria exhibited consistent, non-cholinesterase (i.e., physostigmine-resistant) staining, whereas only the outer membranes of mitochondria in dendrites, perikarya, and Schwann cells were similarly stained. Although no reason could be offered for this difference, it sometimes provided a useful criterion for the identification of isolated neurites.

The finding by direct EM visualization of both AChE and BuChE activities at the plasma membranes of normal ganglion cells and their dendrites is at variance with conclusions reached from indirect evidence several years earlier on the basis of quantitative determinations (2, 3) and light microscopic (LM) histochemical comparisons of normal and preganglionically denervated cat SCG by the highly specific copper thiocyanate method (4, 5). The limited resolution afforded by LM did not permit distinction between pre- and postsynaptic staining in the normal neuropil. The thiocyanate esters are not satisfactory substrates for high-resolution EM because of their poor penetration of tissues and the crystalline reaction product's tendency to diffusion and relatively low electron density.

This report describes the distributions of AChE and BuChE in preganglionically denervated SCG stained by the Au(TA)2 method. The results confirm the nearly complete disappearance of AChE in contrast to the retention of most of the BuChE after denervation, as was indicated by the aforementioned LM studies, and also the EM identification of the AChE-stained myelinated fibers in the normal ganglion as preganglionic.

MATERIALS AND METHODS

The procedures employed for preganglionic denervation of the SCG, selective inactivation of AChE and BuChE in vivo, fixation, the Au(TA)2 histochemical method, physostigmine controls, and copper thiocyanate staining of sections for examination by LM as additional controls have been previously described (1). Ganglia were denervated by resection of a 1.5-2.0-cm segment of the preganglionic cervical sympathetic trunk 6-8 d before removal. For this and the previous study (1), a total of nine normal and 11 denervated SCG were examined, including the preganglionic and postsympathetic trunks of two normal and two denervated ganglia, for the localization of AChE. For the localization of BuChE, six normal and seven denervated SCG were studied, including preganglionic and postganglionic trunks of two normal and two denervated ganglia.

The Au(TA)2 method provides a high degree of resolution for the localization of AChE, BuChE, and other esterases but lacks specificity. Accordingly, essentially complete, selective irreversible inactivation of either enzyme was produced in vivo before the removal and fixation of tissues: BuChE, by the intravenous injection of tetramonoisopropyl pyrophosphorotetramide (iso-OMPA), 3 μmol/kg, and AChE, by the intravenous injection of 10-(α-diethylaminopropionyl) phe-}

nothiazine HCl (Astra 1397), 100 μmol/kg, which reversibly protects BuChE but not AChE against inactivation by the subsequent injection of isopropyl methylphosphonofluoridate (sarin), 2.0 μmol/kg (1). The SCG was cut into 1-mm cubes and the preganglionic and postganglionic internal carotid trunks into 1-mm lengths; these were fixed for 6 h at 2°C in 4% formaldehyde in Krebs-Ringer-calcium solution, then rinsed in Krebs-Ringer-calcium solution overnight. During the 6-h incubation period in Au(TA)2 medium at 5°C, with hourly replacement by fresh medium, control blocks of tissue were incubated similarly in medium also containing physostigmine, 3·10−3 M, which produces selective inhibition of both AChE and BuChE but leaves intact any nonspecific esterases. Sites of AChE activity can be identified with certainty only in tissues in which BuChE has been inactivated in vivo and where staining is blocked by incorporation of physostigmine in vitro; however, the persistence of staining at various sites in the physostigmine controls does not preclude the possible presence of low concentrations of AChE in addition to other esterases. The same principles apply to the identification of sites of definite and questionable staining for BuChE in tissues in which AChE has been inactivated selectively in vivo. As in the preceding study of the normal SCG (1), the essentially complete, selective inactivation of either BuChE or AChE in vivo with retention of the activity of the other enzyme was confirmed in each experiment by staining frozen sections of the stellate ganglion (StG) from the same animal by the highly specific CuThCh method (4) for examination by LM.

RESULTS

Ganglion

In contrast to the pattern observed in the normal SCG, where intense staining for AChE was present at the axolemmas of the myelinated (Fig. 1 a) and unmyelinated (Fig. 1b) portions of presynaptic axons and their terminals (Fig. 1c and d), the denervated ganglion was nearly devoid of AChE staining (Fig. 2). As in the normal SCG, occasional intact myelinated fibers were noted in which the axolemma did not stain for AChE (Fig. 2a). The traces of staining that were present at postsynaptic membranes and in varying intensities at other sites, including the ER of ganglion and Schwann sheath cells, lysosomes, and mitochondria (Fig. 2a and b), were essentially indistinguishable from those in the corresponding physostigmine controls (Fig. 2c); hence, such staining is attributable largely to unspecified esterases, but the presence in addition of small amounts of AChE cannot be excluded. As was noted within dendrites in the preceding study of the normal SCG (1), only the outer membranes of mitochondria were

![Figure 1](image-url)
stained. Within the ganglion, there was little or no evidence of the remnants of degenerated preganglionic fibers, even as early as 6 d after denervation.

Contrary to the nearly complete disappearance of ganglionic AchE that followed preganglionic denervation, there was relatively little change in the intensity or distribution of staining for BuChE. As in the normal ganglion (Fig. 3a), intense, physostigmine-sensitive staining was present at the postsynaptic dendritic and perikaryonal membranes of the denervated ganglia (Fig. 3b); faint physostigmine-resistant staining (Fig. 3c), representing nonspecific esterases, occurred at the same sites as in the physostigmine control denervated SCG stained for AchE.

**Preganglionic Trunk**

Associated with the normal SCG, uniform staining for AchE was noted consistently at the axolemmas of myelinated fibers of the preganglionic cervical sympathetic trunk; in addition, a considerable number (approximately one-half the total) of unmyelinated, unstained neurites were present (Fig. 4a). There was no evidence of BuChE staining in the normal preganglionic trunk (not shown). After preganglionic denervation for 6–8 d, all myelinated axons exhibited pronounced degeneration and no longer stained for AchE (Fig. 4b). Axolysis was essentially complete without recognizable traces of axolemma or intracellular organelles. Myelin sheaths were usually collapsed with lamellae dissociating. The numerous small unmyelinated neurites had almost completely disappeared, presumably also degenerated; however, a few vacuolated areas of Schwann cell (or possibly macrophage) cytoplasm may represent their remnants (Fig. 4c). Rarely, an unmyelinated neurite with normal appearance persisted after denervation (4d). The significance of this is taken up in the Discussion. Traces of staining at other sites, including intraaxonal tubules, mitochondria, and lysosomes, was identical with that in physostigmine controls (not shown).

**Postganglionic Trunk**

Sections of the postganglionic trunk from portions immediately cranial to normal and denervated SCG were practically identical, both morphologically and histochemically. Although no quantitative comparison was made, inspection of a large number of sections indicated that approximately one-tenth of the postganglionic axons were myelinated and the remainder unmyelinated; no staining for AchE was detected in the axolemmas of either type in normal (Fig. 5a) or denervated ganglia. Scattered staining of the mitochondria, axonal tubules, lysosomes, and ER membranes of the axons and Schwann sheath cells was identical in the physostigmine controls (Fig. 5b). The same negative pattern was found in sections of the postganglionic trunk of normal and denervated SCG stained for BuChE and their corresponding physostigmine controls (not shown).

**DISCUSSION**

The absence of AchE-stained myelinated fibers and axonal terminals from the denervated SCG is consistent with earlier LM (6) and EM (7, 8) studies in which it was shown that essentially all the preganglionic axons and their terminals in the SCG of the cat degenerate to the point of nearly complete disappearance within 6 or 7 d after sectioning of the cervical sympathetic trunk.

The present findings of the total disappearance of AchE and retention of most of the BuChE of the neuropil of the SCG after preganglionic denervation are in agreement with earlier LM observations with the highly specific CTHch method (4, 5). It was concluded from the earlier results that AchE is normally confined to the preganglionic fibers and their terminals. However, as was found by direct observation here and in the previous EM study (1), in the normal ganglion, AchE is distributed even more extensively at postsynaptic than at presynaptic sites. Its disappearance from the former sites a few days after preganglionic denervation raises the question of how the enzyme is maintained there under normal conditions. Two possible primary explanations can be offered: (a) AchE is continually released from the presynaptic terminals, as has been demonstrated at various sites (9–11), and diffuses through the intercellular spaces to become localized at the membranes of the ganglion cells, or (b) a trophic factor essential for the synthesis and maintenance of AchE at postsynaptic sites is released by the presynaptic terminals. Several factors appear to mitigate against the former possibility. It is unlikely that large molecules are released in significant amounts from preganglionic terminals (12); as noted above, the distribution of AchE at the dendritic and perikaryonal membranes was extremely regular and intense at sites far remote from areas of synaptic contact, and, hence, it would have entailed the extensive migration of considerable amounts of AchE to be consistent with this explanation. Finally, no definitive staining for

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**Figure 2** Fig. 2a–c shows cat SCG that had been denervated 6–7 d before staining for AchE by the Au(TA)₂ method. Fig. 2c also had physostigmine treatment in vitro to inactivate completely any remaining AchE. (a) An occasional myelinated axon (A) remains after denervation. It may contain some staining of mitochondria (Ma) or ER membranes (ERa), but its axolemma is not stained. The small dendrites nearby also contain stained mitochondria (Md) and ER membranes (ERd), but plasma membranes are unstained. (b) A portion of a perikaryon with moderately stained mitochondrial (Mp) and ER (ERp) membranes, but whose plasmalemma (arrows) is practically devoid of stain. Nearby, small dendrites exhibit some staining of mitochondria (Md) and ER membranes (ERd), but their plasma membranes are unstained. Other items labeled include a stained Schwann cell mitochondrion (Ms) and heavily stained lysosomes (Ly). (c) After in vitro physostigmine treatment, some nonspecific staining persists. In this micrograph, two dendrites (D₁ and D₂) show unstained plasma membranes but light staining of their outer mitochondrial membranes (Md). Some staining also persists in ER membranes (ERs) of Schwann cells. A perikaryon (P) also shows an unstained plasma membrane, though some stain remains on intracellular organelles. Also labeled are a lysosome (Ly) and a Schwann cell nucleus (N). a and b, × 31,000; c, × 31,500. Bar, 0.5 μm.
AChE was observed at intercellular sites (the end product which appears here occasionally is considered more probably a result of diffusion). The alternative explanation is in keeping with several recent reports of the maintenance of AChE at the motor endplate of skeletal muscle in culture by a peptide trophic factor released by the motor fibers (13–16). One additional factor to be considered is that activation of the ganglion cells by preganglionic impulses may contribute to the maintenance of AChE at their membranes, as has been shown recently for skeletal muscle (17).

The presence of BuChE at the dendritic and perikaryonal membranes of the ganglion cells, which largely persisted after preganglionic denervation, also raises a question which cannot be answered readily, namely the source of the enzyme. As noted in Materials and Methods, with the Au(TA)₂₅ procedure employed, the positive identification of AChE or BuChE at any site is dependent upon its complete suppression by 3 · 10⁻⁶ M physostigmine. Hence, the presence of residual staining of the ER of ganglion cells in physostigmine controls of tissues stained for either AChE or BuChE precludes the positive identification there of either enzyme but does not exclude its presence in low concentration in addition to other esterases. In the earlier LM studies of the SCG by the copper thiocholine method, with prolonged incubation light staining for AChE was found in the cytoplasm (presumably the ER) of all ganglion cells in both normal and preganglionically denervated cat SCG (4). However, the cytoplasm in ganglion cells stained for BuChE remained completely blank, which was one of the reasons for ascribing its location to the capsular glial cells. If the BuChE of the plasmalemma of the ganglion cells is not synthesized within their rough ER, what is its site of origin? Possibilities include: (a) the uptake of BuChE from the plasma, (b) its synthesis within the capsular glial cells and subsequent transposition to the plasmalemma of the ganglion cells, because the ER of the former also showed physostigmine-resistant staining for BuChE, or (c) the synthesis by the ganglion cells of a precursor of BuChE, to which it is converted at its ultimate sites of localization. The last mentioned proposal is a corollary to the working hypothesis that the ganglionic BuChE is in turn a precursor of AChE. This was originally suggested by the observation of the identical localization of the two enzymes on the ganglion cells of the cat SCG (1). It is supported by the findings that the selective alkylphosphorylation of BuChE by iso-OMPA causes a decrease in the rate of regeneration of ganglionic AChE in vivo after its inactivation by sarin (18), and an increase in the rate of disappearance of AChE in ganglionic homogenates in vitro (19).

The absence of significant staining for AChE or BuChE in the adrenergic fibers of the postganglionic trunk of both normal and denervated SCG is consistent with LM (4) and EM (20) observations in the cat by the CuThCh method. The scattered, unstained myelinated fibers noted here as well as previously in the normal SCG (1), which constituted ~10% of the fibers of the postganglionic trunk, probably represent adrenergic postganglionic axons (21, 22); according to classical reports (6, 23), afferent fibers, if present at all, must be extremely rare in the cat SCG.

As would be expected, the myelinated fibers of the preganglionic trunk showed considerable degeneration and also loss of their normal axolemmal staining for AChE after decentralization. The normal preganglionic trunk was also found to contain a considerable proportion of unmyelinated fibers that did not stain for AChE. After denervation, most of these disappeared but a very small number persisted. On the basis of the absence of AChE staining from postganglionic adrenergic fibers, noted above, and a considerable number of anatomical and physiological studies (for review see reference 24), it is likely that all these likewise represented adrenergic postganglionic fibers. The fibers that disappeared after denervation presumably originated from centrally displaced ganglion cells, located proximal to the site of sectioning; the small number that survived denervation probably arose from cells within the trunk distal to the site of sectioning or from cells within or near the ganglion whose axons pursued a retrograde course toward the central nervous system.

It was noted in the preceding study of the normal SCG (1) that both inner folded and outer mitochondrial membranes exhibited physostigmine-resistant staining in unmyelinated segments of preganglionic axons and identifiable portions of their terminals, whereas in definitely identifiable dendrites and at other postsynaptic sites only the outer mitochondrial membranes were similarly stained. The conclusion that this feature may provide an empirical means to aid in the identification of isolated neurites in the cat SCG is upheld by the present observations, where essentially no mitochondria with staining of the folded inner membranes were detected in the denervated ganglia. No explanation can be offered for this interesting observation.

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**FIGURE 3.** Fig. 3 a–c were stained by the Au(TA)₂₅ method after administration of Astra 1397/sarin in vivo to inactivate AChE, and, therefore, demonstrate BuChE and possible nonspecific esterases. In addition, b had been denervated 8 d before incubation and c (also denervated) was treated with physostigmine to inactivate BuChE, to demonstrate nonspecific esterases. (a) In the normal cat SCG, the plasma membranes of dendrites (D₁ and D₂) and also of the perikarya (not shown) are markedly stained for BuChE. Dendritic mitochondria (Md) and ER membranes (ERd) and also the same organelles of Schwann cells (Ms and ERs) show some physostigmine resistant staining (see Fig. 3 c). An axon terminal (A) with characteristic staining of inner and outer mitochondrial membranes (Ma) exhibits an unstained axolemma. (b) After denervation, the distribution of BuChE is little changed, i.e., very prominent on the plasma membranes of the perikaryon (P) and dendrites (D). Moderate staining may be present on mitochondria and ER membranes of dendrites, perikarya, or Schwann cells (unlabeled). Small axon terminals or varicosities are absent. (c) Physostigmine treatment in vitro leaves dendritic (D) and perikaryal (not shown) plasma membranes virtually unstained, but allows nonspecific staining to persist on outer mitochondrial membranes of dendrites (Md) or Schwann cells (Ms). The mitochondrion labeled (Md) clearly demonstrates that, when staining is not heavy and diffusion is limited, it is present only on the outer membrane. A few ER membranes (ERs) and possibly some Golgi membranes (G) are also stained in a Schwann cell. a and b, x 31,500; c, x 50,000. Bar, 0.5 μm.
**FIGURE 5** Fig. 5a and b shows the internal carotid branch of the postganglionic trunk stained for AChE by the Au(TA)₂ method in the normal trunk (a) and also after preganglionic denervation plus in vitro physostigmine treatment (b). (a) This illustrates a myelinated axon (A₁) and an unmyelinated (A₂) axon, both with unstained axolemmas (arrows). However, axonal mitochondria (Ma) and ER membranes (ERa) exhibit some staining. A few stained Schwann cell ER membranes (ERs) are also present. (b) After denervation and in vitro physostigmine treatment, a myelinated (A₁) and several unmyelinated axons (A₂) exhibit unstained axolemmas (arrows). However, nonspecific esterases persist in ER membranes (ERa) and Schwann cell mitochondria (Ms). x31,500. Bar, 0.5 μm.

**FIGURE 4** Fig. 4a–d shows the preganglionic cervical sympathetic trunk, illustrating AChE staining in the normal trunk (a) and the lack of AChE staining (b) or BuChE staining (c and d) as well as neuronal degeneration after nerve resection. (a) The axolemma of a myelinated axon (A₁) is well stained for AChE (arrow), but the plasma membrane of a small neurite (A₂), considered to be a ganglion cell axon, is unstained. (b) 6 d after nerve resection, a figure of delaminating and collapsed myelin (My) remains after the axon has completely degenerated. Vacuolated Schwann cell cytoplasm surrounds the myelin remnants. (c) Some normal appearing Schwann cell cytoplasm (Sc₁) is adjacent to a mass of highly vacuolated cytoplasm (Sc₂). It is considered that these vacuoles (arrow) contain remnants of nearly completely degenerated small neurites (ganglion cell axons) that were numerous in the normal ganglion. (d) This shows the delaminating remnants of a myelinated fiber (My) plus a rarely found normal appearing unmyelinated neurite (A), whose significance is considered in the Discussion. a, × 50,500; b–d, × 31,500. Bar, 0.5 μm.
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