Localization of choline acetyltransferase in rat peripheral sympathetic neurons and its coexistence with nitric oxide synthase and neuropeptides

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ABSTRACT Indirect immunofluorescence methods using a mouse monoclonal antibody raised to rat choline acetyltransferase (ChAT) revealed dense networks of ChAT-immunoreactive fibers in the superior cervical ganglion, the stellate ganglion, and the celiac superior mesenteric ganglion of the rat. Numerous and single ChAT-immunoreactive cell bodies were observed in the stellate and superior cervical ganglia, respectively. The majority of ChAT-immunoreactive fibers in the stellate and superior cervical ganglia were nitric oxide synthase (NOS) positive. Some ChAT-immunoreactive fibers contained enkephalin-like immunoreactivity. Virtually all ChAT-positive cell bodies in the stellate ganglion were vasoactive intestinal polypeptide (VIP)-positive, and some were calcitonin gene-related peptide (CGRP)-positive. After transsection of the cervical sympathetic trunk almost all ChAT- and NOS-positive fibers and most enkephalin- and CGRP-positive fibers disappeared in the superior cervical ganglion. The results suggest that most preganglionic fibers are cholinergic and that the majority of these in addition can release nitric oxide, some enkephalin, and a few CGRP. Acetylcholine, VIP, and CGRP are coexisting messenger molecules in some postganglionic sympathetic neurons.

Acetylcholine (ACh) and norepinephrine are the classic transmitters in parasympathetic and preganglionic sympathetic neurons and in postganglionic sympathetic neurons, respectively. The noradrenergic neurons could early on be demonstrated with a specific histochemical fluorescence method developed by Falt et al. (1). Koelle and Friedewald (2) described a histochemical technique for detecting ACh esterase, the enzyme responsible for breakdown of ACh, but this method is not specific for cholinergic neurons (3). Therefore, the purification of the ACh-synthesizing enzyme, choline acetyltransferase (ChAT), by several groups offered considerable promise for tracing cholinergic systems in brain and periphery with immunochemistry, and several mapping studies on central cholinergic systems have been published (see ref. 4). In the peripheral nervous system Costa and coworkers (5, 6) visualized cholinergic cell bodies and fibers in the guinea pig gastrointestinal tract, Lindh et al. (7) found dense ChAT-positive networks in the superior cervical ganglion (SCG) and stellate ganglion (SG) of the guinea pig Kasa et al. (34) and Matthews et al. (35) found ChAT-positive fibers in mouse and rat SCG, respectively, and Suzuki et al. (8) demonstrated ChAT in postganglionic rat parasympathetic neurons. However, the situation with regard to identification of cholinergic neurons in the periphery is less satisfactory than that for the central cholinergic systems.

Autonomic ganglia contain in both the pre- and the postganglionic neurons a number of peptides showing distinct distribution patterns and often coexisting with classical transmitters (9). Moreover, nitric oxide (NO) has during recent years emerged as a messenger candidate in both the central and peripheral nervous systems (10, 36–40), and its synthesizing enzyme, NO synthase (NOS), is present in preganglionic fibers in both prevertebral and paravertebral ganglia (11-13, 41, 42).

We have used a mouse monoclonal antibody raised against purified rat brain ChAT (14) to study sympathetic ganglia of the rat. The results demonstrate that both cholinergic cell bodies and fibers can be distinctly visualized in pre- and some postganglionic sympathetic neurons. Moreover, several coexistence situations of ChAT with NO and peptides are defined.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200–250 g) were used. In three rats the SCG was denervated by cutting the cervical sympathetic trunk 15 mm caudal to the ganglion under sodium pentobarbital anesthesia (Mebumal; 40 mg/kg, i.p.). Five to 7 days later the animals that had undergone this surgery as well as the untreated rats were anesthetized (as above) and perfused with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0). The SCG, the SG, and the celiac superior mesenteric ganglion (CSMG) were dissected out, postfixed, and cut at 14-μm thickness in a cryostat (Microm, Heidelberg). The sections were processed for the indirect immunohistochemical method—that is, incubated in a humid atmosphere at 4°C overnight with a mouse monoclonal antibody raised against rat brain ChAT (diluted 1:1,000) (14) or with one of several rabbit antiserum raised against enkephalin (1:400) (15), vasoactive intestinal polypeptide (VIP) (1:400; Cambridge Research Biochemicals), calcitonin gene-related peptide (CGRP) (1:400; Peninsula Laboratories), the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) (1:1,000) (16), or goat antiserum to NOS (1:2,000; P.E., unpublished work). The ChAT antibody is characterized by a high affinity for the enzyme (3 × 10^11 M⁻¹; C.C. and K.H., unpublished work). All antiserum were diluted in phosphate-buffered saline containing 0.3% Triton X-100 (17). After rinsing, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated sheep (1:20; Amersham) or donkey (1:40; Jackson ImmunoResearch) anti-mouse antibodies for the ChAT antibody and with FITC-conjugated goat anti-rabbit antibodies (1:80; Boehringer-Mannheim) for the other antiserum. The goat NOS antiserum was followed by

Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; CSMG, celiac superior mesenteric ganglion; NOS, nitric oxide synthase; SCG, superior cervical ganglion; SG, stellate ganglion; TH, tyrosine hydroxylase; VIP, vasoactive intestinal polypeptide.

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Lissamine rhodamine sulfonyl chloride (LRSC)-conjugated donkey anti-goat antibodies (1:40; Jackson ImmunoResearch) or FITC-conjugated donkey anti-goat antibodies (1:50; Nordic Lausanne, Switzerland). For colocalization studies, sections processed for visualization of ChAT or NOS were subsequently incubated with one of the above-mentioned antisera followed by LRSC-conjugated goat anti-rabbit antibodies (1:40) or LRSC-conjugated donkey anti-rabbit antibodies (1:40), or aminomethylcoumarin (AMCA)-conjugated donkey anti-goat antibodies (all from Jackson ImmunoResearch). For control purposes all peptide antisera were preabsorbed overnight at 4°C with the respective peptide at 1 μM.

**FIG. 1.** Immunofluorescence micrographs of the SG (a and g–m), CSMG (b), and SCG (c–f) after incubation with antiserum to ChAT (a–c, e, g, j, and l), NOS (d and f), TH (h), VIP (i and k), or CGRP (m). Thus, c/d, e/f, g/h, j/k, and l/m are double-stained sections. ChAT-immunoreactive cell bodies (a, g, j, and l) are found in SG, and dense fiber networks are seen in SG, SCG, and CSMG (a–c and e). In SG and SCG there is mostly a close overlap between ChAT- and NOS-positive fiber networks (c and d), with an almost complete coexistence (e and f), although in restricted areas NOS-positive fibers are absent or express low NOS-like immunoreactivity (asterisk). Note a single ChAT-immunoreactive cell body in SCG (c). SG contains numerous VIP neurons (i), and ChAT (g) coexists with VIP (k) and CGRP (m) but not TH (h). (Bars = 50 μm for the groups a/b, c/d, e/f, and g–m.)
Normal mouse, rabbit, and goat serum were used as control for ChAT, TH, and NOS staining, respectively. The sections were examined in a Nikon Microphot FX epifluorescence microscope equipped with filters for FITC, LRSC, and AMCA fluorescence.

**RESULTS**

In control SG (Fig. 1a), SCG (Fig. 1 c and e; Fig. 2 a, c, e, and i), and CSMG (Fig. 1b), dense networks of ChAT-immunoreactive fibers were observed often surrounding the...
ganglion cell bodies in a basket-like manner as well as forming tracts with multiple parallel fibers. In addition there were in SG numerous ChAT-immunoreactive cell bodies (Fig. 1 a, g, j, and l), whereas only single cells showed this immunoreactivity in SCG (Fig. 1c). The ChAT-immunoreactive cell bodies were TH-negative (Fig. 1 g and h). There were many VIP-immunoreactive cell bodies in the SG (Fig. 1i), and virtually all of them were ChAT-immunoreactive (Fig. 1 j and k). Smaller numbers of ChAT-immunoreactive cells contained CGRP-like immunoreactivity (Fig. 1 l and m). Varying numbers of enkephalin-immunoreactive cell bodies were observed in SG and especially SCG, and they were ChAT-negative and TH-positive. A very dense network of NOS-immunoreactive fibers was observed in all three ganglia (Fig. 1 d and f; Fig. 2g) with a distribution overlapping with the ChAT-immunoreactive fibers (Fig. 1 e and e), and these fibers were mostly identical (Fig. 1 e and f). However, in restricted areas of the SCG, ChAT-immunoreactive fibers were NOS-negative or at most very weakly NOS-immunoreactive (Fig. 1 c and d). There were enkephalin-positive (Fig. 2 b, d, f, h, and n) and CGRP-positive (Fig. 2 g) fibers in SCG (Fig. 2 a–h), SG (Fig. 2 j), and CSMG. Enkephalin-immunoreactive fibers were more numerous in SCG than in SG. Numerous enkephalin-positive fibers formed baskets in the SCG (Fig. 2 b, d, f, and j), and at least some of these fibers were ChAT-immunoreactive (Fig. 2 c, d, e, and f). In the SG, CGRP-immunoreactive fibers formed baskets (Fig. 2q), and they were frequently ChAT-immunoreactive (Fig. 2 g), whereas other CGRP-positive fibers (single or in bundles) lacked ChAT-like immunoreactivity. No coexistence between NOS (Fig. 2 q) and enkephalin (Fig. 2 n) immunoreactivities could be established. After sectioning of the sympathetic trunk caudal to the SCG, virtually all ChAT (Fig. 2 k and l), NOS (Fig. 2 m), and most enkephalin (Fig. 2 n) fibers disappeared in the SCG. The remaining ChAT-positive fibers were NOS-positive but enkephalin- and CGRP-negative. None of the immunoreactivities described above were observed after incubation with control serum.

**DISCUSSION**

Using an antibody raised against purified rat brain ChAT (14), we here demonstrate the presence of this enzyme in the sympathetic autonomic nervous system, in both paravertebral and prevertebral ganglia of the rat. This antibody has previously been used to demonstrate cortical cholinergic systems in rat brain with an excellent sensitivity and resolution (18). The present findings show dense networks of fibers containing ChAT-like immunoreactivity in the SCG, SG, and CSMG. Surgical transection of the sympathetic trunk caused an almost complete disappearance of these fibers in the SCG, confirming their preganglionic nature. The source(s) of the dense fiber networks in the rat SG and CSMG remains to be analyzed. Moreover a population of principal ganglion neurons in the SG, and single neurons in SCG, expressed ChAT but not TH, strongly supporting their cholinergic nature. The large number of ChAT-positive fibers in all parts of the SCG and SG suggests that perhaps all preganglionic fibers in fact are cholinergic, in agreement with numerous physiological studies showing that ACh is the principal transmitter in sympathetic ganglia (19).

The present findings confirm the existence of numerous NOS-positive fibers in paravertebral ganglia (11) and their disappearance after transection of the sympathetic trunk (13), supporting the view of a preganglionic origin in cell bodies in the sympathetic lateral column (13, 20–22). Here we show that virtually all of these NOS fibers in SG and SCG are cholinergic and thus presumably release both ACh and NO. However, there are patches of ChAT-positive fibers which seem to lack, or at least contain only very low levels of, NOS-like immunoreactivity. It is thus possible that a population of the preganglionic fibers release ACh but not NO.

A small population of sympathetic neurons express ACh esterase (23, 24). In the cat these neurons contain VIP and project to salivary glands, where VIP and ACh interact in the control of secretion and blood flow (25, 26). The present findings clearly demonstrate that virtually all VIP-immunoreactive neurons in the rat SG are ChAT-immunoreactive and vice versa, giving final proof for the cholinergic nature of these sympathetic neurons. A few VIP-positive neurons were also observed in SCG (27, 28), and they were also ChAT-positive. Small numbers of CGRP-positive neurons in rat paravertebral ganglia contain VIP and have been suggested to be cholinergic (29). In agreement, the present results show that CGRP- and ChAT-like immunoreactivities coexist in these neurons.

Nerve fibers containing various peptides have been identified in pre- and paravertebral ganglia (see ref. 9). They are particularly abundant in prevertebral ganglia and originate in spinal cord, sensory ganglia, and the gastrointestinal tract (see refs. 9 and 30). Here we focus on enkephalin and CGRP fibers in paravertebral ganglia. At least part of the CGRP fibers seems to be preganglionic (31). Since enkephalin coexists with ChAT in cell bodies in the sympathetic lateral column (32), some enkephalin fibers in the SCG may represent preganglionic cholinergic fibers. In agreement with these views, many CGRP and enkephalin fibers disappear after immunologically induced sympathectomy (33). The enkephalin fibers in the SCG exhibit a patchy distribution (15) overlapping with, but being much more sparse than, the ChAT-immunoreactive ones. Under high-power objectives it was found that in normal ganglia only some enkephalin-immunoreactive terminals in the SCG were clearly ChAT-immunoreactive but that CGRP-immunoreactive baskets in SG often were ChAT-positive. However, to monitor coexistence of ChAT and NOS versus enkephalin and CGRP in single nerves/varicosities may be difficult, partly due to their different intracellular localization: ChAT and NOS are cytoplasmic enzymes and “fill out” the entire axons and nerve endings, whereas the peptides are stored in vesicles and are mainly present in the nerve endings. We may therefore underestimate the degree of coexistence of, for example, ChAT and enkephalin. Finally, enkephalin-like immunoreactivity was not observed in NOS-immunoreactive fibers. Thus, it is possible that ChAT/NOS fibers and ChAT/enkephalin fibers represent two subpopulations of preganglionic fibers. Whether or not there is a population of enkephalin-positive, ChAT-negative, and NOS-negative fibers with an unknown principal transmitter remains to be investigated. Clearly some of the enkephalin fibers in the SCG could arise from enkephalin-containing noradrenergic neurons in this ganglion (15).

**Note Added in Proof.** After submission of this paper, we have become aware of a publication by Sann et al. (43) which provides a detailed study on ChAT-immunoreactive neurons and peptide coexistence in the inferior mesenteric ganglion of several species.

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