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Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles

(vesicular monoamine transporter/immunohistochemistry/small synaptic vesicles/large dense-core vesicles/regulated secretion)

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ABSTRACT **Immunohistochemical visualization of the rat vesicular acetylcholine transporter (VAChT) in cholinergic neurons and nerve terminals has been compared to that for choline acetyltransferase (ChAT), heretofore the most specific marker for cholinergic neurons. VAChT-positive cell bodies were visualized in cerebral cortex, basal forebrain, medial habenula, striatum, brain stem, and spinal cord by using a polyclonal anti-VAChT antiserum. VAChT-immunoreactive fibers and terminals were also visualized in these regions and in hippocampus, at neuromuscular junctions within skeletal muscle, and in sympathetic and parasympathetic autonomic ganglia and target tissues. Cholinergic nerve terminals contain more VAChT than ChAT immunoreactivity after routine fixation, consistent with a concentration of VAChT within terminal neuronal arborizations in which secretory vesicles are clustered. These include VAChT-positive terminals of the median eminence of the hypothalamus, not observed with ChAT antiserum after routine fixation. Subcellular localization of VAChT in specific organelles in neuronal cells was examined by immunoelectron microscopy in a rat neuronal cell line (PC12-c4) expressing VAChT as well as the endocrine and neuronal forms of the vesicular monoamine transporters (VMAT1 and VMAT2). VAChT is targeted to small synaptic vesicles, while VMAT1 is found mainly but not exclusively on large dense-core vesicles. VMAT2 is found on large dense-core vesicles but not on the small synaptic vesicles that contain VAChT in PC12-c4 cells, despite the presence of VMAT2 immunoreactivity in central and peripheral nerve terminals known to contain monoamines in small synaptic vesicles. Thus, VAChT and VMAT2 may be specific markers for "cholinergic" and "adrenergic" small synaptic vesicles, with the latter not expressed in nonstimulated neuronally differentiated PC12-c4 cells.**

Markers for functional presynaptic cholinergic and monoaminergic nerve terminals *per se* would be useful for the study of cholinergic and monoaminergic synapse formation, loss of synapses in neurodegenerative diseases, and neuroanatomical disposition of synapses throughout the central and peripheral nervous systems. Antibodies against biosynthetic enzymes for acetylcholine (ACh) and monoamines have been used previously for this purpose. However, they are cytosolic proteins that are not specifically concentrated at nerve terminals. Two isoforms of the vesicular monoamine transporter (VMAT) responsible for vesicular uptake of monoamines have been functionally characterized, and antibodies against rat VMAT1 and VMAT2 have previously been used to localize these transporters to neuronal and neuroendocrine biogenic amine-containing cells and in particular their terminal processes (1–6).

We have recently cloned and sequenced the rat and human homologs of a putative vesicular acetylcholine (ACh) transporter (VAChT) in *Caenorhabditis elegans* and *Torpedo* (7–9) and demonstrated that the rat VAChT is a functional transporter for ACh (8). VAChT-encoding mRNA is present in all known major cholinergic cell groups of the central and peripheral nervous systems (8, 10, 11). Here, antibodies specific for VAChT were used to visualize cholinergic neurons, in particular cholinergic nerve terminals in the central and peripheral nervous systems. The purpose of these immunohistochemical investigations at the light microscopic level was to determine the suitability of the VAChT antigen as a marker for cholinergic nerve terminals, in which secretory vesicles presumably containing VAChT are specifically concentrated. Results were compared with those obtained by immunohistochemical staining for choline acetyltransferase (ChAT), a cytosolic protein for which visualization in nerve terminals has been problematic (12). The VAChT antigen was also visualized by immunocytochemical electron microscopy (EM) at the ultrastructural level in a cell line expressing both VAChT and VMAT1 and VMAT2. The aim of investigations at the ultrastructural level was to determine if the type of secretory vesicle to which VAChT is targeted is distinct from the secretory vesicles expressing the monoamine transporters in a neuronally differentiated cell line expressing all three transporters.

MATERIALS AND METHODS

Generation of Anti-VAChT Antisera. The peptide sequence Cys-Glu-Asp-Asp-Tyr-Asn-Tyr-Tyr-Ser-Arg-Ser (CEDDYNYYSRS in single-letter code), corresponding to the C terminus of rat VAChT, was conjugated through the N-terminal cysteine to keyhole limpet hemocyanin (KLH) and antisera raised in New Zealand White rabbits as described for antibodies generated against C-terminal peptides of rat VMAT1 and VMAT2 (5). Sera from rabbits immunized with the KLH-CEDDYNYYSRS conjugate were assayed for anti-VAChT immunoreactivity and specificity by staining of CV-1 cells expressing the rat VAChT cDNA (8), as described for antibodies against rat VMAT1 and VMAT2 (5). Representative

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Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; EM, electron microscopy; LDCV, large dense-core vesicle; NGF, nerve growth factor; PBS, phosphate-buffered saline; SSV, small synaptic vesicle; VAChT, vesicular acetylcholine transporter; VMAT, vesicular monoamine transporter; TH, tyrosine hydroxylase.

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immunohistochemical and immunocytochemical EM data using antiserum 80259 are shown below.

Generation of VMAT2-Expressing PC12 Cell Clone PC12-c4. PC12 cells were infected with recombinant virions produced by transfection of the PG-13 packaging cell line with the plasmid pLNCMAT, which contains a *Hin*dIII–*Acc* I (blunted) fragment of the rat VMAT2 cDNA ligated into the vector pLNCX (13) after *Hin*dIII/*Hpa* I double digestion. VMAT2 expression is initiated by a cytomegalovirus promoter. The VMAT2-expressing clone PC12-c4 was obtained via limiting dilution culture in G418 medium.

Culture and Staining of PC12 Cells. PC12 cell clone PC12-G was cultured and treated with nerve growth factor (NGF) as described (14). Cells were grown in four- or eight-well plastic chamber slides on either a Matrigel or poly(L-lysine) matrix for light microscopy, fixed in formalin, and processed for immunohistochemistry by using the rabbit IgG Vectastain ABC kit (Vector Laboratories).

Preembedding Immunocytochemical EM for VAChT, VMAT1, and VMAT2 in PC12 and PC12-c4 Cells. Cells were grown on Matrigel with NGF treatment for 14 days, fixed in 4% formaldehyde (EM grade) for 1 hr at room temperature, washed extensively, and stained for VAChT, VMAT1, or VMAT2 in saponin/phosphate-buffered saline (PBS) at antibody dilutions from 1:500 to 1:1000. VMAT1 and VMAT2 antibodies used were those described previously (5). Cells were then washed and incubated with a secondary antibody conjugated with a 1.4-nm gold probe (Nanoprobe, Stony Brook, NY) followed by silver enhancement (15).

Immunohistochemistry. Immunohistochemistry for VAChT, VMAT2, ChAT, and tyrosine hydroxylase (TH) was carried out essentially as described for VMAT1 and VMAT2

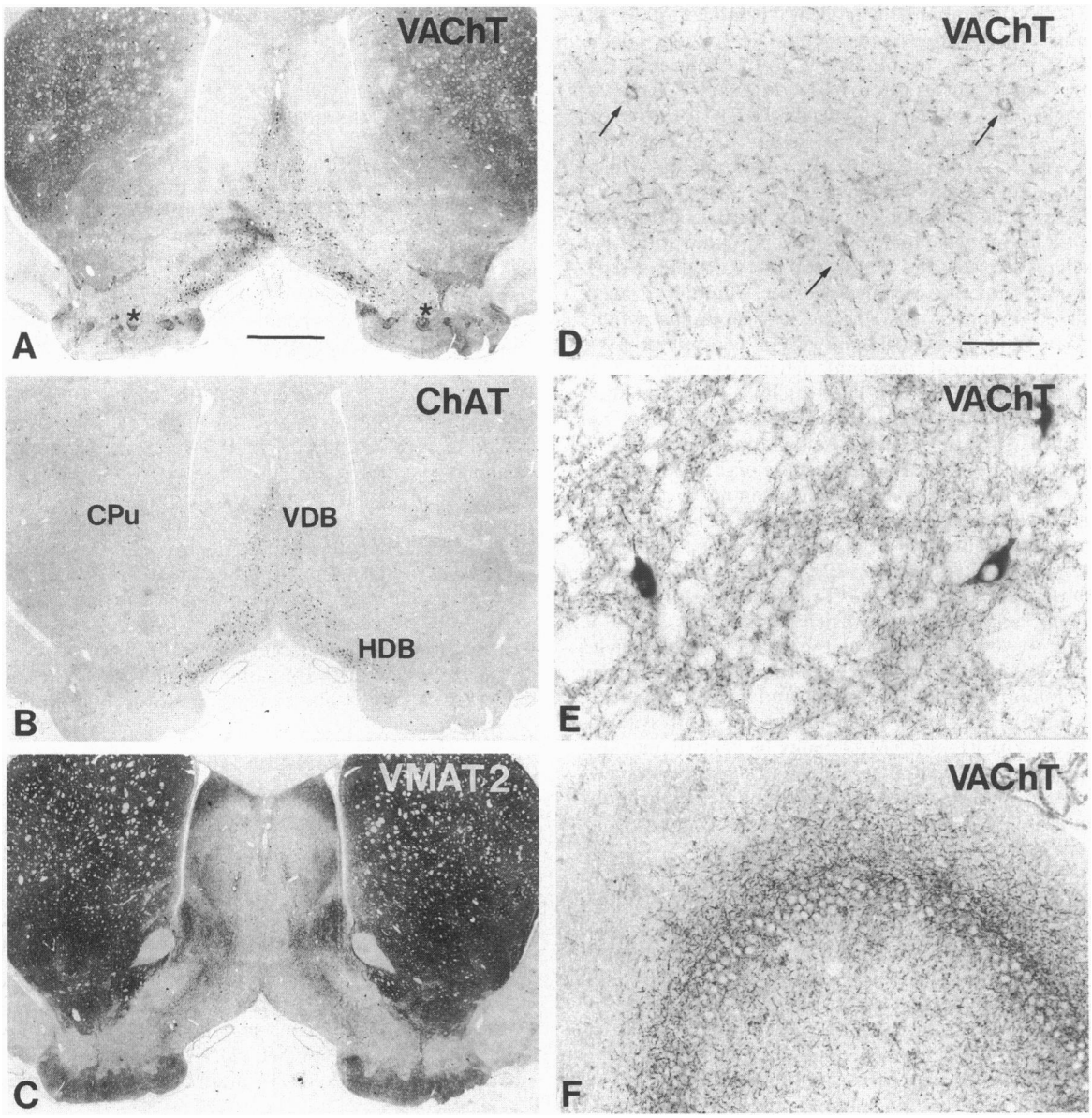

FIG. 1. Comparative distribution of VAChT, ChAT, and VMAT2 immunoreactivity in the rat forebrain. (*A–C*) Adjacent coronal sections of rat brain at the level of the striatum and basal forebrain stained for VAChT (*A*), ChAT (*B*), and VMAT2 (*C*). VAChT immunoreactive neurons and fibers in the islands of Calleja are marked by asterisks in *A*. (*A–C* are at identical magnification. (Bar in *A* = 1000 μm.) (*D* and *E*) VAChT-immunoreactive cell bodies and nerve fibers and terminals in parietal cortex (*D*) and caudate nucleus (*E*). *D* and *E* are at identical magnification. (Bar in *D* = 50 μm.) (*F*) VAChT immunoreactive terminal fibers surrounding pyramidal perikarya in the CA3 region of the hippocampal formation at medium magnification. VDB and HDB, vertical and horizontal limb of the diagonal band of Broca; CPu, caudate-putamen.

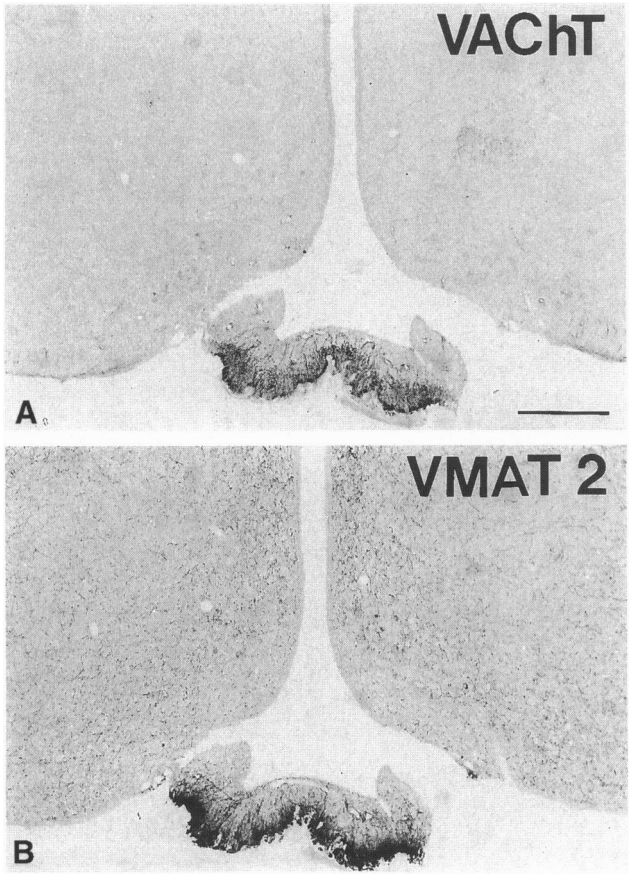

FIG. 2. VAChT and VMAT2 immunoreactivity in the median eminence. (*A*) Strong VAChT immunoreactivity is concentrated in the external layer of the median eminence but also is present in the internal layer. (*B*) VMAT2 immunoreactivity in the infundibular dopaminergic TH-positive neurons that project to the median eminence. (Bar = 100 μm.)

in rat tissues prepared in Bouin–Hollande fixative (5). Antibody dilutions were 1:2000 to 1:3000 for anti-VAChT and 1:2000 for anti-VMAT2. Antibodies against ChAT and TH were used at the dilutions reported previously (16, 17).

RESULTS

The specificity of rat anti-VAChT antibodies was established by staining of CV-1 cells only if expressing exogenous recombinant VAChT and by staining of endogenous VAChT-expressing PC12 cells. The specific staining of PC12 cells was adsorbed by preincubation of antibody with the peptide immunogen against which the antiserum was raised at a concentration of 10 μM (data not shown). All VAChT staining shown in Figs. 1–4 was likewise absent from incubations performed with antiserum preincubated with 10 μM of the peptide immunogen.

Immunohistochemical visualization of VAChT in cholinergic neuronal cell bodies, fibers, and terminals of rat brain is shown in Fig. 1. Staining for either VAChT or ChAT revealed similar numbers of immunoreactive cell bodies in basal forebrain and striatum (Fig. 1 *A* and *B*). However, VAChT staining of neuronal processes was much more intense than that for ChAT (Fig. 1 *A* and *B*). VAChT staining demonstrated intrinsic cholinergic nerve terminals throughout the striatum, roughly coextensive in the caudate nucleus with the distribution of extrinsic dopaminergic innervation demonstrated with VMAT2 (Fig. 1*C*). High-power micrographs of VAChT immunoreactivity in cerebral cortex and striatum demonstrate the presence of relatively weak VAChT immunoreactivity in small cortical neurons (Fig. 1*D*, arrows) and strong VAChT immunoreactivity in the cholinergic interneurons of the caudate nucleus (Fig. 1*E*). Visualization of VAChT-immunoreactive cell bodies in cerebral cortex is consistent with previous reports of sparse intrinsic cholinergic innervation of the cortex in addition to the extrinsic cortical cholinergic innervation arising from the basal forebrain. Note the dense cholinergic terminal nets in the deep layers of parietal cerebral cortex and in the caudate nucleus (Figs. 1 *D* and *E*). In the hippocampus, strong VAChT immunoreactivity in nerve terminals as well as fibers allowed a clear delineation of cholinergic nerve terminal investiture of pyramidal cell bodies in the CA3 region (Fig. 1*F*). VAChT-positive cell bodies were also identified in nuclei of the cholinergic ascending reticular activating system and in the medial habenular nucleus, in which VAChT mRNA was previously detected by *in situ* hybridization histochemistry (11).

Differential visualization of cholinergic terminals by VAChT and ChAT immunoreactivity was most dramatic in the median eminence. Intense VAChT immunoreactivity in median eminence (Fig. 2*A*) is accompanied by only weak staining for ChAT (not shown). The presence of VAChT immunore-

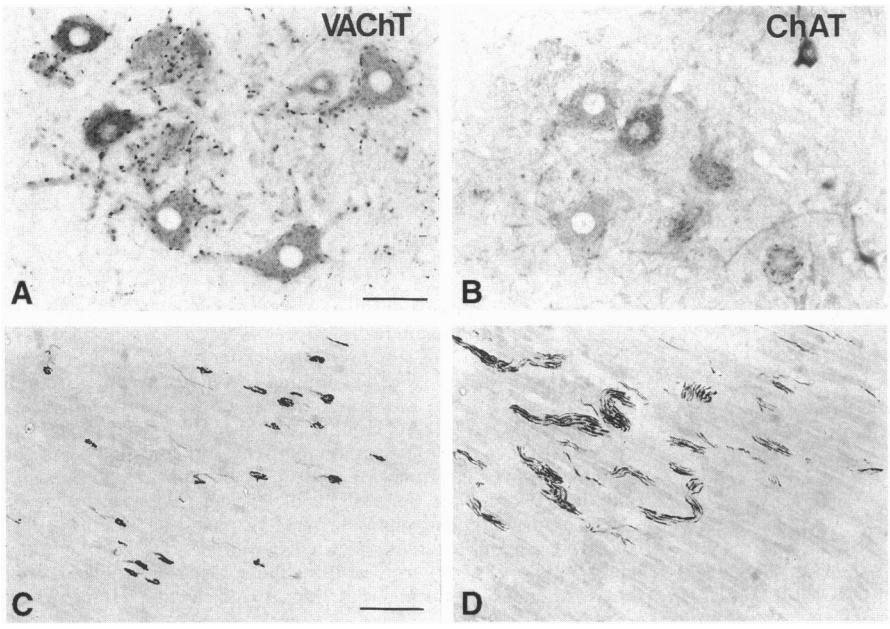

FIG. 3. Comparative distribution of VAChT and ChAT immunoreactivity in the ventral horn and skeletal muscle. (*A* and *B*) Neighboring sections of the ventral horn of the spinal cord stained for VAChT (*A*) or ChAT (*B*) immunoreactivity. [Bar in *A* (for *A* and *B*) = 50 μm.] (*C* and *D*) Adjacent sections of skeletal muscle stained for VAChT (*C*) or ChAT (*D*) reveal preferential localization of strong VAChT immunoreactivity in motoneuron terminals and weak VAChT immunoreactivity in some preterminal axons (*C*). Conversely, strong ChAT immunoreactivity is concentrated in axonal portions of motoneurons and not visualized in the terminal arborization (*D*). [Bar in *C* (for *C* and *D*) = 100 μm.]

activity in the median eminence suggests a copious cholinergic innervation of this important neuroendocrine nexus. VMAT2 staining of terminal arborizations of the dopaminergic tuberoinfundibular system is shown for reference in Fig. 2*B*. VMAT2 staining of monoaminergic nerve terminals of the median eminence was much more intense than staining for the cytosolic biosynthetic enzyme TH (not shown), similar to the relationship between VAChT and ChAT immunoreactivity in the median eminence and in nerve terminals throughout the central and peripheral nervous systems.

Motor neurons of the spinal cord showed intense VAChT immunoreactivity in cell bodies and numerous VAChT-positive nerve terminals near the VAChT-immunoreactive motor neurons (Fig. 3*A*). Whether these are collateral or autosynapses or derive from nonmotoneuron cholinergic afferents to the ventral horn is not known. These terminals were not visualized with anti-ChAT, although motor neuron cell bodies were (Fig. 3*B*). VAChT immunoreactivity is also concentrated at motor end plates in skeletal muscle relative to ChAT (Fig. 3*C*). Staining for VAChT allows visualization of the presynaptic portion of the neuromuscular junction in skeletal muscle in which ChAT immunoreactivity is weak or absent, while ChAT stains motor neuron fibers entering muscle in which VAChT immunoreactivity is weak (Fig. 3*D*). Thus, the complementary visualization of nerve fibers and nerve terminals by ChAT and VAChT observed in cholinergic intrinsic and projection neurons of the central nervous system is found in cholinergic motoneurons as well.

The relationship between VAChT and ChAT immunoreactivity in cholinergic neurons within the autonomic nervous system is shown in Fig. 4. Principal ganglion cells in the superior cervical ganglion are invested with VAChT-positive preganglionic cholinergic nerve terminals (Fig. 4*A*). While multiple VAChT-immunoreactive nerve terminals are found on cell bodies in the superior cervical ganglion, ChAT immunoreactivity is relatively stronger in incoming preganglionic cholinergic fibers than in their terminals on principal ganglion cells (Fig. 4*B*). Occasional VAChT-positive postganglionic cell bodies are found in superior cervical ganglion of the rat (arrow in Fig. 4*A*), in addition to the mainly VMAT2-positive principal ganglion cells (Fig. 4*C*). These VAChT-immunoreactive cells may represent sparse cholinergic projections to sweat glands, skeletal muscle vasculature, or other as yet uncharacterized cholinergic targets. Indeed, cholinergic nerve terminals innervating sweat glands of the paw in the rat (18) are easily visualized via staining for VAChT (Fig. 4*D*).

VAChT localization was extended to the subcellular level to determine if selectivity of expression of the VAChT includes selective targeting to a specific subpopulation of secretory vesicles within nerve terminals. Accordingly, immunocytochemical EM for VAChT and VMAT1 was performed in neuronally differentiated PC12 cells, which contain both large dense-core vesicles (LDCVs) and small synaptic vesicles (SSVs) and express both transporter proteins. Specific VAChT immunoreactivity is clearly preferentially localized to SSV clusters compared to LDCVs (Fig. 5 *A* and *B*). VMAT1 immunoreactivity is present mostly on LDCVs (Fig. 5*C*). VMAT1 staining of SSVs (Fig. 5*C Inset*) may indicate a mixed transporter phenotype for small vesicles in PC12 cells or two populations of SSVs, one containing VAChT and a minor population containing VMAT1.

Endocrine cells *in vivo* expressing VMAT1 contain catecholamines in LDCVs. In contrast, catecholamines are found in both large and small vesicle populations in peripheral nerves, which express only VMAT2, the neuronal isoform of VMAT. A PC12 subclone, PC12-c4, was produced that constitutively expresses VMAT2 resulting in strong immunoreactivity for VMAT2 absent from the parent PC12 cell line (data not shown). VMAT2 was localized by immunocytochemical EM to LDCVs and was largely absent from the abundant

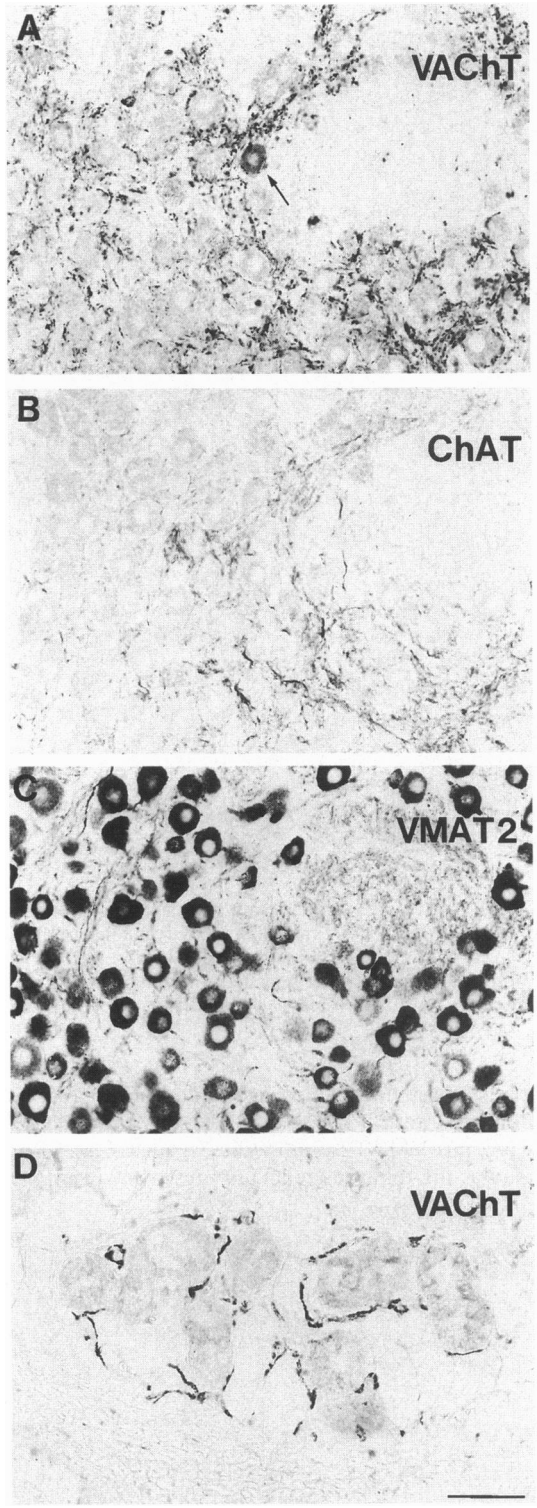

FIG. 4. Comparative distribution of VAChT, ChAT, and VMAT2 immunoreactivity in superior cervical ganglion and the presence of VAChT-immunoreactive nerve terminals in sweat glands. (*A*) Strong VAChT immunoreactivity in present in the preganglionic plexus surrounding positive postganglionic neuron (arrow in *A*). (*B*) ChAT immunoreactivity is concentrated in preterminal preganglionic sympathetic nerve fibers. (*C*) VMAT2 immunoreactivity is displayed in virtually all postganglionic sympathetic neurons. Note the absence of VAChT immunoreactivity (*A*) from areas with postganglionic VMAT2-positive efferents (*C*). (*D*) VAChT immunoreactivity is visualized in presumptive sympathetic postganglionic cholinergic neurons innervating sweat glands of the paw. All micrographs are at identical magnification. (Bar in *D* = 50 μm.)

VAChT-positive SSV clusters present in the NGF-differentiated PC12-c4 cells (Fig. 6*A*). The specificity of immunogold labeling for VMAT2 was demonstrated by the absence of grains above background in the parent PC12 cell line. Thus, VMAT2 is directed to LDCVs present in PC12 cells, in preference to SSVs, indicating that NGF-differentiated PC12 cells containing "cholinergic" SSVs do not, at least under basal conditions, have the capability to produce "adrenergic" SSVs.

DISCUSSION

Antibodies against VAChT have been used here for the first time to visualize cholinergic synapses in the central and peripheral nervous systems. Specific visualization of cholinergic nerve terminals *in vivo* has previously been accomplished with antibodies against ChAT (12). VAChT and ChAT gene expression appears to be tightly linked in mammalian neurons, with both transcripts produced from a common "cholinergic operon" (19). VAChT is localized here by immunohistochemistry to all known major central and peripheral cholinergic systems identified previously with antibodies directed to ChAT (12, 18, 20–22). However, ChAT immunoreactivity is more prominent in cholinergic nerve fibers and cell bodies, while VAChT immunoreactivity is most prominent in nerve terminals, consistent with the cytosolic localization of ChAT and the clustering of VAChT-containing synaptic vesicles at the nerve terminal. Thus, VAChT immunoreactivity clearly delineates extensive investment of hippocampal, spinal motor neuronal, sympathetic principal ganglionic cell bodies, and eccrine sweat glands by cholinergic nerve terminals. VAChT and ChAT are overlapping but complementary markers for cholinergic neurons.

Intense VAChT immunoreactivity in the median eminence of the hypothalamus suggests a neuroanatomical substrate for cholinergic modulation of neuroendocrine function. The cell bodies of origin of this cholinergic innervation of the median eminence are unknown.

Terminal fields of cholinergic motor neurons in skeletal muscle, and intrinsic cholinergic innervation of autonomic targets such as the heart (E.W., unpublished observation) are also visualized with antibodies to the VAChT antigen in greater detail than with antibodies to ChAT. Analogously, the neuronal VMAT2 is preferentially visualized in terminal fields of monoaminergic neurons, compared to the monoaminergic biosynthetic enzyme TH (E.W., unpublished observation). The VAChTs and VMATs may afford a more sensitive visualization of cholinergic and monoaminergic synaptic develop-

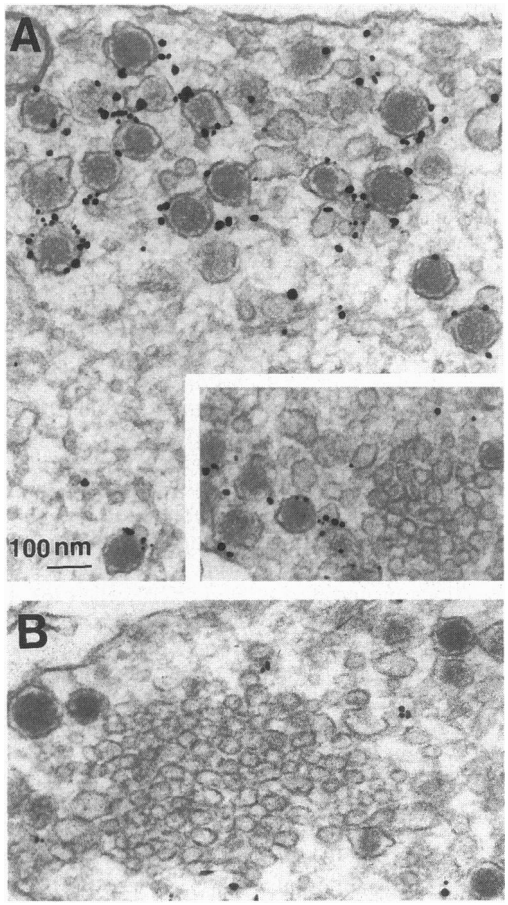

FIG. 6. VMAT2 immunocytochemical EM in PC12-c4 cells. (*A*) VMAT2 is present on LDCVs in PC12-c4 cells. (*Inset*) Absence of VMAT2 staining of SSV clusters. (*B*) Lack of LDCV or SSV staining within PC12 cells, which do not express VMAT2, by anti-VMAT2 immunocytochemical EM.

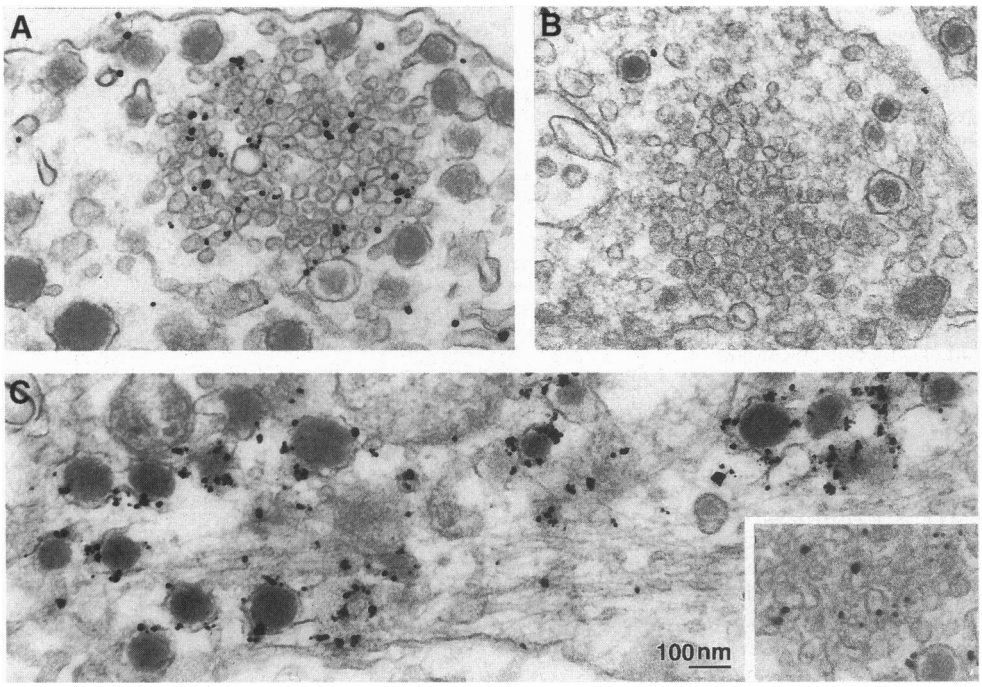

FIG. 5. VAChT and VMAT1 immunocytochemical EM in NGF-treated PC12 cells. (*A*) VAChT immunoreactivity (silver-enhanced immunogold particles) in PC12 cell neurites is confined to the membranes of SSVs with only occasional staining of LDCVs. (*B*) VAChT immunoreactivity at the same dilution as that used in *A* (1:500) but after preadsorption of antiserum no. 80259 with the synthetic C-terminal VAChT peptide fragment used to generate the antiserum. (*C*) VMAT1 immunoreactivity in NGF-treated PC12 cells. Note the presence of VMAT1 immunoreactivity predominantly on LDCVs. (*Inset*) VMAT1 immunoreactivity localizes to a subpopulation of SSVs present in some nerve terminals of NGF-differentiated PC12 cells.

ment and synaptic loss in neurodegenerative disease than previously obtained by immunohistochemical visualization of cytosolic biosynthetic enzymes. Visualization of cholinergic nerve terminals in routinely fixed, paraffin-embedded human tissues with antibodies raised against the human VAChT suggests that these will be useful reagents for assessment of synaptic changes in human diseases in which cholinergic neuronal systems undergo neurodegeneration (23).

The characterization of VAChT and VMATs as neuron-specific antigens has allowed the issue of the subcellular localization and biogenesis of ACh-sequestering versus monoamine-sequestering vesicles to be addressed. PC12 cells treated with NGF acquire morphological and functional characteristics of both cholinergic and adrenergic neurons (24, 25). They possess both LDCV and SSV clusters (15). Preferential targeting of both VAChT and VMATs to individual vesicle types can therefore be examined in these cells. VAChT and VMAT1 are differentially distributed to vesicular subpopulations when examined at the EM level. VMAT1 is preferentially localized to LDCVs in PC12 cells but is also contained to a significant extent on SSVs, consistent with the subcellular localization of VMAT1 to both LDCVs and to some extent to more buoyant vesicle fractions in undifferentiated PC12 cells (26). In contrast, VAChT is highly preferentially localized to SSVs. This is consistent with the preferential uptake of ACh by subcellular fractions of undifferentiated PC12 cells containing synaptic-like microvesicles (27). Distribution of VAChT to SSVs and not LDCVs in PC12 cells suggests that VAChT is also absent from LDCVs in cholinergic neurons *in vivo*, albeit this remains to be documented ultrastructurally. The absence of ACh uptake into LDCVs even in cholinergic neurons may account for the differential release of ACh and vasoactive intestinal polypeptide; mutually exclusively stored in SSVs and LDCVs, respectively, from cholinergic neurons *in vivo* (28).

It is of some interest that PC12 cells differentiated with NGF do not express VMAT2, since the latter is the only VMAT isoform expressed in neurons *in vivo* (5). VMAT2 is expressed both centrally and peripherally in monoaminergic neurons whose nerve terminals contain mainly SSVs about 50 nm in diameter that in the absence of pretreatment with permanganate or chromate salts appear to be electronlucent (ref. 29 and references therein). VMAT2 has been localized by immunocytochemical EM to both LDCVs and SSVs in the same catecholaminergic cells of the nucleus tractus solitarius of the brain stem (30). It has been suggested that SSVs of cholinergic neurons are generated from constitutive vesicles after acquisition of antigens from the early endosome of the nerve terminal (31). Controversy exists over whether monoaminergic SSVs are derived from constitutive vesicles or from LDCVs, since they possess antigens that are contained in both large and small vesicles but not common to both (32).

If small adrenergic vesicles containing VMAT2 are derived from the same organellogenic pathway as cholinergic SSVs containing VAChT, then expression of VMAT2 in VAChT-containing neurons should result in localization of VMAT2 to VAChT-positive vesicles. Surprisingly, VMAT2 is expressed on LDCVs and not on SSVs in NGF-treated PC12-c4 cells. This observation strongly suggests that cholinergic and monoaminergic SSVs differ not only in the transporter expressed in cholinergic and monoaminergic neurons but also in more fundamental ways. The lack of monoaminergic SSVs in PC12-c4 cells could be explained by the absence from PC12 cells of additional proteins or factors, present in adrenergic neurons *in vivo*, which allow VMAT2 to reach the SSV compartment or allow the biogenesis of a specific subpopulation of "adrenergic" SSVs. Alternatively, if monoaminergic SSVs are produced from LDCVs only after fusion of the latter with the plasma membrane, stimulation of LDCV exocytosis, regulated by cell depolarization, may be required for conversion of LDCVs to small monoaminergic vesicles in PC12 cells. In any event, in PC12 cells at least, production of cholinergic and monoaminergic SSVs apparently does not occur by a common biosynthetic pathway. Cholinergic and adrenergic SSVs may represent unique organelles generated by two quite distinct pathways in neuronal cells.

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