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Synthesizing enzymes for four neuroactive substances in motor neurons and neuromuscular junctions: Light and electron microscopic immunocytochemistry

(transmitters/choline acetyltransferase/tyrosine hydroxylase/cysteine sulfinic acid decarboxylase/glutamic acid decarboxylase)

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ABSTRACT **Immunocytochemical evidence is presented for the existence of choline acetyltransferase (ChoAcTase), cysteine sulfinic acid decarboxylase (CSADCase), tyrosine hydroxylase (TyrOHase), and glutamic acid decarboxylase (GluDCase) in large motor neurons of the hypoglossal nucleus and the spinal cord and in nerve terminals of motor end plates in tongue and skeletal muscle of five mammalian species, including man. These enzymes, which are responsible for the synthesis of acetylcholine (AcCho), taurine, dopamine, and γ-aminobutyrate (GABA), respectively, were detected by immunocytochemical studies with monoclonal or polyclonal antibodies raised against the enzymes. Electron microscopy of the neuromuscular junctions showed that the immunoreactivity in each case was confined to the cytoplasmic matrix of presynaptic nerve terminals. Immunoreactivity obtained for each enzyme antibody varied with the species. It was highest in fresh, unfixed muscle and lowest in aldehyde-fixed specimens. Negative controls were obtained with preimmune sera and antisera preabsorbed with pure ChoAcTase, CSADCase, or GluDCase antigen. Double-labeling studies with ChoAcTase antibodies and acetylcholinesterase (AcChoEase) antibodies, AcChoEase enzyme activity, or α-bungarotoxin binding indicated that ChoAcTase, AcChoEase, and AcCho receptors were colocalized at the same end plates.**

Acetylcholine (AcCho) occurs in motor nerve fibers along with the enzyme choline acetyltransferase (ChoAcTase; EC 2.3.1.6) which catalyzes its synthesis. This enzyme has been localized by immunocytochemistry to the motor neurons in the spinal cord and brainstem (1). AcCho is released upon stimulation of the motor nerve and evokes a contraction in the innervated muscle (2, 3). Furthermore, the postsynaptic potentials induced by the nerve impulse and the spontaneous miniature end plate potentials found at the neuromuscular junction can be mimicked by the controlled application of AcCho to the surface of a muscle fiber close to the nerve ending (4). The specific hydrolyzing enzyme acetylcholinesterase (AcChoEase; EC 3.1.1.7) occurs both in the motor neuron and in the synaptic cleft of the neuromuscular junction as demonstrated by histochemical (5, 6) and immunocytochemical methods (7). Finally, the postsynaptic effects of both the nerve impulse and applied AcCho can be blocked by the same specific antagonists (8). Thus, the exclusive role of AcCho at the neuromuscular junction has been established and universally accepted for the last half century.

The apparent chemical uniformity of the somatic motor system contrasts, however, with the heterogeneity that has been discovered in other parts of the nervous system. For example, some neurons in sympathetic ganglia are adrenergic whereas others are cholinergic. Some cells in the raphe nuclei are serotoninergic, whereas others are peptidergic and still others are both (9–11). Recently, it has been shown that cerebellar Purkinje cells are heterogeneous chemically as well as morphologically, some being peptidergic, some containing γ-aminobutyric acid (GABA), some containing taurine, and others containing various combinations of peptides, taurine, and GABA (12–15). Therefore, it is important to inquire whether the motor neuron and its axonal terminals at the neuromuscular junction might also be more diversified than has been thought.

The present paper reports evidence from an immunocytochemical study of five mammalian species (including man) to the effect that motor neurons and nerve terminals at the neuromuscular junction contain not only the enzyme ChoAcTase but also other transmitter synthesizing enzymes: cysteine sulfinic acid decarboxylase (CSADCase; EC 4.1.1.29), which catalyzes the conversion of cysteine sulfinic acid or cysteic acid to taurine; glutamic acid decarboxylase (GluDCase; EC 4.1.1.15), the rate-limiting enzyme in the synthesis of GABA; and the catecholamine biosynthetic enzyme tyrosine 3-hydroxylase (TyrOHase; EC 1.14.16.2).

MATERIALS AND METHODS

The following antibodies and controls were used in this study: monoclonal antibody and polyclonal antibody against ChoAcTase (for specificity see ref. 16); polyclonal antibody against CSADCase (17); polyclonal antibody against GluDCase (18, 19); polyclonal antibody against TyrOHase (20); and monoclonal antibody against AcChoEase (AE-3) (7). Control sera included preimmune rabbit serum and anti-CSADCase, anti-GluDCase, or anti-ChoAcTase preabsorbed with the corresponding purified antigen. The appropriate conjugated second antibodies were used for the indirect peroxidase-labeled second antibody method and, in some instances, followed by the peroxidase-antiperoxidase (PAP) reaction. For the monoclonal antibodies, treatment with peroxidase-labeled goat anti-mouse immunoglobulin was followed by the application of mouse PAP or fluorescein-labeled goat anti-mouse immunoglobulin. For the polyclonal antibodies, peroxidase-labeled goat anti-rabbit immunoglobulin (PGARIg) was followed by rabbit PAP or fluorescein-labeled goat anti-rabbit immunoglobulin. The peroxidase reactions were visualized with diaminobenzidine (DAB).

Central nervous system motor neurons in medullary brainstem and cervical and thoracic spinal cord were examined in Vibratome sections. Swiss albino mice of both sexes (body weight, 6–20 g) were used. Tissues were fixed and Vibratome

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Abbreviations: AcCho, acetylcholine; AcChoEase, acetylcholinesterase; BTX, α-bungarotoxin; ChoAcTase, choline acetyltransferase; CSADCase, cysteine sulfinic acid decarboxylase; DAB, diaminobenzidine; GABA, γ-aminobutyric acid; GluDCase, glutamic acid decarboxylase; PAP, peroxidase-antiperoxidase; PGARIg, peroxidase-labeled goat anti-rabbit immunoglobulin; TyrOHase, tyrosine hydroxylase.

sections were processed for light microscopic immunocytochemical study according to described procedures (15). Animals were injected intraperitoneally with demecolcine (Sigma, 1 mg/kg) or intrathecally with colchicine (1 mg/kg) 16 hr before transcardiac perfusion with 4% formaldehyde in 0.12 M phosphate buffer (first at pH 6.5 and then at pH 11.0) at 4°C. The tissues were left overnight in the same fixative at pH 7.4. Loose 20-μm sections were incubated with antibodies and control sera at dilutions of 1:1,000. Unfixed cryostat sections of mouse spinal cord (injected with colchicine intrathecally) and human muscle were also examined after immunocytochemical processing.

Specimens from different muscles and species were sampled as follows: from mouse and rat—tongue, masseter, omohyoid, sternothyroid, sternocleidomastoid, digastric, gastrocnemius, soleus, anterior tibial, hand and toe extensors, and interossei; from guinea pig—gastrocnemius; and from monkey (*Macaca mulatta*)—biceps, intercostal, and dorsal interosseus. Diagnostic muscle biopsy specimens from six humans were used: deltoid (79-yr-old man), triceps (67-yr-old woman), deltoid (42-yr-old woman), and vastus lateralis (two 24-mo-old boys and one 18-mo-old girl). Serial cryostat sections (5 μm) were cut in transverse, oblique, or longitudinal planes. Mouse tongue and skeletal muscle fixed by cardiac perfusion were also embedded in paraffin, serially sectioned at 5 μm, and examined immunochemically.

Adjacent 5-μm serial sections were incubated overnight at 4°C with the primary antibody (1:400 to 1:1,000 in buffer), rinsed extensively, treated with PGARIg (1:400, 2 hr, room temperature), rinsed, and treated with 0.05% DAB and 0.01% H_2O_2. In double-staining experiments, cholinergic markers were localized as follows: (*a*) AcChoEase in cryostat sections by either the AE-3 antibody or the α-naphthyl acetate method (21) with preincubation in the esterase inhibitor 0.1 mM tetraisopropyl pyrophosphoramide; (*b*) AcCho receptor by using rhodamine-labeled α-bungarotoxin (BTX).

The intensity of immunochemical reactions in the sections was estimated independently by two investigators using a four-step personal grading system for stained elements in comparison to background. Comparisons of staining were made between different muscle groups in the same species (rat, mouse) and between the same muscle groups in the various species. The specificity of the reactions was confirmed by comparison with sections incubated with control sera.

Electron Microscopy. Slips of the first dorsal interosseus muscle, complete from origin to insertion, were removed from an anesthetized monkey; biceps muscle was sampled from an anesthetized patient. The excised muscles were pinned to a wax plate, fixed by injection of 4% formaldehyde/0.2 M sodium cacodylate, pH 7.4, at room temperature and then left in the fixative on ice for 2 hr. The fixed muscle preparations were teased, and end plate regions were located in small samples, as described (22), and treated in the following sequence: 10% goat serum and 0.1% saponin in Tris pH 7.4 buffer (1/2 hr, 4°C); primary antibody or control antibodies (1:100) with 1% goat serum, 2 hr at 4°C; buffer rinse; PGARIg (1:200) in 1% goat serum, 1 hr; buffer rinse; PAP (1:100) in 1% goat serum, 1 hr; buffer rinse; 2% glutaraldehyde/0.1 M cacodylate, pH 7.3, 15 min; 25 mM hydroxylamine, 30 min, to remove excess aldehyde; buffer rinse; 0.05% DAB and 0.01% H_2O_2, 30 min; postfixation in 2% osmium tetroxide, 15 min; staining with 1% uranyl acetate in 10% ethanol, 20 min. The tissues were then rapidly dehydrated through graded ethanols and embedded in Spurr resin. Single semi-thin sections were cut from each block and examined with phase-contrast optics for reactive end plates. Approximately 20 serial thin sections (60–80 nm thick) were cut, mounted on hexagonal copper grids, and examined in a Philips 400 electron microscope without further contrast enhancement.

RESULTS

Large and medium-sized neurons in the hypoglossal nucleus and spinal cord displayed immunoreactivity to the antibodies raised against the transmitter synthesizing enzymes (ChoAcTase, TyrOHase, CSADCase, and GluDCase) as well as to antibodies against AcChoEase. Background staining or reactions obtained with control sera (preimmune, preabsorbed CSADCase, preabsorbed GluDCase, preabsorbed ChoAcTase) were negligible. The results are shown in Table 1 and Figs. 1 and 2.

Both monoclonal and polyclonal ChoAcTase antibodies revealed immunoreactive cell bodies that were also reactive with AE-3 in adjacent serial sections. ChoAcTase antibody consistently produced the most intense reactions in neuronal perikarya, primary dendrites, large- and small-caliber axons, and terminals in the immediate area. The monoclonal antibodies against ChoAcTase and AcChoEase revealed similar numbers of neurons but with less intense reactivity against a very faint background. These antibodies stained approximately 40% of the large hypoglossal, cervical, and thoracic motor neurons. CSADCase antibodies also showed a sharp reaction in approximately 40% of motor neurons in both locations. This immunoreactivity was not evident in material incubated in anti-CSADCase preabsorbed with purified antigen. TyrOHase immunoreactivity was present in about 20% of neurons but was weaker than that for either ChoAcTase or CSADCase. This reaction was enhanced slightly in material obtained after intrathecal colchicine injections. In all cases, GluDCase antibodies evoked weak reactivity in a few cell bodies and large axons which was not much greater than that achieved with preimmune serum but which was distinctly greater than the weak generalized staining with anti-GluDCase preabsorbed with purified GluDCase antigen. Unfixed cryostat sections from mouse and human spinal cord specimens reacted with all six antibodies, corroborating results obtained with fixed Vibratome sections.

Neuromuscular Junctions. In all species examined and with all antibodies tested, unfixed cryostat sections of muscle exhibited greater immunoreactivity than did aldehyde-fixed muscle. Fixation with 4% formaldehyde decreased but did not completely eliminate immunoreactivity. Detectable reactions with all four antibodies were obtained with fixed muscle in both cryostat and paraffin sections. Addition of 0.1% glutaraldehyde to the primary fixation eliminated immunoreactivity.

Neuromuscular junctions from man, monkey, guinea pig, rat, and mouse displayed immunoreactivity for ChoAcTase, CSADCase, TyrOHase, and GluDCase with varying intensity (Fig. 3). These reactivities in every case were stronger than and were readily differentiated from background in the same material and

Table 1. Incidence and intensity of immunoreactivity of six primary antibodies and control sera in mouse motor neurons

	Hypoglossal nucleus		Spinal cord (ventral horn)	
Antibody	Incidence	Intensity	Incidence	Intensity
ChoAcTase (monoclonal)	+	+	+	+
ChoAcTase (polyclonal)	++	+++	++	+++
AE-3	+	+	+	+
CSADCase	++	++	++	++
TyrOHase	+	+	+	+
GluDCase	(+)	(+)	(+)	(+)
Preimmune	(+)	(+)	(+)	(+)
Preabsorbed CSADCase	−	−	−	−
Preabsorbed GluDCase	−	−	−	−
Preabsorbed ChoAcTase	−	−	−	−

PGARIg and PAP were applied to fixed Vibratome sections of spinal cord and medulla. Reaction scale: (+), barely visible; +, light brown; ++, medium brown; +++, dark brown.

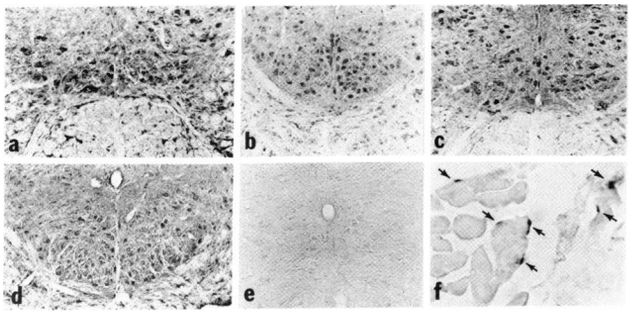

FIG. 1. Vibratome sections (25 μm) of mouse hypoglossal nucleus exposed to anti-ChoAcTase (*a*), anti-AcChoEase (*b*), anti-CSADCase (*c*), and anti-TyrOHase (*d*) or treated with preimmune serum (*e*), followed by PGARIg and PAP. Note that larger motor neurons are reactive in all preparations and negative in the control. (×60.) (*f*) Paraffin sections (5-μm) of mouse tongue exposed to CSADCase. Note the six reactive motor end plates in the field (arrows). (×150.)

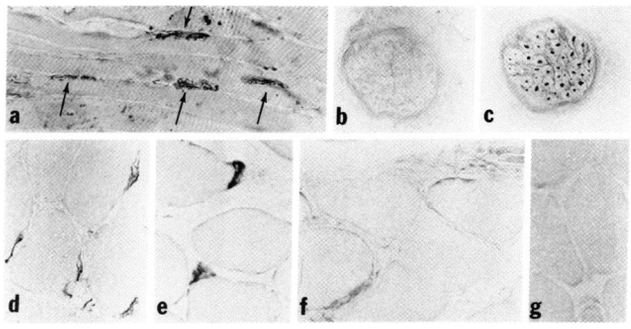

FIG. 3 (*a*) Six CSADCase-reactive motor end plates (arrows) in mouse interosseus muscles. (Paraffin-embedded, 5-μm section, PGARIg/PAP reaction; ×200.) (*b* and *c*) ChoAcTase-reactive myelinated axons (*c*) in a nerve bundle cut in cross section compared to negative control (*b*). (Unfixed cryostat sections; ×300.) (*d–g*) Motor end plates in human skeletal muscle exposed to anti-ChoAcTase (*d*), anti-TyrOHase (*e*), anti-GluDCase (*f*), and preimmune control serum (*g*). (*d*, ×250; *e–g*, ×375.)

were not present with preabsorbed control sera (Fig. 3 *b* and *g*). Each of the four antibodies tested appeared to have a species-specific spectrum of immunoreactivity (Table 2). Reactivity was generally highest in man and monkey. Immunoreactivity for ChoAcTase and CSADCase was strong in axons (Fig. 3*c*) as well as nerve terminals (Fig. 3 *a* and *d*); however, with TyrOHase and GluDCase, more reaction product appeared in the terminals (Fig. 3 *e* and *f*) than in axons. A survey of muscles from the tongue (Figs. 1*f* and 4 *a*) and representative groups of extensors, flexors, and small muscles of the forepaw in rat and mouse (Fig. 3*a*) demonstrated that all end plates in a species reacted consistently. Immunoreactivity to specific antibody did not vary with the muscle group or, in the human specimens, with sex or age of subject.

The specificity of the antibody reaction for the motor end plate was demonstrated in the following way. The end plates in a muscle were first identified in a section stained for AcChoEase by the α-naphthyl acetate method with esterase inhibitor, and adjacent serial sections were then stained with any one of the four antibodies and control serum. The identified group of end plates was located in each of the antibody-treated sections and compared for location, number, and intensity of antibody staining. ChoAcTase-immunoreactive end plates matched locations (*i*) stained by α-naphthyl acetate (Fig. 4), (*ii*) immunoreactive for AcChoEase (Fig. 4*g*), and (*iii*) reactive for BTX labeled with rhodamine (Fig. 4 *b* and *c*). The synthesizing enzymes were localized to the nerve terminals and the esterase reaction, or BTX binding sites, was localized to a separate adjacent site in every case in which section orientation allowed the observation of both reactive partners at the end plate. Double-labeling experiments on muscle preparations treated first with CSADCase antibodies and then with α-naphthyl acetate in the presence of esterase inhibitor (Fig. 4 *h–j*) or with AE-3 antibody against AcChoEase provided similar results. Comparable observations were made with double labeling first with TyrOHase or GluDCase and then with α-naphthyl acetate or AE-3 for confirmation of end plate location. These results show that vertebrate skeletal muscle end plates are associated with nerve fibers and terminals which can contain any of the synthesizing enzymes considered here.

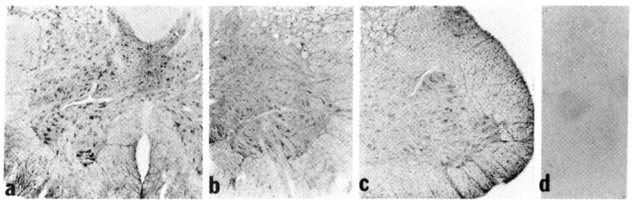

FIG. 2. Vibratome sections (25 μm) through the cervical spinal cord of the mouse exposed to anti-CSADCase (*a*), monoclonal anti-ChoAcTase (*b*), anti-TyrOHase (*c*), and anti-CSADCase preabsorbed with excess antigen (*d*) followed by PGARIg and PAP. Note that large motor neurons in the ventral horn are reactive in all preparations and negative in the control. (×160.)

Electron Microscopy. This study was performed on neuromuscular junctions of monkey interosseus muscle that had been exposed to antibodies against ChoAcTase or CSADCase and on human biceps muscle exposed to antibodies against TyrOHase (Fig. 5). The reaction product in each case was confined to the nerve terminal at the end plate. No reaction product appeared extracellularly or on the postsynaptic membrane. Within the nerve terminal the electron-dense reaction product was associated with cytoplasmic matrix, mitochondria, and synaptic vesicles. In rare instances the content of a single synaptic vesicle (60 nm) was electron dense in sections treated for ChoAcTase.

In the preparations for electron microscopy, the intensity of the reaction was directly related to the effectiveness of the saponin (detergent) treatment, which renders the membranes of intact nerve terminals permeable to immunoreagents.

DISCUSSION

This study provides evidence that the synthesizing enzymes for AcCho, catecholamine, taurine, and GABA are present in neuromuscular junctions of tongue and somatic musculature. These

Table 2. Intensities of immunoreactivity of ChoAcTase, CSADCase, TyrOHase, and GluDCase and control sera in neuromuscular junctions in five species

Antibody	Human	Monkey	Guinea pig	Rat	Mouse
ChoAcTase	+++	+++	+	+	++
CSADCase	++	++	+	+	+
TyrOHase	+++	++	+	+	(+)
GluDCase	+	+	+	(+)	(+)
Preimmune	(±)	(±)	(±)	(±)	(±)
Preabsorbed CSADCase	−	−	−	−	−
Preabsorbed GluDCase	−	−	−	−	−
Preabsorbed ChoAcTase	−	−	−	−	−

The antibodies and PGARIg were applied to fresh cryostat sections of muscle. Reaction scale: (±), equivocal; −, negative; (+), barely visible; +, light brown; ++, medium brown; +++, dark brown.

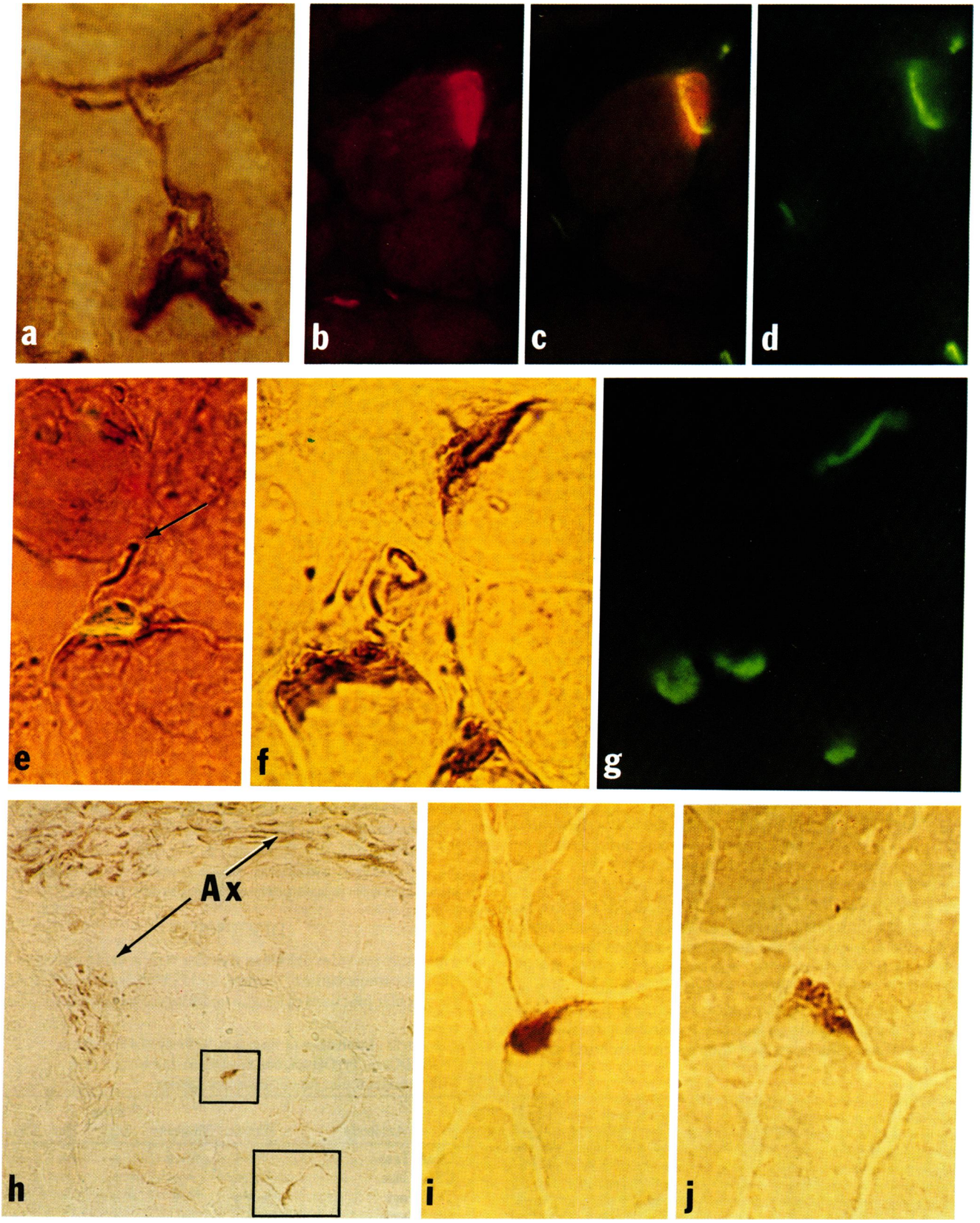

FIG. 4. (*a*) Bifurcating ChoAcTase reactive nerve fiber and terminal at a motor end plate in mouse tongue. The esterase site at the end plate is marked by red AcChoEase reaction product. The ChoAcTase-positive nerve terminal and the esterase sites are adjacent in the same end plate. (Unfixed cryostat section, PAP method; ×1,000.) (*b–d*) Human neuromuscular junction in a single section double-labeled with α-BTX conjugated to rhodamine (*b*) and antibody against ChoAcTase revealed with fluorescein (*d*). Both labels also are simultaneously displayed in a double-exposure photomicrograph (*c*). (Unfixed cryostat section. ×700.) (*e*) Human neuromuscular junction double-labeled in the same section with antibody against ChoAcTase visualized by the PAP reaction (brown) and with antibody against AcChoEase revealed by fluorescein (green). ChoAcTase is present in the nerve fiber (arrow). AcChoEase occurs in the immediately adjacent region. (Unfixed cryostat section. ×700.) (*f* and *g*) Human neuromuscular

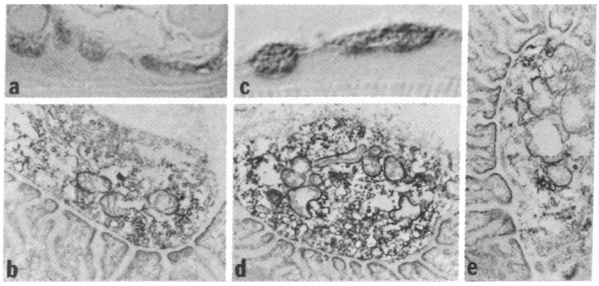

FIG. 5. Human neuromuscular junctions in muscle fibers exposed to antibodies against ChoAcTase (*a* and *b*), CSADCase (*c* and *d*), or TyrOHase (*e*) and then to PGARIg. *a* and *c* are micrographs made with phase-contrast and Nomarski interference optics, respectively, of sections adjacent to the thin sections in electron micrographs *b* and *d*. Note that the label lies in the nerve terminal associated with the cytoplasmic matrix and attached to synaptic vesicles and mitochondria. (*a*, ×1,000; *b*, ×9,500; *c*, ×1,250; *d*, ×8,500; *e*, ×11,000.)

enzymes are ubiquitous in muscles of all groups studied. However, their levels of detectability vary: ChoAcTase > TyrOHase > CSADCase > GluDCase. This variation may be explained by differences either in reactivity of each antibody or in amount of enzyme. In the somata and axons of large motor neurons, the same enzymes are detected in varying amounts, in the order: ChoAcTase > CSADCase > TyrOHase > GluDCase. Perikaryal enzyme levels are highest after pretreatment with colchicine or demecolcine in the living animal. These substances retard axoplasmic flow (23), thus causing accumulation of proteins and rendering the enzymes more readily detectable by immunocytochemistry. Nevertheless, the strong immunoreactivity for ChoAcTase and CSADCase in neuronal perikarya, axons, and motor end plates indicates that these enzymes exist throughout the neuron. However, this does not hold for TyrOHase and GluDCase, which display stronger reactivity in the motor end plates than in the axon and cell body. This difference suggests that more synthesizing enzyme is present in the terminals than in the perikarya, which is consistent with the likelihood that these substances are used at the junction for nerve–muscle communication.

For each antibody raised against the four synthesizing enzymes, a spectrum of species specificity has been demonstrated. This might be explained either by species-dependent variations in enzyme epitopes or by variations in the amount of enzyme present in the neuromuscular junction in the various species. In agreement with others (24, 25), this study indicates that fixation may so change the molecular conformation of epitopes that they are no longer recognized by the antibody molecules. For the light microscopic studies of the nerve–muscle junction we used frozen unfixed muscle, in which detectable enzyme levels are highest. This has not compromised the sharpness of the resulting localization because immunoreactivity remained confined to the motor end plate region. The electron microscopic data unambiguously confirmed the localization of these transmitter synthetic enzymes in the nerve terminal.

Because double-labeling experiments indicated that each of the four synthetic enzymes can be clearly localized to motor end plates displaying AcChoEase activity or AcCho receptors, a fundamental question is raised: Do nerve–muscle junctions contain other neuroactive substances in addition to AcCho? This will be addressed separately (26).

The role of AcCho in nerve–muscle junctions has been scrutinized for almost 5 decades without suspicion that additional neuroactive substances exist. The present demonstration of enzymes synthesizing catecholamine, taurine, and GABA in nerve–muscle junctions directs attention to the role of these substances in the innervation of muscle. They could act as tropic factors, as modulators of neurotransmission, or as possible participants in chemical transmission itself. Finally, the present study provides tools for assessing the relationship of each transmitter-synthesizing enzyme to human muscle diseases.

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junctions with antibody against ChoAcTase shown by the peroxidase reaction (brown) (*f*) and with antibody against AcChoEase shown by fluorescein (green) (*g*) in the same unfixed cryostat section. (×1,000.) (*h–j*) Human nerve fibers in large nerve bundles (Ax) and two neuromuscular junctions (boxes) with CSADCase immunoreactivity. The motor end plates in the lower and upper boxes are shown at higher magnification *i* and *j*, respectively. The CSADCase-positive nerve fiber and terminals are identified by a peroxidase reaction (brown). The red AcChoEase reaction product is juxtaposed at the same end plates. (Unfixed cryostat sections; *h*, ×100; *i* and *j*, ×700.)