Shared antigenic determinant between the *Electrophorus* acetylcholine receptor and a synaptic component on chicken ciliary ganglion neurons

(monoclonal antibodies/ultrastructure/ α -bungarotoxin binding sites/cholinergic)

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Communicated by Theodore H. Bullock, February 2, 1984

ABSTRACT Monoclonal antibodies raised against purified acetylcholine receptor from muscle and electric organ were tested for cross-reaction with surface components on chicken ciliary ganglion neurons. Indirect immunofluorescence indicated that antibodies to a determinant in the “main immunogenic region” of the receptor bind to the neurons in culture. Ultrastructural studies on 16-day embryonic ganglia, using horseradish peroxidase-conjugated monoclonal antibody, revealed that most of the conjugate labeling was associated with synaptic membrane on the neurons. A lesser amount of labeling was associated with the short processes extending from the neuronal somata in the region of preganglionic innervation. The labeling was blocked by coincubation with unlabeled antibodies of appropriate specificity and not by nonimmune serum. The pattern of labeling was clearly different from that previously found for a horseradish peroxidase conjugate of α -bungarotoxin: the toxin conjugate bound extensively to the short processes but not to synaptic membrane on the neurons. The synaptic antigen identified here by the cross-reacting antibodies is a candidate for the synaptic acetylcholine receptor on chicken ciliary ganglion neurons.

Chicken ciliary ganglion neurons have nicotinic acetylcholine (AcCho) receptors that mediate chemical synaptic transmission through the ganglion (1, 2). Efforts to study the regulation and distribution of such receptors on neurons have been limited by the absence of suitable probes. α -Bungarotoxin has been a very useful probe for studying nicotinic AcCho receptors in vertebrate skeletal muscle and electric organ, where the toxin binds tightly and specifically to the receptor and blocks its function (3). Ciliary ganglion neurons also have high-affinity binding sites for α -bungarotoxin, but in this case the binding sites appear to be distinct from synaptic AcCho receptors. Ultrastructural studies demonstrate that toxin binding sites on the neurons are located in the immediate vicinity of presynaptic terminals but are not present in the postsynaptic membrane (4), as would be expected for synaptic receptors. Moreover, α -bungarotoxin does not block AcCho receptor function on the neurons (5, 6), and, when the levels of AcCho sensitivity and toxin binding are compared for the neurons grown under various conditions in cell culture, no simple correlation is found between the two properties (7), as would be expected if they represented the same membrane component.

An alternative approach is to use antibodies against the AcCho receptor as probes. Monoclonal antibodies (mAbs) against AcCho receptors from skeletal muscle and electric organ have been useful in studying the structure and synthesis of these receptors (8–12). Recent studies have shown that mAbs specific for each of the four subunits of AcCho recep-

tors from muscle and electric organ bind to neurons in the lateral spiriform nucleus of chicken brain (13). These neurons do not bind α -bungarotoxin. Best cross-reaction was obtained with mAbs to the “main immunogenic region” (MIR) that is exposed on the extracellular surface of receptor α subunits (14–16).

We report here that mAbs to the MIR preferentially bind to the synaptic membrane on ciliary and choroid neurons in embryonic chicken ganglia. This represents suggestive evidence that the mAbs recognize a class of neuronal AcCho receptors.

MATERIALS AND METHODS

Antibody Preparation. Hybridoma cell lines were obtained by fusing myeloma cell lines with spleen cells from rats immunized with purified AcCho receptor protein. mAbs 6, 35, and 203 were raised against receptors from *Torpedo* (14) and *Electrophorus* electric organ (15) and human skeletal muscle (16), respectively. mAb stocks were prepared by ammonium sulfate precipitation of hybridoma culture media. mAb 35 was affinity purified by using goat anti-rat IgG coupled to agarose. Horseradish peroxidase-conjugated affinity-purified mAb 35 (HRP-35) was prepared as described (17). Affinity-purified goat anti-rat IgG was labeled with fluorescein using fluorescein isothiocyanate and contained 6.9 fluorescein molecules per goat IgG (17).

Cell Culture. Ciliary ganglion neurons from 8-day White Leghorn chicken embryos were grown in dissociated cell culture for 1 wk at 10^4 neurons per dish as described (18) with the following modification: 35-mm Falcon tissue culture plastic dishes were used containing three 12-mm Propper glass coverslips coated with collagen and lysed fibroblast residue to promote attachment of the neurons to the glass coverslip for subsequent fluorescence microscopy.

Fluorescence Microscopy. Ciliary ganglion neurons cultured on glass coverslips were labeled by indirect immunofluorescence (19) and examined by fluorescence microscopy as described (5). Briefly, the coverslip cultures were incubated for 1 hr at room temperature in 0.05 ml of Eagle's minimal essential medium (ME medium) plus 10% (vol/vol) horse serum and buffered with 5 mM Hepes instead of sodium bicarbonate (ME medium/Hepes) containing 0.1 μ M mAb and 17% (vol/vol) preimmune goat serum IgG. The cultures were rinsed with several changes of ME medium/Hepes, incubated in 0.05 ml of ME medium/Hepes containing 0.1 μ M fluorescein-conjugated goat anti-rat IgG for 0.5 hr at room temperature, rinsed in phosphate-buffered saline containing divalent cations and 2 mg of bovine serum albumin per ml, fixed in 5% acetic acid/95% ethanol for 10 min at

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Abbreviations: AcCho, acetylcholine; mAb, monoclonal antibody; HRP, horseradish peroxidase; HRP-35, HRP-conjugated mAb 35; MIR, “main immunogenic region” of the α subunit of AcCho receptor from muscle and electric organ.

–20°C, and rinsed again. The coverslips were mounted in glycerol containing 5% *n*-propyl gallate to reduce fluorescence fading due to photobleaching (20) and examined with a Leitz Diavert fluorescence microscope.

Electron Microscopy. Ciliary ganglia from 16-day chicken embryos were dissected, incubated in 0.1 μ M HRP-35, rinsed, fixed, reacted for peroxidase activity, processed, and examined with a Philips 300 electron microscope as described (4, 21).

Materials. Culture medium consisted of ME medium containing 10% (vol/vol) heat-inactivated horse serum and 3% (vol/vol) embryonic chicken eye extract (18). Fluorescein isothiocyanate, HRP, and *n*-propyl gallate were obtained from Sigma.

RESULTS

Antibody Binding in Culture. Chicken ciliary ganglion neurons, grown on glass coverslips, were incubated with mAbs to AcCho receptors from skeletal muscle and electric organ. Binding was detected by using fluorescein-conjugated goat anti-rat IgG. Neurons are the main cell type present in such cultures (18) and can be easily distinguished by their rounded, phase-bright somas and extended neurites. Labeling of the neurons was obtained with mAbs 6, 35, and 203, all of which are directed at the MIR (Fig. 1*a*). All three mAbs gave similar patterns of labeling with thin fluorescent patches apparent on the neuronal somas, particularly in regions including neurites. Distal portions of the neurites were generally unlabeled but occasionally light punctate labeling was present on a small portion of their surfaces. Little fluorescence was associated with the substratum and the few non-neuronal cells in the culture. Substitution of nonimmune rat serum at a comparable concentration of immunoglobulin for the mAb did not result in significant labeling of the neurons (Fig. 1*b*).

Ultrastructural Distribution of the Neuronal Determinant. HRP-35 was prepared to determine the distribution of the mAb 35 antigen on ciliary ganglion neurons at the ultrastructural level. Ganglia from 16-day chicken embryos were used for these studies to facilitate identification of mAb binding at differentiated synapses. Clearly identifiable synapses on ciliary ganglion neurons are characterized by a parallel arrangement and thickening of the pre- and postsynaptic membranes, a widened synaptic cleft, an accumulation of synap-

tic vesicles along the presynaptic membrane, and an enhanced postsynaptic density (22–24).

Ganglia were incubated with HRP-35, reacted for peroxidase activity, and examined with the electron microscope. Dense HRP reaction product was present on synaptic membranes and filled the clefts of synapses on both ciliary and choroid neurons (Figs. 2 and 3). Labeled synapses were present both on the smooth portion of the cell soma and on the short processes emerging from the postsynaptic cell in the region of preganglionic innervation. Reaction product did not extend beyond the specialized synaptic contact zones in areas of pre- and postganglionic cell apposition.

To quantitate the proportion of labeled synapses in 16-day embryonic ganglia, cells were selected that had at least some labeling associated with their surface membrane. This ensured that the antibody conjugate was able to penetrate the ganglion in the region occupied by the cells. Of the clearly identifiable synapses present on 38 such neurons, 82% (132 of 162) were heavily labeled.

Dense reaction product was also present on a portion of the numerous small processes that emerge from the postsynaptic cell in the vicinity of the presynaptic terminals (Figs. 2*a*, 3*b* and *c*, and 4). Usually only a small fraction of the surface membrane of the processes was labeled. HRP reaction product was not present elsewhere on the neuron surface membrane and was not associated with other cell types in the ganglion.

Both synaptic and process labeling by HRP-35 was specific in that it was blocked by other mAbs of the same specificity. Incubation of the ganglia in a 2- to 28-fold excess of mAb 6, 35, or 203 prior to and along with 0.1 μ M HRP-35 prevented labeling (Fig. 5). Nonimmune serum at a 28-fold excess concentration of immunoglobulin did not prevent the labeling obtained with HRP-35. Little if any endogenous labeling of the tissue was apparent when nonimmune serum was substituted for HRP-35 in the incubation.

DISCUSSION

The findings reported here demonstrate that at least one antigenic determinant present in the MIR of skeletal muscle and electric organ AcCho receptors is also present on the surface of chicken ciliary ganglion neurons. Furthermore, the antigen is preferentially located in the synaptic membrane on the neurons. Since the antibody labeling was car-

FIG. 1. Binding of anti-MIR mAbs to a surface component on ciliary ganglion neurons in culture. Neurons were grown for 6 days on glass coverslips and then incubated for 1 hr either in 0.1 μ M mAb 203 (*a*) or in nonimmune rat serum at an equivalent concentration of immunoglobulin (*b*). After washing, the cultures were incubated 0.5 hr at room temperature with fluorescein-conjugated goat anti-rat IgG antiserum, rinsed, fixed, and examined with fluorescence microscopy. Thin patches of fluorescent staining are present along a portion of the surfaces of the two bright rounded neuron cell bodies in *a*. The portion of the neuron surface that is stained often includes neurites (arrows). Distal portions of the neurites are not labeled (arrowheads). There is essentially no fluorescent staining present on the surfaces of neurons (double arrows) in *b*. ($\times 285$.)

FIG. 2. Ultrastructural distribution of HRP-35 binding on choroid and ciliary neurons in embryonic ganglia. The synaptic membranes (arrows) of a ciliary (*a*) and a choroid (*b*) neuron from a 16-day embryonic chicken ganglion are heavily labeled following incubation in $0.1 \mu\text{M}$ HRP-35 and HRP reaction. Labeling does not extend beyond the specialized synaptic zones (see text) in areas of pre- and postganglionic cell apposition. Reaction product is also present along a small portion of the surface of the small processes (arrowhead) that project out from the ciliary neuron (*a*) in the vicinity of the large presynaptic terminal or calyx. The surface of the small processes emerging from this particular choroid neuron (*b*) are not labeled. (*a*, $\times 50,000$; *b*, $\times 81,900$.)

FIG. 3. Binding of HRP-35 to synaptic membranes. A 16-day embryonic chicken ciliary ganglion was incubated with $0.1 \mu\text{M}$ HRP-35 and examined. Heavy labeling is associated with synapses (arrows) on the smooth portion of the somata (*a-c*) and on the small processes (*c*) of the ciliary neurons shown, filling the synaptic clefts. Labeling is also present on a small portion of the surface membranes of the small processes that project out from the postsynaptic ciliary cells (arrowheads in *b* and *c*). (*a*, $\times 46,400$; *b*, $\times 38,200$; *c*, $\times 65,100$.)

FIG. 4. Binding of HRP-35 to neuronal processes. A 16-day embryonic ciliary ganglion was incubated with HRP-35, reacted for peroxidase activity, and examined. Heavy labeling is associated with a portion of the surface membrane of the numerous small processes (p) of a ciliary neuron (arrowheads). The processes emerge from the postsynaptic ciliary cell in the region of the preganglionic innervation (i). Most of the surface membrane of the processes is not labeled. ($\times 41,000$.)

ried out on intact cells, only surface binding would have been expected. No information is available from these studies about possible intracellular sites as suggested by the heavy labeling of perikaryal cytoplasm seen with frozen sections of the chicken lateral spiriform nucleus (13).

In receptors from muscle and electric organ the MIR is located on the extracellular surface of the α subunit, but it is different from the AcCho binding site and does not depend on the carbohydrate portion (8). mAb binding to the determinant does not block AcCho receptor function either in electric organ (8) or on ciliary ganglion neurons (unpublished results). Lack of an effect on receptor function makes it premature to conclude that the neuronal determinant is in fact associated with the neuronal AcCho receptor. It will be important to determine whether other antigenic determinants from muscle and electric organ AcCho receptors can be

identified in the ganglion as they are in the lateral spiriform nucleus of chicken brain (13). It will also be important to examine and understand the relationship between levels of AcCho sensitivity on the neurons and numbers of antibody binding sites.

Precedents for shared determinants between neuronal AcCho receptors and AcCho receptor of electric organ come from studies with polyclonal sera. Patrick and Stallcup (25) found that a rabbit antiserum raised against AcCho receptor purified from *Electrophorus* was able to inhibit AcCho receptor function on the pheochromocytoma cell line PC12. Marshall (26) found that a rabbit antiserum raised against AcCho receptor purified from *Torpedo* cross-reacted with a synaptic component on frog sympathetic neurons. Cross-reactivity has also been reported recently for a component in chicken sympathetic neurons with two mAbs that recognize the ligand binding site of *Torpedo* AcCho receptor (12).

The significance of HRP-35 binding on the small processes extending from the neuronal somata is unclear. The labeling represents a small fraction of the total HRP-35 binding and is far less than the labeling seen on the processes when the ganglion is incubated with HRP-conjugated α -bungarotoxin (4). Process labeling is not only much more extensive with the toxin conjugate but in fact represents the major portion of toxin sites in the ganglion revealed by this conjugate. Synaptic membranes on the neurons are not labeled by HRP-conjugated α -bungarotoxin, in contrast to the dense labeling of synaptic membranes by HRP-35. Both HRP-35 and the toxin conjugate should have had ample access to all surface membranes on the neurons since an HRP conjugate of a mAb thought to recognize the Na^+, K^+ -dependent ATPase (kindly provided by J. Lippincott-Schwartz and D. Fambrough) heavily labels the entire surface of the neurons *in situ*, including soma, process, and synaptic membranes (4). These results are consistent with those reported by Swanson *et al.* (13) on chicken brain and suggest that at least a substantial fraction of the mAb 35 surface determinants on the ganglionic neurons represents a class of molecules separate from those associated with α -bungarotoxin binding. This is in contrast to AcCho receptors in muscle and electric organ, where the MIR and the toxin binding site are both associated with the α subunit (8).

Efforts to identify shared antigenic determinants between neuronal α -bungarotoxin binding sites and AcCho receptors from muscle and electric organ have met with mixed results.

FIG. 5. Specificity of the HRP-35 labeling in ciliary ganglia. Sixteen-day embryonic ganglia were incubated with an excess of mAb 35 (a) or mAb 203 (b) in addition to $0.1 \mu\text{M}$ HRP-35 and were processed and examined. mAbs 35 and 203 are known to compete for binding to the MIR of muscle and electric organ AcCho receptors (15, 16). Synapses (arrows) and processes (arrowheads) on the neurons are not labeled. A dense subsynaptic bar is present parallel to and beneath the postsynaptic density in a synapse on the soma in a. (a, $\times 53,500$; b, $\times 51,200$.)

Partial cross-reaction has been reported for antisera raised against purified *Torpedo* AcCho receptor when tested against an α -bungarotoxin binding component in rat brain (27, 28) and chicken retina (29). Partial cross-reaction has also been reported between chicken brain α -bungarotoxin binding sites and an antiserum raised against purified denervated muscle AcCho receptor from cat (30). On the other hand, no cross-reaction was found between a series of mono- and polyclonal antibodies raised against AcCho receptor purified from a variety of muscle and electric tissue sources and tested against α -bungarotoxin binding components in rat brain (31). Similarly, an early study failed to find cross-reaction between the α -bungarotoxin binding site on PC12 cells and an antiserum raised against purified eel AcCho receptor that did block AcCho receptor function on the cells (25).

The relationship between the nicotinic AcCho receptor on ciliary ganglion neurons, the α -bungarotoxin binding site, and now the MIR determinant is intriguing and clearly different from that found for muscle. The synaptic component binding the anti-MIR mAbs on the neurons is a candidate for the synaptic AcCho receptor. The significance of the α -bungarotoxin binding component remains unknown.

We thank Dr. Albee Messing for valuable discussions and for describing to us his related immunofluorescent studies on antibody binding to ciliary ganglion neurons. Expert technical assistance was provided by Marie Ryder and Alfredo Franco, Jr. This work was supported by research grants from the National Institutes of Health (NS 12601 and NS 11323), the Muscular Dystrophy Association, and the American Heart Association with funds contributed in part by the California Heart Association.

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