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Characterization and Partial Purification of the Acetylcholine Receptor from *Torpedo* Electroplax

(acetylcholine binding/acetylcholinesterase/chemical modification/electrofocusing)

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ABSTRACT Binding of acetylcholine in the concentration range 1 nM–1 μM was measured by equilibrium dialysis to a particulate preparation of *Torpedo* electroplax, without or with prior treatment of the tissue with one of three chemical modifying reagents. Significant reduction in binding of acetylcholine resulted after treatment with 1,4-dithiothreitol, *p*-chloromercuribenzoate, or *p*-(trimethylammonium)-benzenediazonium fluoroborate. Partial reversal of the reduction in binding occurred when dialysis was performed in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid) or potassium ferricyanide (in the case of treatment with dithiothreitol), and 2-mercaptoethanol (in the case of treatment with *p*-chloromercuribenzoate). It is concluded that the functional acetylcholine-receptor macromolecule of *Torpedo* electroplax has disulfide bond(s), sulfhydryl group(s), and one or more of the amino acids vulnerable to diazotization by *p*-(trimethylammonium)-benzenediazonium fluoroborate. This, plus the effect of phospholipase C (EC 3.1.4.3) in elimination of detectable binding of acetylcholine after electrofocusing, is additional evidence that the functional acetylcholine receptor is a phospholipoprotein or a phospholipid–protein complex, which has a low isoelectric point of 4.5 ± 0.2, yet is denatured by exposure to low pH for 24 hr. Due to this adverse effect, recovery of binding of acetylcholine after electrofocusing, as detected by equilibrium dialysis or ultrafiltration, is only 23% and, so far, only 6.3-fold purification of functional acetylcholine receptors by this technique is possible.

Three or two forms of acetylcholinesterase (EC 3.1.1.7), whose peaks have isoelectric points ranging from 4.3 to 7.2, appear after electrofocusing of *Torpedo* extracted with 1% Triton X-100 or Lubrol, respectively. The major peak in either preparation has an isoelectric point of 5. Although the peaks of the functional acetylcholine receptors and of acetylcholinesterase of *Torpedo* electroplax are separable by electrofocusing, it has not been possible to isolate fractions that contain functional receptors but that are free of acetylcholinesterase. The opposite is possible.

The key to isolation of the acetylcholine (ACh) receptors is to identify which of the multitude of macromolecules present in tissue homogenates are the receptors. We have presented evidence that ACh receptors are present in subcellular preparations of electric tissues, based on the reversible binding of several cholinergic ligands (1–6). For purification of ACh receptors, we selected electric tissue of the electric ray, *Torpedo marmorata*, as the source because the concentrations of the receptors in tissues of *T. marmorata* are 25 times higher than in tissues of the electric eel, *Electrophorus electricus* (7). Although acetylcholine, nicotine, and muscarone exhibited similar binding characteristics, binding of ACh was chosen as the index of receptor activity because it is the natural transmitter. Use of other criteria, e.g., neurotoxin binding, may result in isolation of a part of a receptor, or a nonfunctional ACh receptor i.e, one that has lost the ability to bind its transmitter. As a first step toward purification, we solubilized tissues with several detergents. The receptor solubilized with Lubrol retained its drug specificity, as judged by the strong blocking effect of ACh-binding by 11 nicotinic drugs, and the neurotoxins, α-bungarotoxin and cobrotoxin, the little or no effect of six muscarinic drugs, and the lack of blocking effect by 21 noncholinergic drugs (8).

The use of chromatography on Sepharose 6B or of ultrafiltration resulted in elimination of much of the proteins, as well as in partial loss of ACh-binding, so that in fact no purification of specific functional ACh receptor, i.e., ACh-binding per mg of protein, was achieved (8). For successful purification of ACh receptor, better understanding of its chemical nature is essential. The present study deals with the *in vitro* identification of chemical groups in the ACh-receptor macromolecule, and the use of electrofocusing for its purification.

Abbreviations: S_2threitol, dithiothreitol; pI, isoelectric point; ACh, acetylcholine.

MATERIALS AND METHODS

Binding was measured by equilibrium dialysis (at 4° for 16 hr) in 100-ml volumes of a modified Krebs–Ringer solution (pH 7.4 and ionic strength 0.2 μ) containing various concentrations of [^{3}H]ACh (50 Ci/mol; from New England Nuclear Corp.). The organophosphate Tetram (*O,O*-diethyl *S*-diethylaminoethyl phosphorothiolate) was added (to a final concentration of 0.1 mM) to the tissue preparation 20 min before start of dialysis and also to the dialysis medium; in this way, acetylcholinesterase (EC 3.1.1.7) was totally inhibited throughout the experiment (4).

After dialysis, the excess radioactivity found in samples of the tissue over equal volumes of Ringer bath represented the amount of bound ACh. Each experiment was run three times and triplicate 0.1-ml samples were taken. Details of the procedure and preparation of the lyophilized pellet of 12,000 × *g* of *Torpedo* electroplax (9.3 mg of protein/g of tissue, used at a concentration of 0.5 g of tissue/ml) were described (1, 2).

For the study of the effect of chemical modifiers on ACh-binding by the particulate preparation, each reagent was added to the tissue preparation, which was then shaken intermittently for 30 min at room temperature (24°) before start of dialysis. In experiments where reversal of the action

of the reagent was attempted, the reversing chemical was added only to the Ringer bath used in dialysis.

In electrofocusing, we used the LKB Produkter column (110-ml capacity), which is attached to an ultraviolet (UV)-monitor and a refrigerated LKB fraction collector. After solubilization of the electroplax tissue with 1% Triton X-100 or Lubrol WX in water, the 10-ml supernatant at 100,000 × g for 1 hr mixed with Ampholine (with a spectrum of isoelectric points ranging from 3 to 10) was fed into the column in conjunction with a continuous sucrose gradient. The concentration of Ampholine was 1% and the sucrose gradient was from 0 to 50%. The voltage applied was about 300 and the run lasted 18 hr at 4°. 2-ml Fractions were then collected, and 10 ml of Ringer solution (containing 0.1 mM Tetram) was used to wash each 1 ml in an Amicon 8-MC ultrafiltration cell (Diaflo PM-10 nonionic membrane), so as to greatly reduce the concentration of sucrose and Ampholine. Binding of ACh was measured by equilibrium dialysis or by ultrafiltration in the above cell by a batch-addition method as described (9).

Acetylcholinesterase activity and protein content (after elimination of the Ampholine) were assayed by the methods of Ellman (10) and Lowry (11), respectively.

RESULTS

Chemical modification of the acetylcholine receptor

We measured binding of ACh to the particulate preparation of *Torpedo* electroplax over a wide range of ligand concentrations, in the absence or presence of each of the three following reagents:

1,4-Dithiothreitol (S_2threitol). Treatment of the tissue preparation with 1 mM S_2threitol, a reagent that reduces disulfide bonds, lowered the amount of ACh bound at all ligand concentrations tested (Fig. 1). Reduction was almost constant (35–50%) throughout the concentration range of ACh used. This inhibitory effect of S_2threitol on binding of ACh was partially reversed when the oxidizing Ellman reagent [5,5′-dithio bis-(2-nitrobenzoic acid); 10 μM] was added to the dialysis bath. Increasing the concentration of the reagent to 1 mM improved the recovery of binding slightly, but did not totally reverse the effect of S_2threitol. Potassium ferricyanide (at a concentration of 1 mM) produced similar recovery of binding to that detected when Ellman reagent was added. Neither of these chemicals (at concentrations of 1 mM) affected binding of ACh when S_2threitol was not previously used. On the other hand, the presence of 1 mM S_2threitol in the dialysis bath with ACh, without prior treatment of the tissue preparation with S_2threitol, did not reduce the binding of ACh.

p-Chloromercuribenzoate. This sulfhydryl reagent (at a concentration of 1 mM) had different effects on binding of ACh at low than at high concentrations. There was a significant reduction of ACh-binding only at ACh concentrations above 0.01 μM (Fig. 1), with a maximum reduction of 40% at a concentration of 0.1 μM ACh. This effect was partially reversed when 2-mercaptoethanol (at a concentration of 5 mM) was added to the dialysis bath.

p-(Trimethylammonium)-benzenediazonium fluoroborate. 30-min exposure of the electroplax preparation to the diazotizing agent *p*-(trimethylammonium)-benzenediazonium fluoroborate (at a concentration of 0.1 mM) caused about 50% irreversible reduction in ACh-binding through the whole

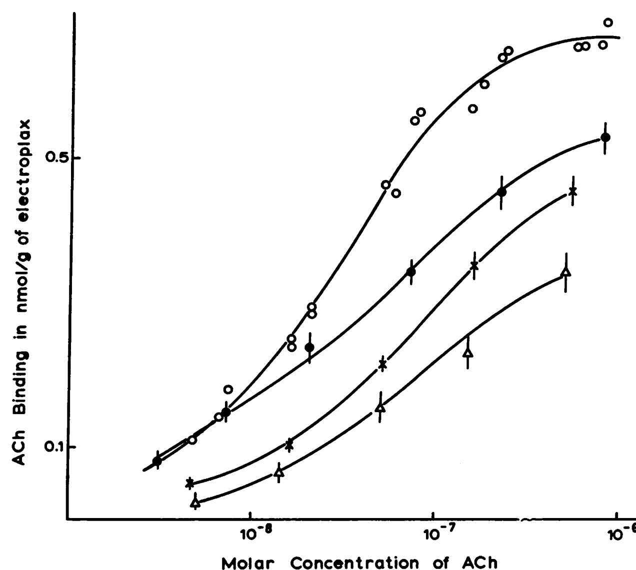

FIG. 1. Binding of ACh to a particulate preparation of *Torpedo* electroplax and reduction of this binding by prior treatment of the preparation with different reagents. The *vertical lines* at each point represent the standard deviation of nine test points. O—O, control; X——X, S_2threitol; ●——●, *p*-chloromercuribenzoate; Δ——Δ, *p*-(trimethylammonium)-benzenediazonium fluoroborate

concentration range (Fig. 1). At a concentration of 0.3 mM of the diazotizing agent, inhibition of ACh-binding was almost complete.

Hill plots of ACh-binding gave Hill coefficients of 1, 0.62, 0.61, and 0.68 for the control, and preparations treated with S_2threitol, *p*-chloromercuribenzoate, or *p*-(trimethylammonium)-benzenediazonium fluoroborate, respectively.

Electrofocusing of the acetylcholine receptor

When we electrofocused the clear 1% Triton extract of *Torpedo* electroplax, several precipitated bands appeared in the pH range from 4 to 5.2 (Fig. 2). Elevation of the Triton concentration in the column from 0.1 to 0.5% did not reduce the precipitation, but precipitates were solubilized by elevation of the pH of the collected fractions to 7 (with sodium hydroxide).

A disadvantage of using Triton is its strong absorption at 280 nm, so that protein absorption is totally masked (Fig. 3*a*). Analysis for acetylcholinesterase in the fractions showed a major peak with an isoelectric point (pI) of 5, preceeded by a smaller peak with a pI of 4.3. There was also a broad peak of acetylcholinesterase activity in the neutral range, showing maximum activity at pH 7.2. The fractions with pI from 4.2 to 4.8 (Fig. 3*a*) bound ACh. Measurement of ACh-binding, by ultrafiltration or equilibrium dialysis (after removal of most of the Ampholine and sucrose present), demonstrated the presence of the two binding sites. Their total concentration was 1.25 nmol/mg of protein, compared to 0.2 nmol/mg of protein for the Triton extract and 0.1 nmol/mg of protein for the particulate preparation. Thus, electrofocusing alone gave 6.3-fold purification, but the recovery of total ACh-binding was only 23%. Prior treatment of the electroplax extract with phospholipase C (EC 3.1.4.3; Worthington) before electrofocusing eliminated detectable ACh-binding by the fractions and reduced the amount of precipitation in the column.

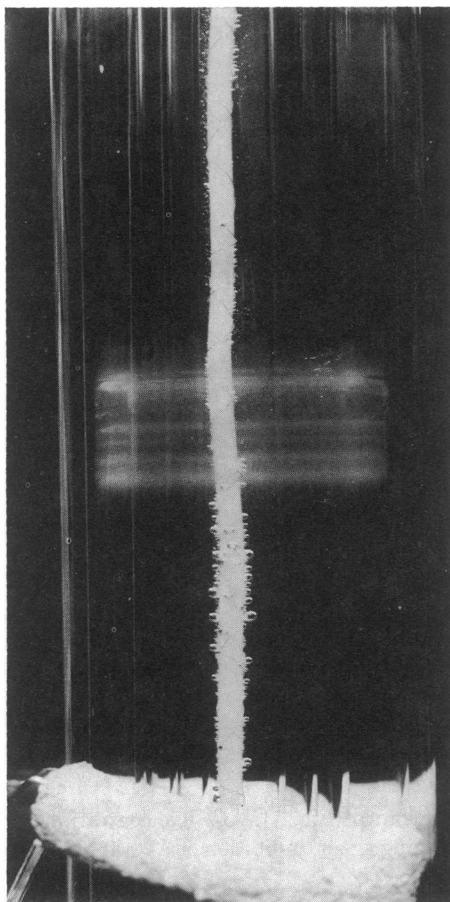

FIG. 2. Part of the electrofocusing column showing several precipitated bands (at pH range from 4 to 5.2) at the end of a run of a 1% Triton extract of *Torpedo* electroplax.

To obtain a fast estimate of the protein concentration in the fractions from their absorption at 280 nm, we had to overcome the high absorption of Triton X-100 at 280 nm and found a good substitute in Lubrol WX, which showed no absorption when present at a concentration of 0.1%. As shown in Fig. 3*b*, four protein peaks appeared at pH values ranging from 3.5 to 5.5. There was a single large peak of acetylcholinesterase with a pI of 5, and a much smaller one at higher pH. Fractions with pI ranging from 4.2 to 4.8 bound ACh as in the Triton experiment.

Suspecting that the low recoveries of ACh-binding were due to denaturation of ACh receptor as a result of the long exposure to low pH during electrofocusing, we tested the stability of ACh-binding to the Lubrol-solubilized preparation by leaving it in low-pH phosphate–citrate buffers at 4° for 20 hr before measuring ACh-binding in phosphate Ringer (pH 7.4). As shown in Table 1, low pH had a strong denaturing effect, but the presence of 30% sucrose gave partial protection. When electrofocusing was run for an additional 24 hr, no ACh-binding was detectable.

DISCUSSION

There are two sites that bind ACh at the concentrations used in this preparation of *Torpedo* electroplax: one is present at a concentration of 0.1 nmol/g of electroplax and has a dissociation constant (K) of 8 nM, and the other is present at a concentration of 0.83 8nmol/g of electroplax and has a K of 68 nM (5). The similar pharmacology of both sites (3), and the change in the Hill coefficient by solubilization (8), or by the present treatments with chemical agents, suggest that both sites are on the same macromolecule. It is interesting to note that the sites believed to be on ACh receptors in this tissue, but detected by α-[^{131}I]bungarotoxin binding, were found to be present in a concentration (1.1 nmol/g electroplax, ref 12) similar to that found for the additive concentration of ACh-binding sites (0.93 nmol/g of electroplax). It is evident that the active site of either acetylcholinesterase (which is phosphorylated by Tetram before and during dialysis, refs. 4 and 5) or choline acetyltransferase (whose activity is undetectable in this preparation pelleted at 12,000 × *g*, ref. 8) are not involved in the binding of ACh (at 0.05 μM) to acetylcholinesterase from bovine erythrocytes (Winthrop) at a concentration giving equivalent enzymic activity to that in the electroplax used (5, 8). The reagents used are known to affect many macromolecules, but since we are studying their effect on the macromolecule that binds ACh, which is believed to be ACh receptor, only the effects of the reagents on the specific receptors are monitored.

The results presented do not prove whether the groups that react with the reagents are in, near, or far from the active sites, for these reagents could affect binding by causing conformational changes in the ACh receptor macromolecules, or causing the dissociation of the receptor into subunits, as was found with *p*-chloromercuribenzoate on aspartate transcarbamoylase (13). When the tissue is not previously treated with S_2threitol, but the S_2threitol is added to the dialysis bath (at a concentration of 1 mM) along with ACh (see *Methods*) or muscarone (1), the binding of ACh or muscarone is unaffected. This insensitivity could be caused by the bound ligand making disulfide bond(s) inaccessible to attack by S_2threitol, either by steric hindrance or through conformational change of the receptor.

The earlier findings (13) that depolarization of the innervated membrane of the electroplax of electric eel by cholinergic ligands was reduced by prior treatment with S_2threitol, *p*-chloromercuribenzoate, or *p*-(trimethylammonium)-benzenediazonium fluoroborate led to the conclusion that ACh receptor or another component in the permeability chain is a protein. The presently observed effects of these reagents on the binding of ACh *in vitro* to a particulate preparations of *Torpedo* electroplax are qualitatively similar to their *in situ* effects on the depolarization of unicellular preparations of eel electroplax. This is despite the fact that these tissues—while having similar pharmacological properties (15)—are from two different species. However, there are

TABLE 1. *Recovery of ACh-binding (at 0.1 μM) by receptor from Lubrol-solubilized preparations after exposure to low pH, in 0.1 M phosphate–citrate buffer for 24 hr*

	% Recovery of ACh-binding	
pH	In absence of sucrose	In presence of 30% sucrose
4.5	0	5
5.5	12.3	29
6.5	22.6	30.5

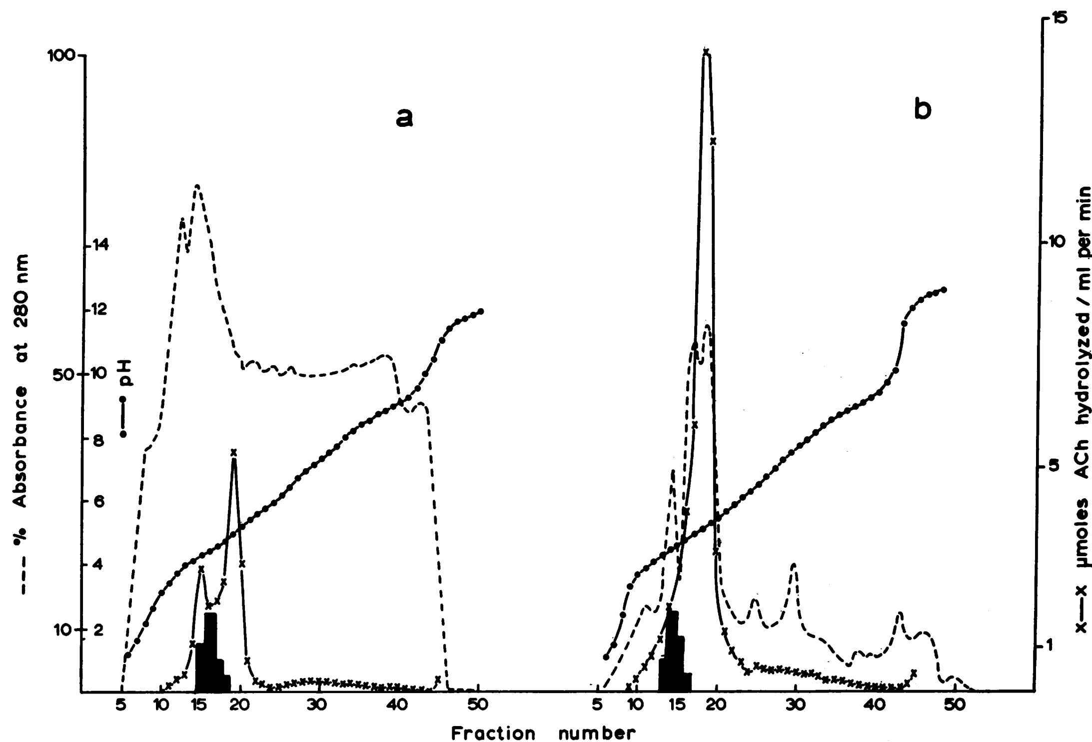

FIG. 3. Electrofocusing of (*a*) 1% Triton extract and (*b*) 1% Lubrol extract of *Torpedo* electroplax. The final concentration of detergent in the column is 0.1%. The *solid histograms* represent binding of ACh. The absorption at 280 nm is due mostly to the Triton in (*a*), but to the protein in (*b*).

quantitative differences, and the milder action of these reagents *in vitro* as compared to their *in situ* action may be due to their effects, in the latter case, on other components in the chain of events between binding and depolarization, in addition to their effect on ACh-binding.

Several conclusions can be drawn with regards to the chemical nature of these ACh-binding macromolecules. They have disulfide bond(s) and sulfhydryl group(s), as judged by the effects on ACh-binding of S_2threitol and *p*-chloromercuribenzoate, respectively (Fig. 1). Reduction of binding by *p*-(trimethylammonium)-benzenediazonium fluoroborate indicates that they also have one or more amino acids vulnerable to diazotization by this reagent (e.g., tyrosine, histidine, or lysine). This also indicates their partial protein nature, which we had previously suggested based on the effect of proteolytic enzymes (3, 5). In addition, their phospholipid nature is demonstrated by the disappearance of ACh-binding after treatment with phospholipase C [presently shown after electrofocusing and previously found for the particulate (3, 5) and solubilized ACh receptors 8]. This conclusion must be tentative in view of the fact that commercial enzymes, which might contain impurities, are used.

One may conclude that the ability to bind ACh may be dependent upon the integrity of a phospholipoprotein complex, and neither of its components is capable, by itself, of binding ACh. These ACh receptor macromolecules are also acidic, with a pI of 4.5 ± 0.2 as shown by electrofocusing (Fig. 3). This value is comparable to those of 4.7 and 5.15 found for the suspected ACh receptor and ACh receptor–α-bungarotoxin complex from eel electroplax, respectively (16). A similar value of 4.8 was also reported for the suspected ACh receptor–acetyl-α-bungarotoxin complex from cerebral cortex of guinea pigs (17). It is possible that this low pI is due to association of the phospholipids with the protein through hydrophobic interaction between the hydrocarbon chains of the phospholipid and the predominantly hydrophobic inner parts of the protein, leaving the polar, charged groups of both protein and phospholipids exposed, as was found for chloroplast and mitochondrial membranes (18). Alternatively, they could imply association with acid proteins, such as sialoproteins, that are common components of membranes. The fact that neuraminidase does not affect ligand binding by ACh receptors (1) does not rule out the possibility of a structural association of receptor with sialoprotein.

Three forms of active acetylcholinesterase, with various pI values, appear in the Triton extract (Fig. 3*a*), but although much more of the enzyme is extracted by Lubrol, only two forms appear (Fig. 3*b*). The third form, with the lowest pI of 4.3, may be present in the Lubrol extract, but is masked by the much higher amount of the main peak of acetylcholinesterase activity The isoelectric point of the major peak of acetylcholinesterase activity is 5 (Fig. 3), which agrees with the value of 5.35 reported for purified acetylcholinesterase of eel (19). It is interesting to note that the two forms of acetylcholinesterase we found in smaller quantities (Fig. 3*a*) have pI values (4.3 and 7) similar to those of the carboxamidomethylated (4.8 and 7) β and α noncatalytic subunits of eel acetylcholinesterase (20).

Although the peaks of functional ACh receptors and of acetylcholinesterase of *Torpedo* electroplax are separable by electrofocusing (Fig. 3), we have been unsuccessful in the isolation of fractions that contain functional receptors but are free of acetylcholinesterase activity. The opposite is feasible

by electrofocusing (Fig. 3) or by gel chromatography (8). On the other hand, macromolecules that bind α-bungarotoxin (suggested to be ACh receptors) were totally separated by gel chromatography from acetylcholinesterase in the presence of sodium dodecyl sulfate (12). It has also been possible to partially purify macromolecules that bind α-bungarotoxin from various tissues without apparent loss in specific binding (12, 16, 17). But, it seems that binding of the neurotoxin does not require as sensitive (i.e., active) a receptor as does ACh-binding, for even boiled brain extracts retain the ability to bind acetylated-α-bungarotoxin (17). Purification of a functional ACh receptor that is capable of binding ACh has been a difficult task, due mainly to the instability of these macromolecules. For example, Lubrol extraction eliminated 70% of the proteins present in particulate preparations of *Torpedo* electroplax, but only 2-fold purification was obtained (8). Neither did gel chromatography on Sepharose 6B improve the recovery of specific ACh-binding, despite partial separation from acetylcholinesterase (8). The ACh receptor macromolecules are also highly sensitive to low pH (Table 1), and this may account for the 6.3-fold purification achieved by electrofocusing instead of the expected 27-fold, based on protein recoveries. There are two possibilities to improve this technique; one is by reactivation of ACh-binding after exposure to low pH. The other is to remove some of the acid groups in the macromolecule (maybe by enzyme treatment) in order to increase the pI, and then reconstitute the subunits after electrofocusing. If either approach is successful, electrofocusing will become very useful in purification of functional ACh receptors.

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