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Affinity Labeling of the Acetylcholine Receptor in the Electroplax: Electrophoretic Separation in Sodium Dodecyl Sulfate

(electric eel/synapse/membrane proteins/molecular weight)

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ABSTRACT **Electroplax, single cells dissected from electric tissue of *Electrophorus*, are labeled in a two-step procedure: reduction by dithiothreitol followed by alkylation by the affinity label 4-(*N*-maleimido)-α-benzyltri-[methyl-^{3}H]methylammonium iodide, either alone or in combination with [2,3-^{14}C]*N*-ethylmaleimide. Electrophoresis in sodium dodecyl sulfate on polyacrylamide gel of an extract, prepared with this detergent, of single-labeled or of double-labeled cells results in a major peak of ^{3}H activity, with a mobility corresponding to a polypeptide of molecular weight 42,000. In addition, in the double-labeled samples, there is a unique peak in the ratio of ^{3}H to ^{14}C that is coincident with the ^{3}H peak. The electrophoretic patterns of extracts of cells in which affinity alkylation of the reduced receptor has been suppressed by dithiobischoline, an affinity oxidizing agent, by cobratoxin, an irreversible ligand, or by hexamethonium, a reversible ligand, show a considerably diminished peak of ^{3}H activity in the region of molecular weight 42,000. This is the predominant difference between the electrophoretic patterns of extracts of unprotected and of protected cells. Furthermore, extracts of cells protected with dithiobischoline before labeling with both tritiated affinity label and [^{14}C]*N*-ethylmaleimide do not show the peak in the ^{3}H to ^{14}C ratio seen in the absence of protection. Thus, by several diverse criteria, the peak of ^{3}H activity corresponding to a molecular weight of 42,000 contains affinity-labeled acetylcholine receptor or receptor subunit.**

The receptor for acetylcholine in the electroplax of *Electrophorus electricus* appears from physiological evidence to be reduced *in situ* by dithiothreitol, and a sulfhydryl group thereby formed appears to be subsequently alkylated with considerable specificity by 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide (MBTA) (1). It appears that MBTA alkylates the reduced receptor *in situ* 1000-fold as rapidly as does the neutral *N*-ethylmaleimide (NEM), due to the affinity of the benzyltrimethylammonium moiety for the receptor (1). Corroborating the physiological evidence is the result that the extent of reaction of 4-(*N*-maleimido)-α-benzyltri[methyl-^{3}H]methylammonium iodide ([^{3}H]-MBTA) with the dithiothreitol-reduced electroplax under physiological conditions is decreased either by the application before alkylation by [^{3}H]MBTA of dithiobischoline, an affinity-oxidizing agent of the reduced receptor (2), or by the presence, during the alkylation, of hexamethonium, a reversible ligand of the receptor (3–5). These decrements in the extent of the labeling reaction approach asymptotic limits with increasing concentrations of [^{3}H]MBTA, and the quantity of receptor in the electroplax has been estimated from these limits (4, 5). Comparison of the extent of labeling of the protectible SH-groups (presumably receptor) and of non-protectible SH-groups corroborates the inferred 1000-fold greater rate of reaction of MBTA with receptor SH-groups; the 10–20% overall specificity of the labeling results from the about 10^4-fold greater quantity of available nonreceptor SH-groups (5).

The present work suggests that a single polypeptide component of the electroplax has the properties previously inferred for the receptor; i.e. after reduction, its rate of reaction with MBTA compared with NEM is enhanced, and it is protected by dithiobischoline and by hexamethonium against alkylation by MBTA. In addition, a third protecting agent, cobratoxin, has been introduced. This polypeptide component of the venom of *Naja naja siamensis* is structurally very similar to the neurotoxins from other elapid snakes. Such neurotoxins appear from physiological evidence to block irreversibly the acetylcholine receptor in vertebrate neuromuscular junctions and electroplax synapses (6–8; and manuscript in preparation). Cobratoxin appears to block the labeling by [^{3}H]MBTA of the same component as does dithiobischoline and hexamethonium.

MATERIALS AND METHODS

Labeling. Single cells (electroplax) dissected from the organ of Sachs of *Electrophorus electricus* were labeled (5) with [methyl-^{3}H]MBTA (2 Ci/mmol). In each experiment, two groups of about 15 cells each were treated in parallel. One group was labeled without protection (*A*) and one group was labeled with protection (*B*, *C*, or *D*) as follows: both groups were treated with 0.2 mM dithiothreitol in a Tris–Ringer's solution [165 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Tris (pH 8.0)] for 10 min, then washed for 10 min in a phosphate–Ringer's solution [as above, except 1.5 mM phosphate (pH 7.1) replaces Tris] containing 10 mM glucose (RG buffer); thereafter, one group was treated according to Procedure *A* and one according to *B*, *C*, or *D:*

(*A*) RG buffer (20 min); 12 nM [^{3}H]MBTA (10 min);

(*B*) RG buffer (5 min); 0.5 μM dithiobischoline (5 min); RG buffer (10 min); 12 nM [^{3}H]MBTA (10 min);

Abbreviations: MBTA, 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

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(*C*) RG buffer (15 min); 1 mM hexamethonium (5 min); 12 nM [^{3}H]MBTA in 1 mM hexamethonium (10 min);

(*D*) 0.28 μM cobratoxin (10 min); RG buffer (10 min); 12 nM [^{3}H]MBTA (10 min).

Finally, both groups were washed for 25 min with five changes of RG buffer, blotted, and weighed. All solutions other than dithiothreitol were made up in phosphate–Ringer's solution (pH 7.1). Purified cobratoxin from *Naja naja siamensis* was a gift from Drs. D. Cooper and E. Reich. Groups of cells were double-labeled in parallel according to schemes *A* and *B*, except that alkylation was by a mixture of 12 nM [^{3}H]MBTA and 9.6 μM [2,3-^{14}C]NEM (2 Ci/mol).

Extraction of Single-Labeled Cells. To each group of blotted, single-labeled cells, an equal volume of 2% sodium dodecyl sulfate (SDS)–10 mM dithiothreitol–20 mM Tris-acetate (pH 8.0) was added. This mixture was then kept at 50° for 2 hr and centrifuged; the supernatant was removed for counting and electrophoresis.

Fractionation and Extraction of Double-Labeled Cells. About 1 g each of double-labeled cells from procedures *A* and *B* were treated in parallel as follows: the cells were dispersed with a Ten Broeck tissue grinder in Ringer's solution (pH 7.1) (final volume 8 ml); the homogenate was sedimented in a Spinco 65 rotor for 15 min at 50,000 rpm (4°).

The pellet was suspended in 8 ml of 1 mM sodium-EDTA (pH 7.2) and sedimented as before. The pellet was then suspended in 1 ml of 1% SDS–5 mM dithiothreitol–10 mM Tris-acetate (pH 8.0), maintained at 50° for 2 hr with frequent mixing, and sedimentated at 30,000 rpm for 15 min (20°). Samples of the supernatant were electrophoresed.

Polyacrylamide Gel Electrophoresis. Gels were prepared in 1% SDS-100 mM Tris-acetate (9), and were run for 1 hr with the buffer containing 1% SDS–2 mM DTT–100 mM Tris-acetate (pH 8.0) before use for an analysis. Typically, 0.2 ml of extract, to which sucrose and bromphenol blue had been added, was layered on the gels, which were then run at a constant current of 3 mA/tube until the bromphenol blue front had migrated to within 1–2 cm of the end of the gel. Duplicate or triplicate gels were run for each extract. After removal from the tubes, gels were fractionated according to the technique of Maizel (10).

Molecular weight standards were prepared by treatment of bovine serum albumin (Sigma Chemical Co.), ovalbumin (Sigma Chemical Co.), and cytochrome *c* (Boehringer-Mannheim) with [^{14}C]dimethylsulfate (40–58 Ci/mol) (11). The labeled and unlabeled proteins had indistinguishable electrophoretic mobilities in SDS.

Counting. The gel fractions were dried at 50°, then digested in 0.2 ml of water and 1 ml of NCS (Amersham/Searle) at 50° for 2 hr. Aliquots of extracts were similarly made up to 0.2 ml with water, 1 ml of NCS was added, and the resulting solutions were digested at 50° for 2 hr. 10 ml of PPO–dimethyl POPOP–toluene were added, and all vials were counted in a Packard scintillation spectrometer for 20 min. Counting efficiencies were 20% for ^{3}H alone, and 17 and 46% for ^{3}H and ^{14}C together. The single-label and double-label counting data were processed by a computer program similar to that described in ref. 12.

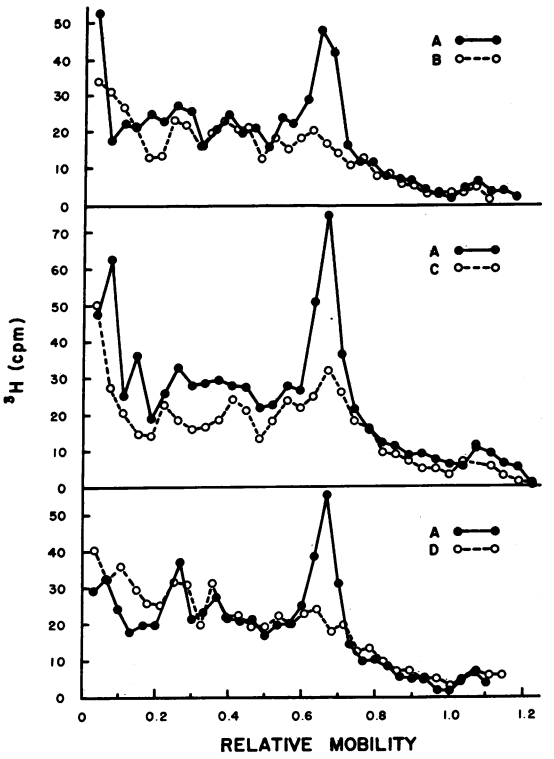

FIG. 1. The electrophoretic distribution on 7.5% acrylamide gel, in 1% SDS, of ^{3}H activity in extracts of [^{3}H]MBTA-labeled electroplax. Typical parallel gels from three experiments are shown. In each experiment, one group of electroplax cells was labeled (see *Methods*) according to procedure *A* (dithiothreitol, [^{3}H]MBTA) and one group according to either procedure *B* (dithiothreitol, dithiobischoline, [^{3}H]MBTA), *C* (dithiothreitol, hexamethonium, [^{3}H]MBTA in hexamethonium), or *D* (dithiothreitol, cobratoxin, [^{3}H]MBTA). The labeled electroplax were extracted in SDS–dithiothreitol–Tris-acetate solution and 200-μl samples of the extracts, containing about 500 μg of protein and 1200 net cpm, were layered over 12 × 0.6-cm gels. Mobility is calculated relative to that of the bromphenol blue front.

RESULTS

Single-labeled cells

Electrophoresis of the SDS extract of electroplax labeled without protection according to procedure *A* results in a major peak of ^{3}H activity that is considerably diminished in extracts of electroplax labeled with protection according to procedures *B*, *C*, and *D* (Fig. 1). Similar results are obtained on 5% (Fig. 2) and on 7.5% acrylamide gels (Fig. 1). The predominant difference between the electrophoretic distributions of the extracts of unprotected (*A*) and of protected (*B*, *C*, and *D*) cells is in the region of the major peak, as shown by the average differences between distributions of percent of total ^{3}H activity per vial (Fig. 3). In extracts of cells protected with hexamethonium (*C*) and with cobratoxin (*D*) there are, in addition, differences at the top of the gels, where the percent ^{3}H activity in *A* is greater than in *C*, and less than in *D*.

The SDS extracts contain 80% of the total radioactivity of the cells. The difference in radioactivity between the extracts of unprotected and protected cells accounts for 90% of the total differences between the two groups of cells. The average recovery of radioactivity on the gels is 90 ± 10%.

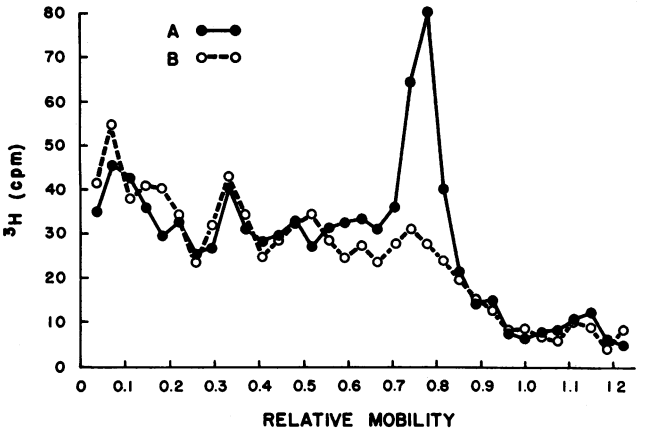

FIG. 2. The electrophoretic distributions on 5% acrylamide gel in 1% SDS of ^{3}H activity in extracts of electroplax labeled with [^{3}H]MBTA according to procedures *A* and *B*. Details as in Fig. 1.

Double-labeled cells

From previous work, it was expected that NEM would have no specificity for receptor SH-groups (1) and would serve as a general label for cellular SH-groups. In addition, if dithiobischoline specifically reoxidizes the SH-groups of the reduced receptor (2, 5), then this agent should have an insignificant effect on the labeling of SH-groups by [^{14}C]NEM, since only a small fraction of the total available SH-groups are receptor SH-groups (5). In fact, cells labeled by procedure *A* and by procedure *B* with a mixture of 12 nM [^{3}H]MBTA and 9.6 μM [^{14}C]NEM differ by 13 $\pm$ 4% (n = 6) in ^{3}H activity, not an appreciably different result than that obtained with [^{3}H]MBTA alone. (The extents of reaction in *A* are 1.4×10^{-14} mol of [^{3}H]MBTA/mg of cells and 1.7×10^{-11} mol of [^{14}C]NEM/mg of cells.) Dithiobischoline has no significant effect on the labeling by [^{14}C]NEM, and the presence of [^{14}C]NEM has no effect on the depression by dithiobischoline of the labeling by [^{3}H]MBTA. After disruption of double-labeled cells in Ringer's solution, about 80% of the ^{3}H activity is sedimentable, whereas about 80% of the ^{14}C activity is soluble. Suspension of the particulate fraction in 1 mM EDTA solubilizes an additional 1% of the ^{3}H activity and 2% of the ^{14}C activity. Finally, 80% of the ^{3}H activity and 75% of the ^{14}C activity sedimentable after EDTA treatment are solubilized by 1% SDS. This extract is enriched about 4-fold with respect to ^{3}H to ^{14}C ratio over whole cells.

The gel-electrophoretic distribution of ^{3}H activity of the SDS extract of the particulate fraction of unprotected double-labeled cells (*A*) shows a major peak at the same position as in comparable single-labeled gels, and this peak is considerably diminished in the extract of the particulate fraction of protected cells (*B*) (Fig. 4). Insignificant differences are seen in the distributions of ^{14}C activity. There is only one significant peak in the ratio of ^{3}H to ^{14}C. It is coincident with the ^{3}H peak, and is virtually eliminated by dithiobischoline. An elevation in ^{3}H to ^{14}C ratio seen just past the bromphenol blue front is associated with less than 1% of the total ^{3}H activity, and may be due to hydrolysis of reacted [^{3}H]MBTA. The average differences in the distributions of ^{3}H to ^{14}C suggest that the only significant effect of dithiobischoline is to eliminate the preferential labeling by [^{3}H]-MBTA of a component in the major peak (Fig. 5).

Molecular weight

For a given concentration of acrylamide, there is, over a wide range, a linear relationship between log of molecular weight and electrophoretic mobility of many reduced proteins in SDS-containing buffers (13, 14). The mobility of the major ^{3}H peak relative to that of bromphenol blue is compared to the mobilities of ^{14}C-labeled molecular weight standards treated similarly to the extracts and coelectrophoresed on 5, 7.5, and 10% acrylamide gels (Fig. 6). Assuming that the components of the peak are reduced polypeptides saturated with SDS (15), we obtain molecular weight estimates, respectively, of 39,900, 42,500, and 43,300, with a mean of 42,000, for the three concentrations of acrylamide.

DISCUSSION

The labeling of the electroplax by [^{3}H]MBTA has been analyzed by polyacrylamide gel electrophoresis in SDS, a technique that separates proteins into molecular weight classes. A major peak of ^{3}H activity, with a mobility corre-

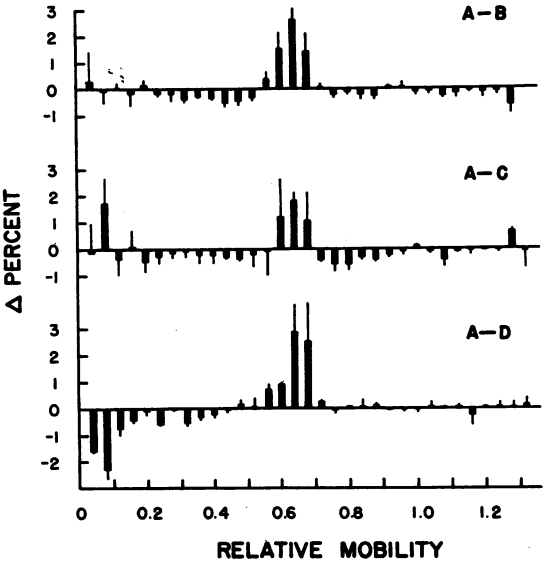

FIG. 3. Average differences in the distributions of percent ^{3}H activity between gels from extracts produced by procedure *A* and by procedures *B*, *C*, and *D*. All experiments in which gels from procedure *A* and *B* (n = 12), *C* (n = 4), or *D* (n = 5) (7.5% acrylamide) were run in parallel are included. In the original data, the number of fractions to the bromphenol blue front ranged from 24 to 29. For the purposes of averaging the distributions, a new distribution was calculated for each gel by interpolation; the radioactivity was partitioned into fractions that each corresponded to a mobility range of 0.04; i.e. the front in the new distribution is in fraction number 25. For each experiment, the calculated distributions of radioactivity of replicate gels were then summed fraction by fraction, and the sums were divided by the overall total radioactivity to yield the average distribution of percent of total radioactivity. The fraction by fraction differences between the average distribution by procedure *A* and the average distribution by procedures *B*, *C*, and *D* were calculated for each experiment. These differences were then averaged over comparable experiments. The mean difference and standard error of the mean or range are presented. The sum of the differences (100 – 100%) is zero.

sponding to a polypeptide of molecular weight 42,000, is found in the electrophoretic distribution of the SDS extract of [^{3}H]MBTA-labeled electroplax. The activity found in this peak region is depressed in extracts of electroplax labeled after protection with three diverse agents (Figs. 1, 2, and 3). Two of these, dithiobischoline (2, 5) and cobratoxin (ref. 8 and unpublished results), are applied at the low concentrations at which they are physiologically effective, and probably highly specific for the receptor. The third agent, hexamethonium, is applied at the relatively high concentration required for this reversible ligand to retard the irreversible reaction of [^{3}H]MBTA with the receptor, and is probably less specific in its interactions (5). The differences between procedure *A* and *C* at the top of the gels (Fig. 3) may be due to the protection by hexamethonium of some high-molecular-weight nonreceptor proteins, a possibility also suggested by the higher than expected overall protection against labeling afforded by hexamethonium (5). The difference in the same region, but in the opposite direction, between gels of extracts treated by procedures *A* and *D* (Fig. 3) may be due to the facilitation by cobratoxin of the reaction of [^{3}H]MBTA with some high molecular weight components, or to the prevention by cobratoxin of the dissociation of labeled

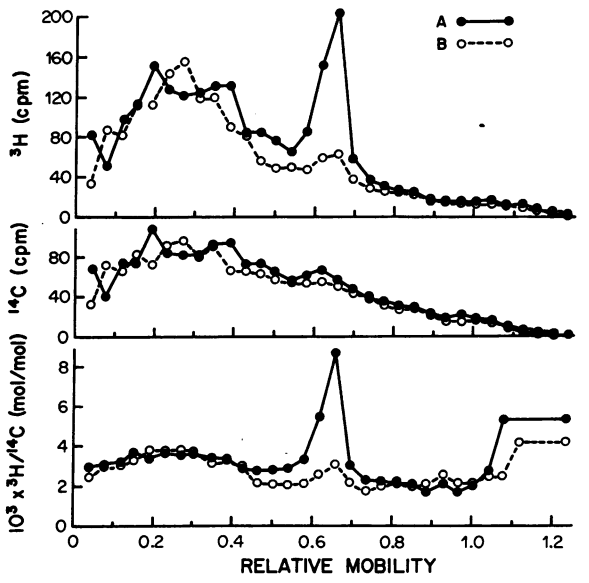

FIG. 4. The electrophoretic distributions on 7.5% acrylamide gel in SDS of ^{3}H activity, ^{14}C activity, and ^{3}H to ^{14}C ratio in extracts of electroplax labeled with [^{3}H]MBTA and [^{14}C]NEM according to procedures *A* and *B*. Electroplax labeled with (*B*) and without (*A*) protection by dithiobischoline (see *Methods*) were homogenized in Ringer's solution, and a particulate fraction was prepared. The particulate fraction was extracted with SDS–dithiothreitol–Tris-acetate solution, and 200-μl samples of the extract, containing about 250 μg of protein, 2400 net cpm of ^{3}H, and 1800 net cpm of ^{14}C, were layered over 12 × 0.6-cm gels. The distributions of typical gels run in parallel are shown. The statistical counting error at the end of the gel, where there are few counts, is relatively large, and where the coefficient of variance of the ^{3}H to ^{14}C ratio exceeded 20%, the counts of successive fractions were combined for the calculation of the ratio (compare ref. 12). This combination step was only necessary in the last few fractions of the gels. The coefficient of variance of the ratio at the peak is 5% in procedure *A* and 7% in procedure *B*.

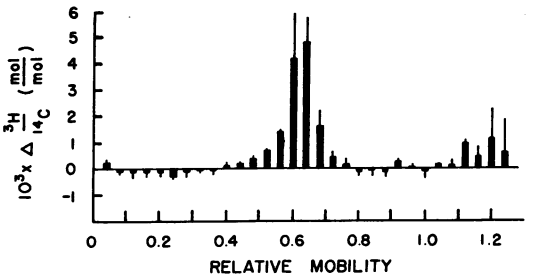

FIG. 5. Average differences in the ^{3}H to ^{14}C ratio on double-labeled gels from extracts prepared by procedures *A* and *B*. The ^{3}H and ^{14}C distributions of all gels such as those in Fig. 4 were partitioned as in Fig. 3. Within an experiment the new distributions of the replicate gels were averaged, and the average ^{3}H to ^{14}C ratios and the differences in the ratios between procedures *A* and *B* were calculated. These differences were then averaged over three comparable experiments. The mean and the standard error of the mean is presented.

receptor in SDS. Notwithstanding the possibly incomplete specificity of the protecting agents, the predominant and overlapping effect of dithiobischoline, hexamethonium, and cobratoxin is on a component of the major peak. Furthermore, a component of the major peak is preferentially labeled with the affinity-alkylating agent [^{3}H]MBTA relative to the nonaffinity-alkylating agent [^{14}C]NEM, and this preferential labeling is uniquely eliminated by application before labeling of dithiobischoline (Fig. 4 and 5). Significant preferential labeling is found only in the major peak area. By several different criteria, therefore, a component of the major peak is affinity-labeled receptor or receptor subunit.

The polypeptide nature of this labeled component is supported by the consistency of the molecular weight estimates obtained at three different gel concentrations when colinearity with protein standards is assumed (13–15, and compare ref. 16). The chemistry of the labeling process suggests that a disulfide is being reduced and alkylated, and the physiological effects of the reduction and of several affinity reactions suggest that the disulfide is about 1 nm from the negative subsite of the acetylcholine-binding site of the receptor

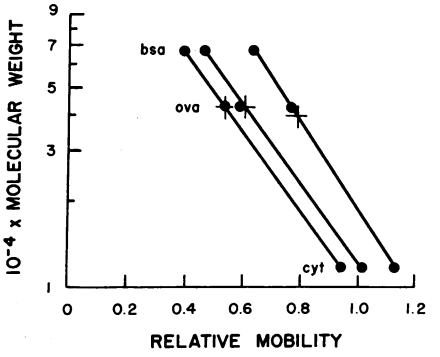

FIG. 6. Molecular weight estimation of the major peak (+). Electroplax labeled with [^{3}H]MBTA according to procedure *A* were extracted with SDS–dithiothreitol–Tris-acetate solution, and the extract was coelectrophoresed with ^{14}C-labeled bovine serum albumin (*bsa*), ovalbumin (*ova*), and cytochrome *c* (*cyt*), treated similarly. Mobility is given relative to that of bromphenol blue. From left to right, the acrylamide concentrations are 10, 7.5, and 5%.

(1, 4, 5). Thus, it is likely that the specifically labeled polypeptide component is either the receptor or a receptor subunit containing all or part of the acetylcholine-binding site.

The portion of the radioactivity of the major peak that is due to labeled receptor can be estimated from the distributions expressed in percent of total activity, to minimize differences in loading and in recovery. If we assume that all labeled receptor in gels from extracts prepared by procedure A is in the major peak, that gels of extracts prepared by procedure B contain no labeled receptor, and that the distributions of labeled nonreceptor components are equivalent in extracts prepared by procedures A and B, then the fraction of radioactivity in the peak from an extract labeled by procedure A that is labeled receptor is given by $(a - b)/a(1 - b)$, where a and b are the fraction of the total ^3H activity in the peak region in parallel gels from extracts prepared by procedures A and B, respectively. The three vials that contained the maximal counts in the peak from procedure A contain $17.2 \pm 0.6\%$ ($n = 15$) of the total recovered activity in procedure A, and the three corresponding vials in procedure B contain $11.1 \pm 0.3\%$ ($n = 15$) of the total recovered activity in procedure B. The average fraction of ^3H activity in the procedure A peak (3 vials) due to labeled receptor is $40 \pm 3\%$.

The results of the fractionation of double-labeled cells support the expectation that [^3H]MBTA, being positively charged, penetrates the cell membrane relatively slowly, reacting predominantly with externally accessible components, whereas [^{14}C]NEM penetrates rapidly, reacting with both external and internal components. The [^{14}C]NEM-labeled components of the total particulate fraction are likely to be representative of the membrane proteins of the cell capable of reacting with maleimide. The receptor accounts for a small fraction of the total of such proteins migrating to the region of the major peak. On the assumption that, except for the contribution of the receptor on gels from extracts prepared by procedure A, the distribution of the ^3H to ^{14}C ratio is equivalent on gels prepared by procedures A and B (see Fig. 5), the ratio of ^3H-labeled receptor to ^{14}C-labeled protein in the peak is given by the ^3H to ^{14}C ratio in the peak from procedure A minus the ^3H to ^{14}C ratio in the corresponding region in the peak from procedure B. For the peak vial, the average difference is $6.1(\pm 1.6) \times 10^{-3}$ mol of [^3H]receptor/mol of [^{14}C]protein. (The peak difference seen in Fig. 5 is slightly less due to the peak-broadening effect of the averaging of distributions.) The overall purification of [^3H]receptor relative to [^{14}C]protein can be estimated from the differences in the ^3H to ^{14}C ratio from procedures A and B at the different stages: 0.1×10^{-3} for whole cells; 0.4×10^{-3} for the SDS extract of the particulate fraction; and 6×10^{-3} mol of ^3H/mol of ^{14}C for the peak vial. Therefore, the purifications achieved are 4-fold by fractionation of the cells, 15-fold by gel electrophoresis, and 60-fold overall.

Others have identified the receptor as a component that tightly binds elapid snake neurotoxins similar to cobratoxin (17, 18). A component to which α-bungarotoxin is tightly bound has been extracted from the electric tissue of *Torpedo* with Triton X-100. In SDS, two components, which have approximate molecular weights as determined by gel filtration of 88,000 and of 180,000 (17), bind the toxin. A component to which the α-toxin of *Naja nigricollis* is tightly bound has been solubilized from the electric tissue of *Electrophorus* with deoxycholate, and is said to have a molecular weight of about 50,000 by SDS–gel electrophoresis (18). The latter molecular weight estimate is close to ours after the molecular weight of one toxin molecule [6800 (19)], presumably still bound, is subtracted. As previously noted, a molecular weight of about 40,000 for the receptor protomer is consistent with its occupancy of 5% of the subaxonal membrane area in the electroplax (5).

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