ACETYLCOLINE RECEPTORS OF CULTURED MUSCLE CELLS DEMONSTRATED WITH FERRITIN-α-BUNGAROTOXIN CONJUGATES


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SUMMARY

α-Bungarotoxin-ferritin conjugates were used to visualize by freeze-fracture and thin-section electron microscopy toxin-binding sites, presumably acetylcholine (ACh) receptors, in membranes of muscle cells grown in tissue culture. Toxin conjugated to ferritin by a glutaraldehyde reaction and purified by column chromatography in a buffer of high ionic strength remains active in blocking the effect of iontophoretically applied ACh. The potency of the conjugates was decreased 5-10 times compared to native α-bungarotoxin. Toxin-ferritin conjugates were identified in small clusters which were not uniformly distributed over the surface membrane. Binding was inhibited by preincubation in d-tubocurare or unconjugated toxin. The relation of the clusters to the non-uniform distribution of ACh sensitivity and α-bungarotoxin binding on cultured muscle fibres is discussed.

INTRODUCTION

Alpha-bungarotoxin (α-BGT), a peptide of mol. wt. 8000 isolated from the venom of the snake Bungarus multicinctus, binds specifically and essentially irreversibly to nicotinic acetylcholine (ACh) receptors in a variety of cells, including muscle fibres that form in vitro from dissociated myoblasts. We here report that α-BGT covalently linked to ferritin binds to cultured muscle cells and describe the electron-microscopic appearance of the conjugates with freeze-fracture and thin-section techniques. ACh receptors are apparently not distributed uniformly over the surface of cultured fibres. Sharp peaks of sensitivity have been found by focal, iontophoresis of ACh and dense patches of grains have been described in autoradiographs of cultures exposed to 125I-α-BGT (Cohen & Fischbach, 1971; Fischbach & Cohen, 1973; Vogel, Sytkowski & Nirenberg, 1972). Toxin-ferritin conjugates provide a high-resolution tag for ACh receptors that may be useful in further analysis of these specialized regions or 'hot spots'.

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MATERIALS AND METHODS

Coupling and purification of ferritin-α-bungarotoxin conjugates

Reagents were 6 x crystallized ferritin (Pentex, Miles Laboratories), glutaraldehyde EM grade (Polysciences), Sephadex G-200 (Pharmacia), and d-tubocurare (Sigma). Alpha-bungarotoxin (α-BGT) purified from the crude venom and 110-α-BGT (sp.act. 40 Ci/mM) prepared by the chloramine T method (Hunter & Greenwood, 1962) were kindly provided by Dr John Whysner.

α-BGT was coupled to ferritin with glutaraldehyde (Avrameas, 1969). The 1-ml reaction mixture consisted of 675 μl distilled water, 100 μl 0.05 M NaH2PO4 pH 7.2, 125 μl ferritin (92 mg/ml), 40 μl α-BGT (0.5 mg) and 3-6 μCi 110-α-BGT in 10 μl. After mixing, 50 μl of 1 % glutaraldehyde were added over a period of 5 min and the reaction mixture incubated for 2 h at 25 °C; 29 mg NaCl were then added (0.5 M NaCl) and the solution was applied to a Sephadex G-200 column equilibrated in 0.05 M NaH2PO4 (pH = 7.2) and 0.5 M NaCl. The column was eluted in the above buffer and 10-μl aliquots of fractions were counted in 10 ml scintillation fluid (toluene containing 10 % Fluorolloy, 12.5 % Biosolve) in a Beckman LS 250 liquid scintillation counter. Peaks were collated and dialysed overnight against Earle’s balanced salt solution (BSS) containing 0.025 % sodium azide. Dialysates were stored at 4 °C until used.

Acetylcholine-blocking activity of Sephadex G-200 peaks

The efficacy of α-BGT-ferritin conjugates in blocking ACh receptors was tested in cultures of chick muscle fibres. The method of preparing sparse, relatively pure muscle cell cultures has been described (Fischbach, 1972). Mononucleated myogenic cells were obtained from pectoral muscles of 11-day embryos. They fused and the muscle fibres matured in Eagle’s Minimum Essential Medium supplemented with horse serum (10 %, v/v) and chick embryo extract (2 %, v/v). Fibroblasts were virtually eliminated from the cultures by addition of cytosine arabinoside (10^-5 M) to the medium for 48 h beginning on the third day after plating. Thus, all of the membranes examined by electron microscopy were muscle cell membranes. Intracellular microelectrode recording techniques and micro-iontophoresis of ACh have also been described (Fischbach, 1972; Fischbach & Cohen, 1973). The ACh sensitivity, measured in terms of millivolts depolarization/nanocoulomb of charge ejected from the ACh microelectrode, was determined at several points on one or two fibres before and after addition of toxin-ferritin conjugates. Following the control measurements, the recording electrode was withdrawn and the entire bath (ca. 2 ml) changed. The same cells were repenetrated and the previously identified points were tested every 1-2 min for the next 30-60 min.

Electron-microscopic studies

For freeze etch, duplicate 7- to 10-day-old chick embryo muscle cell cultures in 60-mm Falcon Petri dishes were washed twice with 3 ml Earle’s salt solution (BSS) containing 2 % bovine serum albumin, and once with 3 ml BSS. Media were removed and cultures incubated with 3 ml of BSS-diluted Sephadex G-200 dialysates (2-4 x 10^-9 M α-BGT) at 37 °C in 10 % CO2 for 30 min. Cultures were washed twice with 5 ml BSS, twice with 5 ml cold PBS and fixed with 1 % glutaraldehyde in phosphate-buffered saline (PBS) for 30 min at 10 °C. Fixative was removed and cultures successively washed with BSS, PBS and 0.05 M Tris-HCl, pH 7.4. Cells were collected by gentle scraping of culture plates with a rubber policeman and cell clumps placed on 3-mm copper planchets. Specimens were rapidly frozen in liquid nitrogen, freeze-fractured and deep etched as previously described (Tillack & Marchesi, 1970). Platinum replicas were examined in a Philips EM 200 electron microscope. For thin sections, cultures were washed 4 times with BSS, fixed in 2.5 % glutaraldehyde in 0.15 M cacodylate buffer, post-fixed with OsO4, block stained with uranyl acetate and embedded in Epon.
RESULTS AND DISCUSSION

Sephadex G-200 gel filtration of the reaction mixture is depicted in Fig. 1. Peak I appeared just after the void volume, contained virtually all the ferritin applied to the column, and accounted for 24–31% (3 experiments) of the bungarotoxin. SDS gel electrophoresis of pooled peak II fractions resulted in multiple bands which suggests that the broad peak was comprised of different-size cross-linked aggregates of α-BGT.

α-BGT-ferritin conjugates blocked ACh receptors of cultured muscle fibres. 8 × 10⁻⁸ M α-BGT from peak I of the preparation shown in Fig. 1A reduced the

![Graph](image-url)

**Fig. 1.** A, gel filtration of α-BGT-ferritin conjugates on Sephadex G-200 equilibrated in 0.05 M NaH₂PO₄ and 0.5 M NaCl. Fraction volume = 1 ml. Ordinate: cpm derived from ³¹¹I-α-BGT added to the reaction mixture (see text).

B, depression of ACh sensitivity measured as mV depolarization/nC of charge ejected following addition of conjugates (2.5 × 10⁻⁷ M in α-BGT) to the bath at zero time. Insets show representative responses elicited by 0.09, 0.11 and 0.53 nC as indicated.
muscle depolarization in response to iontophoretically applied ACh to less than 20% of control in 15 min. The effect of $2.5 \times 10^{-7} \text{ M} \alpha$-BGT conjugated to ferritin in another preparation is shown in Fig. 1B. Similar curves have been obtained with unconjugated toxin at $2.5-5.0 \times 10^{-8} \text{ M}$ (E. Whysner & G. D. Fischbach, unpublished observations). Thus, the conjugates are apparently 5-10 times less potent than unconjugated $\alpha$-BGT.

Small clusters of ferritin molecules were identified on the surface of muscle fibres exposed to $\alpha$-BGT-ferritin conjugates in replicas of freeze-fractured and deep-etched preparations (Fig. 2A) and in thin sections (Fig. 2B). Ferritin was detected on only about 20% of the etched surfaces examined (areas of different surfaces range between 1 and 5 $\mu$m$^2$) and the density of ferritin clusters varied widely within this group. It is not yet clear whether this heterogeneity depicts the actual distribution of receptors or simply reflects slow diffusion of conjugates to relatively inaccessible sites. The latter seems unlikely, since wheat germ agglutinin–ferritin and lysine–ferritin conjugates (neither of which blocked the ACh response) were distributed diffusely over every exposed surface (Fig. 3). Another possibility is that some of the $\alpha$-BGT-ferritin conjugates were removed from the membrane during the washing prior to fixation. Preliminary experiments indicate that significant sensitivity to ACh reappears after 5–6 washes over a 30-min period.

Most of the conjugates were bound specifically to ACh receptors. No ferritin was detected in cultures that were preincubated in $5 \times 10^{-8} \text{ M}$ unconjugated $\alpha$-BGT or in an equivalent concentration of toxin from peak II and only rare, isolated clusters were found after preincubation in the specific competitive inhibitor $\alpha$-tubocurare ($5.0 \times 10^{-5} \text{ M}$). Fig. 4 shows a representative, clean membrane exposed to $\alpha$-BGT-ferritin in the presence of $\alpha$-tubocurare. No clusters were found after incubation with ferritin alone or with ferritin exposed to glutaraldehyde or with material from peak II.

In early experiments, gel filtration was performed in 0.05 $\text{ M}$ Na$_2$HPO$_4$ alone without added salt. Peak I obtained under these conditions accounted for 60–70% of the total $\alpha$-BGT and samples blocked the ACh response at concentrations that were nearly identical to uncoupled $\alpha$-BGT. Ferritin could not be found by electron microscopy, however, and a non-covalent (presumably electrostatic) interaction between $\alpha$-BGT and ferritin was suspected. More than 50% of the counts were removed from peak I on addition of 0.5 $\text{ M}$ NaCl. It seems likely that, in these experiments, non-covalently bound toxin dissociated from the ferritin and competitively inhibited the binding of the conjugated toxin. Thus, the high salt concentration was necessary for the separation and accurate assessment of the ACh blocking activity of $\alpha$-BGT covalently coupled to ferritin.

Ferritin molecules always appeared in clusters even when low concentrations of the conjugates, which reduced the ACh response by less than 20%, were employed. From the elution profile shown in Fig. 1A, it seems unlikely that large numbers of clusters were present in the initial ferritin solution or were produced by exposure to glutaraldehyde. Furthermore, negative staining of material from peak I with 2% phosphotungstic acid revealed individual ferritin molecules and only occasional clusters of 2–3 particles. It is possible that ACh receptors are mobile within the membrane and aggregate after they bind polyvalent $\alpha$-BGT-ferritin conjugates (cf. Karnovsky,
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Unanue & Levinthal, 1972]. Alternatively, ACh receptors may be organized in small clusters under normal conditions. Studies of conjugate binding at low temperature or to prefixed cells may clarify this point.

In some thin sections ferritin molecules were arranged in a single layer adjacent to the membrane but in most, the molecules appeared stacked in several layers that extended up to 0.1 μm from the cell surface (Fig. 2B). Stacked molecules usually overlay electron-dense patches which may represent membrane (perhaps over extensions of the surface) cut tangentially within the plane of the section. This layering is similar to that observed by Karnovsky et al. (1972) on lymphocytes exposed to anti-H-2-ferritin conjugates at 4 °C or to anti-immunoglobulin-ferritin conjugates at 37 °C. They suggested that antigens may be partially avulsed from the membrane after binding polyvalent conjugates and that explanation may apply to ACh receptors as well.

Intramembranous particles were diffusely distributed (Figs. 2A, 3, 4). A few distinctive clusters of particles were found, but there was no obvious correlation between these patches within the membrane and the clusters of α-BGT-ferritin conjugates on the exposed outer surface.

The distinction between relative peaks of sensitivity detected by ACh iontophoresis or patches of α-BGT grains seen in light-microscopic autoradiographs and the small clusters of α-BGT-ferritin particles reported here should be stressed. The area of membrane assayed by either the physiologic or autoradiographic technique is probably several times greater than the area exposed by deep etching in this study. Perhaps a region of membrane with a high density of clusters corresponds to a hot spot. In any case, α-BGT-ferritin conjugates may prove useful in further analysis of the distribution of ACh receptors at the electron-microscopic level.

REFERENCES


(Received 29 March 1974)
Fig. 2. A, replica of a freeze-cleaved and deep-etched muscle cell exposed to α-BGT-ferritin conjugates. Clusters of ferritin particles are indicated by arrows, ff, fracture face; s, outer surface of the membrane. B, thin section showing clusters of ferritin on the surface of the membrane. × 30 000 approx.
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Fig. 3. Replica of a freeze-cleaved and etched muscle cell exposed to wheat germ agglutinin-ferritin conjugates. Note the relatively uniform distribution of ferritin over the exposed outer membrane. $ff$, fracture face; $s$, outer surface of the membrane.

Fig. 4. Replica of a freeze-cleaved and deep-etched muscle cell exposed to $\alpha$-BGT-ferritin conjugates in the presence of $5 \times 10^{-4}$ M $\alpha$-tubocurare. The cells were preincubated in curare for 30 min prior to addition of the conjugates in the presence of the inhibitor.