Journal of Supramolecular Structure 10:39–50 (1979)
Transmembrane Signaling 467–478

A Possible Modulation of Acetylcholine Receptors of Embryonic Chick Muscle Cells by α-Bungarotoxin

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Acetylcholine receptors were assayed with α-bugarotoxin on embryonic chick skeletal muscle growing in primary cell culture. Toxin was bound specifically to muscle cells and could be competed with D-tubocurarine. Two dissociation constants were obtained by equilibrium binding: 7.2×10^{-9}M and 2.7×10^{-7}M at 25°C. Two sets of rate constants were also obtained from dissociation kinetics. There are five times more low affinity sites on cells than high affinity sites. The average density of high-affinity receptors is about $200/\mu m^2$.

A time course of toxin binding to receptors at 37°C vs 25°C in growth medium revealed that under conditions permitting growth and metabolism, toxin bound to cells was lost. The possibility that the growth medium was inactivating toxin molecules was ruled out by showing that unbound toxin molecules in the medium were fully capable of binding to fresh cultures.

Key words: muscle, acetylcholine, acetylcholine receptors, α-Bungarotoxin, chick, modulation

The acetylcholine receptors (AChR) on muscle cells are modulated in several ways. In the classical definition by P. Weiss [1], as applied to a developing cell, modulation is a reversible histological or behavioral change brought about by the particular local environment in which a cell finds itself. It is part of the cell's "response repertory." If one can extend the definition to molecules, the acetylcholine receptors have at least three types of responses which can fit into this definition of modulation. The first is desensitization, in which a prolonged dose of acetylcholine or some analogs can leave receptors in a less active state [2]. Another type of response is to denervation. Here, a loss of neural stimulation or activity of a muscle cell will produce a quantitative increase of the AChR, along with their appearance at extrajunctional regions of the membrane [3]. These extrajunctional receptors have different properties from junctional receptors [4]. It is not clear whether they

Received April 6, 1978; accepted September 15, 1978.

0091-7419/79/1001-0039$02.30

are coupled to other membrane factors in different ways from the junctional AChR or whether they are different proteins. A third form of modulation is seen in the reverse of denervation, or innervation of muscle cells. Innervation, or muscle activity, results in a loss of total AChR, with a concomitant concentration of receptors at the neuromuscular junction [5].

In the formation of the neuromuscular junction there are several events involved which may have different mechanisms. An early event is the recognition of an appropriate muscle cell by a nerve terminal, which is followed by formation of an enduring contact. When embryonic muscle cells were studied in culture, it was found that the AChR existed in two distribution states, diffuse and clustered [6] in the absence of neurons. It is not known whether or not one of the patches will become the site of a neuromuscular junction. If so, it may have been produced by the muscle cell as part of its normal differentiation program. Alternatively, the clusters may have been produced by factors provided in the very complex tissue culture growth medium. Once a neuromuscular junction is formed, a subsequent step must be the loss of other patches and the diffuse extrajunctional receptors, or a down-modulation of AChR. By an as yet unknown mechanism, the receptors are removed from the plasma membrane and possibly degraded. Muscle activity, either depolarization of the membrane to give an altered AChR state, or contraction, can reduce the AChR concentration. Alternatively, down-modulation could be caused by binding of a soluble factor to the receptors, in which case the process would closely resemble the down-modulation observed for receptors of epidermal growth factor (EGF), insulin and antibodies [7]. It is also possible that degradation is a continuous process and that in fact AChR synthesis is modulated [8].

The present study provides additional evidence for two populations of AChR on embryonic skeletal muscle cells, these being on cultured primary chick embryo cells. Studies using α-bungarotoxin (α-Bt) indicate that there are two classes of binding sites in these cultures. A fraction of the total AChR population was lost upon prolonged incubation in α-Bt under conditions permitting growth and metabolism. It is possible that the type of down-modulation noted by others may also be occurring with α-Bt when it binds to the AChR, and that this binding affects the AChR in some way which simulates the signaling mechanism preceeding loss of extrajunctional AChR during formation of neuromuscular junctions.

METHODS

Primary cultures of trypsin-dissociated skeletal muscle cells were prepared from ten day chick embryo breast tissue by the method of Bischoff and Holtzer and grown in medium 8:1:1 [9]. Cells were plated onto collagen-coated petri dishes at a density of 1.4×10^4 cells/cm^2 and the plates incubated at 37°C with 7.5% CO_2. The medium was changed daily. Medium components were purchased from Flow Laboratories.

Cells were harvested by rinsing the dishes in phosphate buffered saline (PBS) three times and taking up the cells in 0.01 N NaOH. The lysate was incubated at 40°C for 30 min and aliquots were counted for radioactivity in duplicate or triplicate. Protein was determined from duplicate aliquots by a modification of the method of Lowry et al [10] using bovine serum albumin as a standard.

α-Bungarotoxin (α-Bt) was prepared from the venom of Bungarus multicinctus (Sigma) by the method of Bosmann [11]. Figure 1 shows the elution pattern from a Sephadex CM 50 (Pharmacia) column. Peak V was purified on Sephadex G50 F (Pharmacia) and identified as α-Bt by several criteria. On SDS-polyacrylamide gel electrophoresis, it ran as a single band at 8,000 amu (Fig. 2). Its amino acid composition [13] and N-terminal end

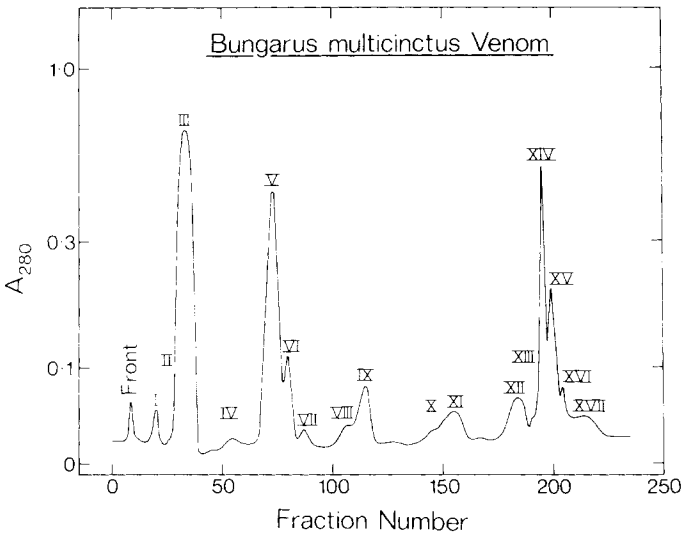

Fig. 1. Elution pattern of the venom of bungarus multicinctus. 500 mg of venom were applied to a column containing Sephadex CM 50 equilibrated with 0.02 M sodium phosphate buffer, pH 7.5 and 10^{-3}M phenylmethylsulfonyl fluoride. The fractions were eluted with a gradient of 0 to 1 M ammonium acetate and monitored continuously at 280 nm. Fractions of 6 ml were collected and the peaks were pooled and lyophilized.

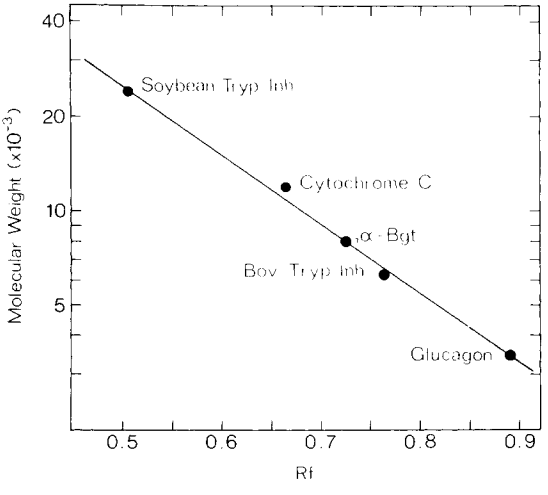

Fig. 2. Polyacrylamide gel electrophoresis of purified α-Bungarotoxin. Gels containing 15% acrylamide were prepared, run and stained according to Laemmli [12]. Standards used to obtain molecular weights were soybean trypsin inhibitor (24,000 amu), cytochrome C (12,000 amu), bovine trypsin inhibitor (6,200 amu) and glucagon (3,460 amu).

analysis with 5-dimethylamino-1-naphthalene sulfonyl chloride (dansyl chloride) [14] corresponded to α-Bt. The purified material produced an irreversible blockage of a rat diaphragm-phrenic nerve preparation.

The toxin was acetylated with ^{3}H-acetic anhydride (ICN, 7×10^{3} mCi/mmole). as described by Bosmann [11]. Labeled α-Bt was repurified on Sephadex G25. The final

specific activity was 4.77×10^4 mCi/mmole. Acetylation did not alter the binding properties of α-Bt to muscle cells. This was shown by diluting the acetylated ^{3}H-α-Bt preparation two-fold with the original unreacted α-Bt. A series of concentrations (from 10^{-12}–10^{-6}M) of diluted and undiluted ^{3}H-α-Bt were bound to 3 day cells for 5.5 h at room temperature in PBS. For a given total concentration, the same number of α-Bt molecules were bound per cell in each case, that is, the two binding vs concentration curves coincided.

RESULTS

Binding of α-Bt and its Specificity

When muscle cultures were incubated with ^{3}H-α-Bt in PBS at 25°C, binding of the toxin saturated by about three hours (Fig. 3). The level of binding was 1.5 pmole/mg cell protein. It was found that 10^6 freshly prepared cells contained 46 μg protein, so that 1 mg protein equals 3.6×10^{-14} mmoles cells. If all the protein assayed in the culture is cell protein, then about 4×10^4 α-Bt were bound per myoblast cell-protein equivalent on 4-day-old cultures. Since some fraction of the culture protein is extracellular, and since there were fibroblasts in the culture, the true number of toxins per muscle cell is higher. This is similar to the values in primary chick cultures of 0.15–1.0 pmole/mg found by Vogel et al [15].

The specificity of α-Bt for muscle cells was determined. Table I presents data on the binding of α-Bt to different types of embryonic chick cells, showing that α-Bt bound preferentially to muscle cells, as expected. The muscle culture in this experiment was confluent and contained many fibroblasts. It can be seen that chick erythrocytes bind only 4.3% as much toxin as muscle cells. Fibroblasts obtained from muscle tissue by allowing only 40 minutes for cell attachment and rinsing off unattached myoblasts [16] bind only 13.6% as

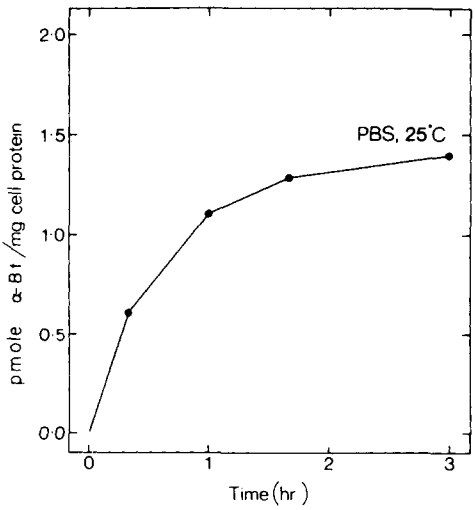

Fig. 3. Binding of ^{3}H-α-Bt to 4 day muscle cell cultures. ^{3}H-α-Bt was used at a final concentration of 5.5×10^{-8}M. The cells were incubated at 25°C in PBS for the intervals indicated. The petri dishes were rinsed with PBS several times and the bound radioactivity was assayed. These are the averages of duplicate determinations. One standard deviation for each of the data was, in order of time of incubation, 7.3%, 8.2%, 6.6% and 9.6% of the indicated value.

TABLE I. Binding of α-Bt to Different Embryonic Chick Cell Types in PBS at Room Temperature

Cell or tissue type	α-Bt bound (picomole/mg cell protein)
a. Breast muscle cells	1.27 ± 0.035
b. Fibroblasts from breast muscle	0.16 ± 0.03
c. Erythrocytes	0.055 ± 0.005
d. Skin over breast muscle	0.48 ± 0.05

^{3}H-α-Bt, 1×10^{-8}M, was incubated with the indicated cells in PBS at 22°C for 90 min. The plated cells were a. confluent muscle cultures growing for four days; b. confluent fibroblasts obtained from breast muscle tissue by the preplating technique of Yaffe [16] and growing in culture one day; d. embryonic chick skin cells obtained by passing breast skin of 10 day chick embryos through the same procedure used for obtaining muscle cells, and cultured for one day; and c. chick erythrocytes obtained from 10 day embryonic chick blood and spun twice through PBS. These blood cells were incubated at a concentration of 10^{7} cells/ml. The incubations were terminated by two rinses in PBS containing 10^{-7}M unlabeled α-Bt. A blank collagenized petri dish had 0.2% as many counts as were bound by the muscle culture.

TABLE II. Competition of ^{3}H-α-Bt Binding by D-tubocurarine

dTC (M)	α-Bt bound (%)
10^{-6}	100 ± 6
10^{-5}	48 ± 3
10^{-4}	47 ± 3
10^{-3}	33 ± 2

Muscle cells which had been cultured for 5 days were rinsed twice with PBS and incubated for 5 min at 22°C in PBS containing the concentration of dTC indicated or just PBS (control). They were then incubated an additional 5 min at 22°C in dTC and 1.5×10^{-9}M ^{3}H-α-Bt. Incubations were terminated by rinsing with PBS containing 10^{-7}M unlabeled α-Bt.

much toxin as muscle cells do. Fibroblasts prepared this way are still slightly contaminated by myoblasts, but it was felt that they served as a better control for primary muscle cell cultures (which contain primary fibroblasts) than pure secondary fibroblasts would. Cells prepared from skin over breast muscle bound a fair amount of toxin (38%). These cells were mostly epithelial-like in morphology but may have been heterogeneous and included populations of cells with acetylcholine receptors.

A five minute incubation of cells with various concentrations of D-tubocurarine (dTC), an acetylcholine receptor antagonist, is shown in Table II. Approximately two-thirds of the toxin-binding sites could be protected by dTC under these conditions.

The competition results are in agreement with protection levels obtained for rat diaphragm [17].

In these experiments, no correction was made for specific binding, by subtracting the amount bound in dTC, because early embryonic chick AChR's have not yet been fully characterized in terms of their pharmacological properties. There is evidence in rat and frog muscle that extrajunctional receptors are considerably less sensitive to dTC than junctional AChR [18].

Binding Properties of the Receptors

A Scatchard plot [19] of the binding data obtained at 25°C with a range of ^{3}H-α-Bt concentrations is shown in Figure 4. The results give the following binding constants. The high affinity sites have a dissociation constant of $K_{D(1)} = 7.2 \times 10^{-9}$M and are present at a concentration of $R_1 = 7.9 \times 10^{-10}$M in 3.75-day muscle cultures containing 0.28 mg protein. This comes to 4×10^4 receptors per myoblast protein equivalent. The weaker binding sites have a dissociation constant of $K_{D(2)} = 2.7 \times 10^{-7}$M and are present at a concentration of $R_2 = 4.0 \times 10^{-9}$M, or 2×10^5 weak binding sites per myoblast protein equivalent. It can be seen that there are five times more weakly binding receptors in the culture than tightly binding ones. The high affinity receptors, however, bind α-Bt about 40 times more strongly than the lower affinity receptors. The orders of magnitude for both the high- and low-affinity sites make them both still relatively tightly bound toxin-receptor complexes.

The biphasic plot could indicate two different populations of receptors (either allosteric or chemically different) or that the sites are not independent [19]. Negative cooperativity, that is, filling of some sites leading to lower affinity at other sites, could produce this binding curve [20].

The magnitude of the K_D's are in agreement with those generally obtained. Since this experiment was done at 25°C to avoid metabolic effects (discussed below) the actual K_D's at 37°C may be different.

Freshly trypsinized myoblasts had a diameter of 7.5 ± 2.5 μm, resulting in a spherical surface area of almost 200 μm^2. This is a minimal figure, since the surface area could be larger if the membrane is convoluted. Given this surface area, the average density of high-affinity and low-affinity receptors is 200 and 1,000/μm^2, respectively. Since nonmuscle protein was included in the estimate of number of receptors per myoblast protein equivalent, the true values of these densities may be higher. Burden [8] finds that the majority of the

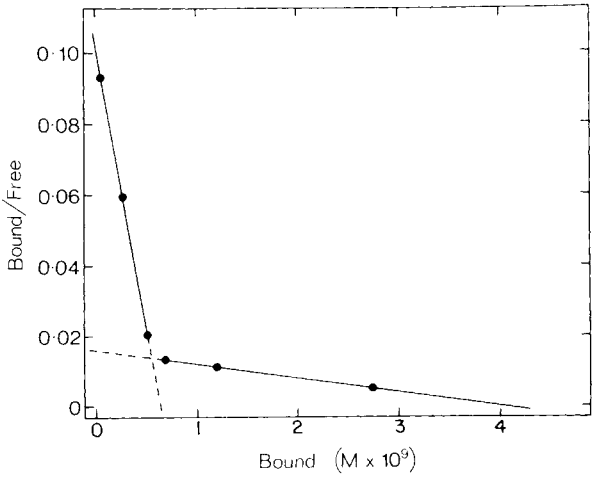

Fig. 4. Scatchard plot of binding data. ^{3}H-α-Bt was bound at various concentrations to muscle cultures which had been growing for 3.75 days. The cells were rinsed twice in PBS and incubated in PBS and toxin at 25°C for three hours. Cells were rinsed with PBS and harvested. The cultures contained 0.28 ± 0.07 mg protein.

α-Bt binding sites in embryonic chick muscle fibers are extrajunctional and occur at a density of 250/μm^2.

Reversibility of α-Bt was demonstrated by binding ^{3}H-α-Bt, rinsing off unbound toxin and incubating further in PBS. In Figure 5, cells which had bound toxin for 3 h were rinsed in PBS and then incubated in PBS at 24°C for up to 17.5 additional hours.

The dissociation does not follow simple first-order kinetics. One can analyze the dissociation data by assuming that there are two parallel first order reactions with a common product. Let $R_{(1)}$ be the total concentration of the high affinity receptor and $R_{(2)}$ the total concentration of low affinity receptor; if R_f is the concentration of free unbound receptor, B_f is the free α-Bt and BR is the toxin-receptor complex:

$$R_{(1)} = BR_{(1)} + R_{f(1)} \qquad R_{(2)} = BR_{(2)} + R_{f(2)}$$

$$BR_{(1)} \underset{k_a}{\overset{k_d}{\rightleftharpoons}} B_f + R_{f(1)} \qquad BR_{(2)} \underset{k_a}{\overset{k_d}{\rightleftharpoons}} B_f + R_{f(2)}$$

By assuming that the low-affinity receptor-toxin complex $BR_{(2)}$ dissociates more rapidly than $BR_{(1)}$ and that no $BR_{(2)}$ remained at 17.5 hours, the following kinetic values were obtained at 24°C. The association rate constants k_a were calculated from the relationship $K_D = k_d/k_a$.

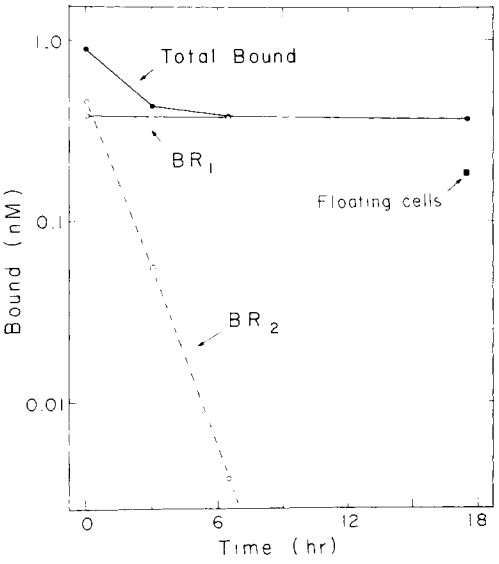

Fig. 5. Reversibility of binding of ^{3}H-α-Bt. Muscle cultures grown 4 days were incubated in 1 × 10^{-8}M ^{3}H-α-Bt for 3 h at 24°C. The cultures were rinsed twice with PBS and assayed. Other cultures which had been incubated for 3 h were washed for a total of 3 min in PBS and then incubated in PBS without toxin for the indicated times. For harvesting, they were rinsed twice in PBS and assayed. Some of the muscle cells which were incubated a total of 20.5 h at room temperature had started to detach from the dish. The medium from these cultures was spun, the pellets were washed twice in PBS and assayed separately. The amount bound to the attahced cells was used for calculations; that bound to floating cells is shown on the graph. The zero-time cultures contained 0.86 ± 0.05 mg protein. (–●–) total α-Bt bound/0.86 mg; (–○–) calculated α-Bt bound to R(1); (- - ○ - -) calculated α-Bt bound to R(2). The data are averages of duplicate determinations. In order of time of incubation, one standard deviation of each point was 4.8%, 6.6%, 9.7% and 9.0% of its value.

	High affinity (slow)	Low affinity (fast)
k_a	72 M^{-1} sec^{-1}	811 M^{-1} sec^{-1}
k_d	5.2 × 10^{-7} sec^{-1}	2.1 × 10^{-4} sec^{-1}
$t_{1/2}$	370 h	0.93 h

From these half-lives, it would appear that complexes formed with $R_{(1)}$ are 400 times more long-lived than those formed with receptors $R_{(2)}$.

Biphasic dissociation of toxin-AChR complexes have also been observed by others [4, 21]. Their values were obtained at 37°C rather than 25°C.

The toxin found on cells which remained attached to the dish declined at a rate of only 0.14% per h, or with a half-life of 370 h or 15 days. The toxin molecules bound to cells which had detached and were floating was lost at a rate of 2.3% per h, or with a half-life of 22 h. Only 24% of the bound toxin was in this floating population of cells after 17.5 h in PBS at 24°C. Such a long incubation in a non-growth supporting medium is likely to produce dying cells, and it may well be that a substantial portion of these floating cells was dying. The 22-hour half-life obtained for the toxin-receptor complex on this group would be a very approximate number for a possibly heterogeneous population. The significance of the value is only that it is much more rapid than the half-life of 15 days found at 24°C in the attached, stable population of cells. These results suggest that there is a more rapid loss of AChR in deteriorating cells, which is consistent with previous observations of more rapid membrane turnover under nongrowth conditions [22].

Other investigators, working at different temperatures, find a more rapid half-life than that of the high affinity receptor-toxin complex and a slower half-life than that of the low affinity receptor-toxin complex reported here. An AChR half-life of 22–24 h was found in skeletal muscle cultures from chick embryos [23], 17 h in cultures from foetal calf [24] and 30 h for embryonic chick skeletal muscle in vivo [8]. Burden [8] finds that several weeks after hatching, chick AChR have a half-life of greater than 5 days.

Down-Modulation of α-Bt Receptors

Table III shows that a decline in bound α-Bt molecules can be observed in cells cultured only one day and assayed at 37°C in their growth medium 8:1:1. About half of the α-Bt bound at 30 min was lost from muscle cultures by 60 minutes. The toxin molecules bound to enriched primary fibroblast cultures, which contain some muscle cells, also decline slightly.

TABLE III. Binding of ^{3}H-α-Bt to Cells at 37°C in Medium 8:1:1

Cells	α-Bt bound (picomole/mg cell protein) 30 min	60 min	% Lost
Myoblasts	2.97 ± 0.11	1.46 ± 0.27	51
Fibroblasts	1.17 ± 0.06	0.92 ± 0.06	21

Cells were prepared as described in the legend to Table I and plated for only 24 h. Myoblasts were contaminated by less than 50% fibroblasts. Fibroblasts contained about 10–20% myoblasts. The cells were incubated with 5.0 × 10^{-7}M ^{3}H-α-Bt in 8:1:1 at 37°C. The amount of protein per culture was: muscle: 0.062 mg; fibroblasts 0.165 mg. At 30 or 60 min, incubations were terminated by rinsing in 8:1:1 followed by PBS.

^{3}H-α-Bt was also bound to 4-day-old muscle cultures in their complete growth medium 8:1:1 at 25°C or 37°C for various times (Fig. 6). In 8:1:1 at 25°C binding saturated by 2 h. When the cells were kept in 8:1:1 at 37°C, however, bound α-Bt again declined after 30 min of incubation.

The loss of bound α-Bt was not due to a degradation of free α-Bt by a component in the 8:1:1 growth medium. This was shown by taking the medium from the cells incubated for 60 min at 37°C and transferring it to a fresh culture at 37°C. The same medium was then transferred to a third culture at 37°C. The two incubations gave α-Bt binding as good as the original incubation.

Although superficially the data in Table III and Figure 6 suggest a down-modulation similar to that seen in other membrane receptor systems [7] several caveats are in order. The first is that receptors are being assayed indirectly by the amount of bound α-Bt. A membrane surveillance mechanism may in fact be removing bound α-Bt without affecting the AChR. One could test for receptor decline by first binding with unradioactive α-Bt, waiting an hour, rinsing and then challenging the cells with labeled α-Bt to assay for AChR. If the amount of binding is lower than that seen in the absence of unlabeled α-Bt then receptors may have decreased (although the decrease could represent activity loss as well as physical receptor loss). Hartzell and Fambrough obtained this result [25, Fig. 5] although they did not discuss it. Presumably, they consider it due to irreversible binding. They did not see a decline when binding at 37°C in their HEPES- buffered incubation medium, and did not see reversible binding. Their extensive eight-rinse half-hour washing procedure may have removed the toxins bound to lower affinity sites. If this is the case, it would suggest that the lower-affinity receptors are the ones modulated here.

The physiological role of these sites is now speculative, but they have a very significant K_D of 2.7×10^{-7}M at 25°, suggesting specific binding.

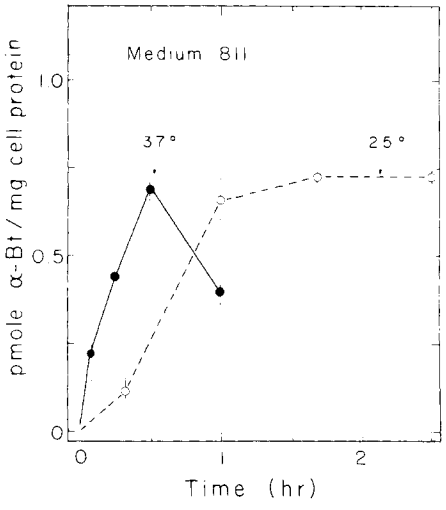

Fig. 6. Binding ^{3}H-α-Bt at 37°C vs 25°C in medium 8:1:1. Four day old cultures were incubated in 8:1:1 with ^{3}H-α-Bt, 5.5×10^{-8}M, for various times at the indicated temperatures. Error bars indicate the range of the data. The cultures contained 0.72 ± 0.05 mg protein. Duplicate determinations were performed.

DISCUSSION

It appears from these results that cultured embryonic chick muscle cells which are not innervated may contain two populations of acetylcholine receptors and that in metabolically active cells a fraction of the total population of receptors may be lost after binding α-bungarotoxin.

Several ways in which α-Bt molecules may be lost from the cells have been illustrated here.

A. Equilibrium. The reversibility of bound toxins under non-metabolizing conditions was due to the binding being an equilibrium process, and when reactants were removed the product dissociated.

B. Turnover. The observation that floating, detached cells lose toxin sixteen times faster than attached cells would indicate that a dying population of cells has a loss of bound toxins not explained by simple equilibrium reversibility, but probably by a manifest membrane degradation in non-growth conditions, as observed previously [22].

C. Down-modulation. Under growth-permitting conditions, a loss of bound toxins in the continued presence of toxin resembling down-modulation of hormone receptors was seen. The mechanism of this metabolic removal of α-Bt is not known. Toxin-receptor complexes could be internalized or released into the medium, or both [26].

Since a loss of AChR was not monitored directly here, but inferred from the lost bound α-Bt, it is also possible that receptors were not lost, but that there is a form of membrane surveillance which removes unwanted bound molecules from the cell surface without removing the receptors. Also, receptors could have been inactivated by α-Bt.

Other investigators have also noted a turnover of bound α-Bt [27]. Rat diaphragms in organ culture lost bound α-Bt, and the loss was prevented by NaCN and to some extent cycloheximide, suggesting that the loss requires metabolic activity. The authors also found that toxins bound to extrajunctional receptors were lost four times more rapidly than those bound to junctional receptors in a 24-h period, suggesting that these two classes of AChR have different turnover properties.

A small decline in bound α-Bt was seen in the chick embryo pectoral muscle after a single injection of 25 μg/g α-Bt into an 11-day-chick embryo [8]. This pulse of α-Bt did not affect concentrations of AChR in chick muscle several days later.

In vivo studies with α-Bt in the toad [28] and rat [29] suggest a loss in ACh receptors following α-Bt binding. Binding α-Bt results in long-term effects preventing maintenance and reformation of functional synapses.

Conversely, chronic exposure to α-Bt in vivo in adult rats, over a period of 3 days, resulted in an increase in AChR in the rat diaphragm similar to that which occurs after denervation [30].

Down-modulation of AChR was produced by AChR antibodies [31]. Receptors for β-adrenergic neurotransmitters have also been found to be down-regulated [32].

The loss of α-Bt binding seen here required optimal growth conditions, medium 8:1:1 at 37°C, suggesting that a metabolic process was involved. It was produced by an AChR antagonist (a molecule which competes with ACh for the receptor but does not depolarize the muscle membrane). It was found in 24-h unfused myoblasts and also in 4-day heterogeneous myotube/myoblast cultures. Older cultures were not investigated.

If the loss of bound α-Bt seen here was produced by a type of desensitization which converts the AChR to an inactive state, then this conversion has an activation requiring 37°C and therefore does not occur at 25°C.

The function of down-modulation in general is still speculative. The AChR are indeed down-modulated during the normal development of nerve–nerve and nerve–muscle junctions. It remains to be seen if the mechanism for down-modulation of extrajunctional AChR is the same as the one involved in the loss of α-Bt binding seen here.

ACKNOWLEDGMENTS

The author appreciates help with protein chemistry from Chris Bruton and Bob Jack, wishes to thank Dennis Bray and Sybil Holtzer for training in muscle cell cultures, and Lincoln Potter, Alan Fersht and Sydney Brenner for valuable discussions. This work was supported by the Arthritis Foundation at the MRC Laboratory of Molecular Biology, Cambridge, England, and by the Muscular Dystrophy Association.

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