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Appearance of Acetylcholine Receptors During Differentiation of a Myogenic Cell Line

(snake venom/hyperpolarizing response/neurotoxin)

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ABSTRACT **Acquisition of acetylcholine receptors during differentiation of a clonal myoblast cell line was monitored with a neurotoxin isolated from venom of the Indian Cobra *Naja naja*. Toxin bound specifically and reversibly to acetylcholine receptors of the differentiated cells. Specificity of the binding reaction was assayed by measurement of the ability of various cholinergic agonists and antagonists to compete with neurotoxin for its binding site. The rate of toxin binding paralleled the rate of inactivation of functional acetylcholine receptors, as measured by iontophoretic application of acetylcholine. Bound toxin was released from the cells with a half-life of about 7 hr. This release was not associated with a decrease in the total number of toxin-binding sites. A slow hyperpolarizing response to acetylcholine seen in myoblasts was insensitive to toxin; the appearance of toxin-binding sites parallels the appearance of fused fibers during differentiation of the muscle cells in tissue culture.**

Our understanding of the functioning of the acetylcholine receptor during neuromuscular transmission (1), of the regulation of its distribution (2), or of its postulated role in mediating nerve–muscle trophic interactions (3) has not been limited by lack of interest. Rather, the limitations in our current understanding are a consequence of inadequate means of identification and manipulation of the receptor and a lack of biological preparations amenable to biochemical analysis. There has recently appeared, however, evidence that one type of neurotoxin isolated from the venom of elapid snakes binds specifically to the acetylcholine receptor. A neurotoxin, α-bungarotoxin, isolated from the venom of *Bungarus multicinictus* blocks neuromuscular transmission (4) and chemical excitation in the electroplax preparation (5). Binding of the radioactively labeled toxin has been demonstrated in membrane preparations derived from the eel electroplax (6, 7) and from guinea-pig brain (8). Binding to rat (9, 10) and mouse (11) skeletal muscle has also been reported. Binding specificity has been inferred either from the ability of high concentrations of receptor ligands to compete with toxin or from the enhanced binding seen at regions of muscle known to contain synapses.

To investigate both the function and regulation of the acetylcholine receptor, we have examined the interaction of a neurotoxin, isolated from the venom of the Indian Cobra, *Naja naja*, with a muscle-cell line in tissue culture. This line was chosen because it exhibits many of the differentiative steps seen in muscle cells *in vivo* (12), and because it interacts with nerve cells in tissue culture in a manner reminiscent of nerve–muscle interactions seen *in vivo* (13). In this paper, we define the conditions for binding of the neurotoxin to the muscle cells in which the toxin is considered to react specifically with the acetylcholine receptor. We also distinguish between two different acetylcholine receptors and present the time course of appearance, during muscle differentiation, of the class of acetylcholine receptors responsible for the fast depolarizing response to acetylcholine.

Abbreviation: dTC, *d*-tubocurarine.

MATERIALS AND METHODS

Cell Lines. A clone, L6, of the muscle cell line isolated by Yaffe (12) was used in all experiments. The cells were grown in 60-mm Falcon tissue culture dishes in modified Eagle's medium containing 20% fetal-calf serum (GIBCO), and incubated in a 12% carbon dioxide–88% air incubator (14). The line was propagated as myoblasts until needed, at which time dishes containing unfused cells were treated with 0.05% Viokase (GIBCO), harvested, washed, and replated at a density of 5×10^4 per dish. Under these conditions the cells divide for about 6 days and then fuse to form multinucleate myotubules. Binding experiments were done on cells within 2 weeks of fusion.

Preparation of Toxin. Neurotoxin was purified from the venom of *Naja naja* (Ross Island Reptile Institute) by the techniques described by Karlsson (15) for the purification of a neurotoxin from the venom of *Naja nigricollis*. The purified toxin formed a single symmetrical peak on chromatography through Sephadex G-75 and on ion exchange chromatography on Amberlite CG-50, and migrated as a single band on electrophoresis on polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The purified toxin was iodinated with ^{125}I by the chloramine-T method (16) and separated from unincorporated iodine by gel filtration through Sephadex G-25. The resulting ^{125}I-labeled toxin was chromatographed again through Sephadex G-75 for removal of aggregated material that appeared during iodination. The purified, iodinated toxin used in these experiments had a specific activity of 4800 Ci/mol.

The Binding Assay. Binding of neurotoxin to myotubules was performed in the tissue culture dish in which the cells were grown. The growth medium was removed; cells were washed once with prep medium (modified Eagle's medium in which the bicarbonate was replaced with 1.08 mM Na_2HPO_4–1.15 mM KH_2PO_4) containing 0.2% fetal-calf serum tested by

immunoprecipitation (GIBCO), and then covered with 2.0 ml of the same medium. If applicable, compounds to be tested for inhibition of binding were added and incubated for 10 min. Labeled toxin was then added and allowed to react for the desired time at room temperature (19–22°). The incubation medium was removed by suction; cells were washed four times with prep medium, removed from the surface of the dish with a rubber policeman, and collected by suction on cellotate filters (Millipore). The filters were then washed twice with 4 ml of phosphate buffer solution (pH 0.0), dried, and counted in a liquid scintillation counter. All results are averages of duplicate assays.

Electrophysiological Recording. Recording electrodes were filled with methanol by boiling under reduced pressure at room temperature. The methanol was replaced first with water and then with 3 M KCl. Acceptable electrodes had resistances in the range of 50–150 mΩ. Acetylcholine electrodes were made by boiling in water and then replacing the water with a 0.5 g/ml solution of acetylcholine. Acceptable electrodes filled with acetylcholine had resistances of 150–300 MΩ.

Iontophoretic application of acetylcholine was accomplished by connection of the acetylcholine pipette to a constant current source with a feedback circuit (17). A bias current, which was determined for each position on the cell, was applied to the acetylcholine electrode to prevent leakage of acetylcholine from the electrode. Due to the fact that it is not possible to estimate what proportion of the current pulse is capacitive current, we cannot estimate how many acetylcholine molecules are released from the pipette, but for our purposes it is only necessary that the same amount of acetylcholine be released from a given pipette with each current pulse of the same intensity and duration. That this is indeed the case is shown by the fact that a given pulse of current delivered through the acetylcholine pipette always gives rise to the same amount of depolarization (±10%) of the muscle fiber in the absence of toxin.

The muscle fibers were visualized under water-immersion (×400) phase optics for microelectrode penetration. The

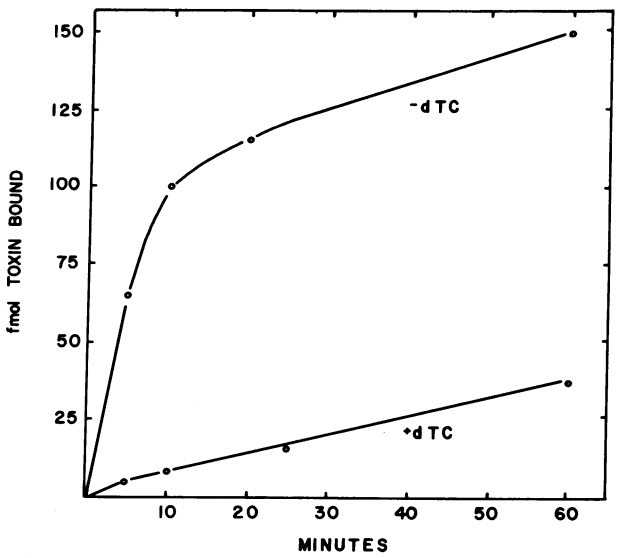

FIG. 1 Binding of ^{125}I-labeled toxin to muscle cells. Cells were incubated in 15 nM toxin with or without 0.2 mM dTC. The amount of toxin bound per plate at the indicated times was determined as described in *Methods*.

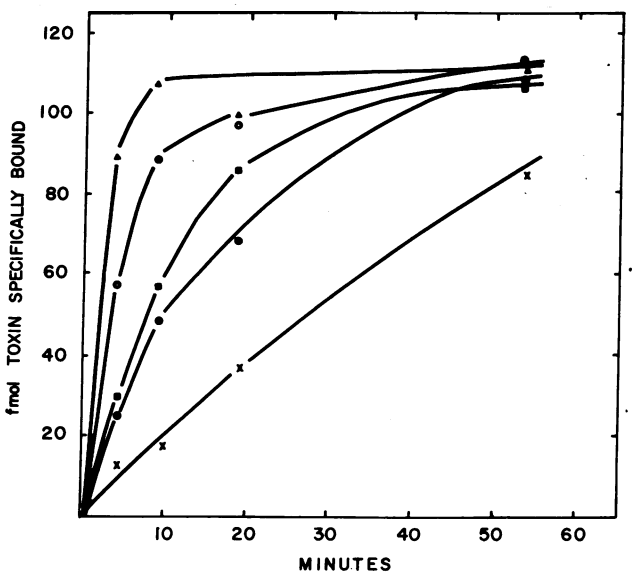

FIG. 2. Concentration dependence of binding of ^{125}I-labeled toxin to muscle cells. Cells were incubated in various concentrations of toxin for the indicated times with or without 0.2 mM dTC. The amount of toxin bound in the absence of dTC minus that bound in the presence of dTC is considered specific and is plotted as a function of incubation time. The initial rates are directly proportional to toxin concentrations. The calculated second-order rate constant is $9 \times 10^4 M^{-1}s^{-1}$. ×——×, 3 nM; ●——●, 6 nM, ■——■, 9 nM; ○——○, 15 nM; ▲——▲, 30 nM.

cells were grown as described above, and experiments were performed in prep medium supplemented with 0.2% fetal-calf serum at room temperature.

An experiment was performed by penetration of a cell with a recording electrode in the presence or absence of toxin (9 nM). The acetylcholine pipette was then placed on the surface of the muscle, within 30 μm of the recording electrode, and the current pulse was adjusted to give a depolarization of about 3 mV. The electrodes were left in place for the duration of the experiment. A pulse of acetylcholine was applied every 2 min, and the response was recorded through the intracellular recording pipette. In the absence of toxin, the response was constant (±10%) for up to 90 min.

RESULTS

When myotubules are incubated in the presence of 15 nM toxin, the amount of toxin bound to the cells increases rapidly for about 15 min and then proceeds to increase at a lower rate. Addition of 0.2 mM *d*-tubocurarine (dTC), a known antagonist of acetylcholine receptors, essentially eliminates the fast binding component leaving only the slow component (Fig. 1). The difference between the amount of toxin bound in the presence or absence of 0.2 mM dTC can be considered bound to the acetylcholine receptor. The neurotoxin binding to muscle cells that is protected by dTC, occurred in a manner consistent with overall second-order kinetics. At a given concentration of toxin, where toxin is present in a large molar excess with respect to receptors, the time course may be described as pseudo first-order with respect to available receptor sites. Fig. 2 shows typical time courses of binding at various toxin concentrations. At low neurotoxin concentrations, the initial rates are linearly related to the toxin con-

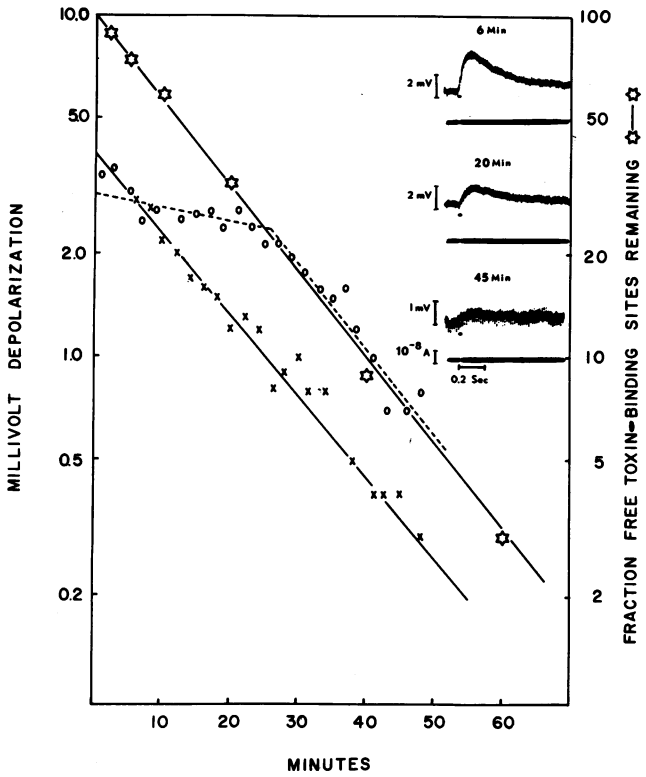

FIG. 3. Rates of toxin binding and inactivation of the cellular response to iontophoretically applied acetylcholine. Toxin was added at time zero to a concentration of 9 nM, and the response to acetylcholine was determined at the indicated times. The *circles* and *crosses* represent responses with two different cells. The *stars* represent the loss of toxin-binding sites as measured in a separate experiment with the same concentration of toxin. The *insert* shows representative traces of the electrophysiological responses of the cells at the indicated times after the application of toxin.

centration. The plateau was independent of neurotoxin concentration over the range tested. No attempt was made to investigate the binding reaction at neurotoxin concentrations less than 3 nM.

Specificity of the binding reaction was investigated by two methods. In the first, we simply asked whether the affinities of various receptor agonists and antagonists for the toxin-binding site were similar to their affinities for the acetylcholine receptor as measured in other preparations. Four compounds, two agonists and two antagonists, were chosen on the basis of their widely different affinities. In other systems, the apparent dissociation constants for these compounds range from 0.01 to 30 μM. If we assume that at the concentration equal to their apparent dissociation constant these compounds bind 50% of the available receptors, then, at this concentration, each agonist and antagonist should decrease the initial rate of toxin binding by 50%, but all should decrease the rate to the same extent at saturating concentrations. Cells were incubated with 9 nM toxin for 5 min in the absence of protector, in the presence of saturating protector, or in the presence of concentrations of protector equal to their apparent dissociation constant. As seen in Table 1, all compounds gave essentially the same protection at saturating concentrations and all gave partial protection at 50% occupancy. The variations around the expected 50% inhibition are within the variations seen between different experiments. The fact that partial protection is exhibited in proportion to the affinities expected of these four compounds argues that the toxin is binding to a site with the binding characteristics of the acetylcholine receptor.

In the second instance, we asked whether the dTC protectable binding was associated with binding at functional acetylcholine receptors. In this case we compared the rate of loss of toxin-binding sites to the rate of loss of free receptors as measured electrophysiologically. The results of these experiments (Fig. 3) demonstrate that the rates are indeed the same, but that there is sometimes a lag before any inactivation is seen. A lag was seen in three of the six preparations tested. The fact that there are instances in which there is no lag and the fact that the rate of inactivation after the lag is the same as the toxin-binding rate argues that the protectable binding is, in fact, binding to acetylcholine receptors.

The lag in inactivation of acetylcholine sensitivity is the property of cells showing high toxin-binding capacity. A reasonable explanation for the lag in toxin inactivation of acetylcholine sensitivity is that the response to iontophoretically applied acetylcholine is not always limited by the number of available receptors. In the experiments on two cells from different preparations shown in Fig. 3, the preparation that showed a lag had 3.4 times more protectable toxin-binding sites. If we look at the curve showing the lag we see that extrapolation of the linear portion of the inactivation curve extrapolates to a 10 mV depolarization at time zero. This value is about three times the average maximum depolarization seen in the two cells. Although this observation is consistent with the idea of excess receptors, it does not eliminate other interpretations. It should be noted, however, that in examination of many myotubule preparations those that had low-binding capacity did not show a lag but gave simple first-order inactivation kinetics.

Binding of neurotoxin to the acetylcholine receptor has been considered irreversible (5–7, 9, 10), although reversible binding to electroplax membrane fragments has been demonstrated (submitted for publication). We examined the reversibility of toxin binding to muscle cells that had been labeled to saturation. Cells were labeled with toxin in the presence or absence of dTC, washed extensively on the plate and returned to the incubator at 37° in their normal growth medium with or without 0.2 mM dTC. The amount of toxin remaining bound was then determined at various times. The amount of specifically bound toxin was determined by subtracting from each measurement the amount of toxin remaining bound to

TABLE 1. *Inhibition of toxin binding by cholinergic ligands**

Ligand	Concentration (μM)	Counts bound	% Protection
None	—	352	—
Carbamylcholine	1000	39	89
Carbamylcholine	20	260	29
Decamethonium	1000	30	92
Decamethonium	2	238	37
d-Tubocurarine	200	31	91
d-Tubocurarine	0.2	153	62
Benzaquinonium	200	36	90
Benzaquinonium	0.01	224	40

* Toxin concentration was 9 nM.

cells labeled in the presence of dTC. As seen in Fig. 4, the amount of bound toxin decreases with time, the rate of toxin release being 8–10 times faster in the presence of 0.2 mM dTC.

The results of the toxin release experiments do not eliminate the possibility that the binding of toxin to receptor is, in fact, irreversible and that the observed release of toxin is actually release of a stable toxin–receptor complex. The fact that dTC increases the rate of release makes this idea unlikely. To further substantiate this conclusion, we measured the number of toxin-binding sites remaining after release of toxin was nearly complete. Since the presence of receptor could be due either to dissociation of the toxin–receptor complex or to resynthesis of receptor, we performed the experiment in the presence of cycloheximide (50 μg/ml), which we found to reduce the rate of protein synthesis by more than 99%. We assumed that cycloheximide inhibits receptor synthesis to the same extent that it inhibits protein synthesis. Under these conditions, the loss of more than 80% of the bound toxin from the cells was associated with a loss of less than 10% of the acetylcholine receptors initially detected.

There remains the possibility that toxin dissociated from an acetylcoline receptor molecule in a manner that left unchanged the toxin-binding site, but irreversibly altered its ability to respond to acetylcholine. This hypothesis became untenable when it was found that sensitivity to iontophoretically applied acetylcholine reappeared after dissociation of the toxin–receptor complex, even under conditions in which protein synthesis was inhibited by cycloheximide (Heinemann, in preparation).

Having established the extent to which toxin binding is specific for the acetylcholine receptor, we proceeded to examine the relationship between the toxin-binding capacity of myoblasts and myotubules and their physiological response to

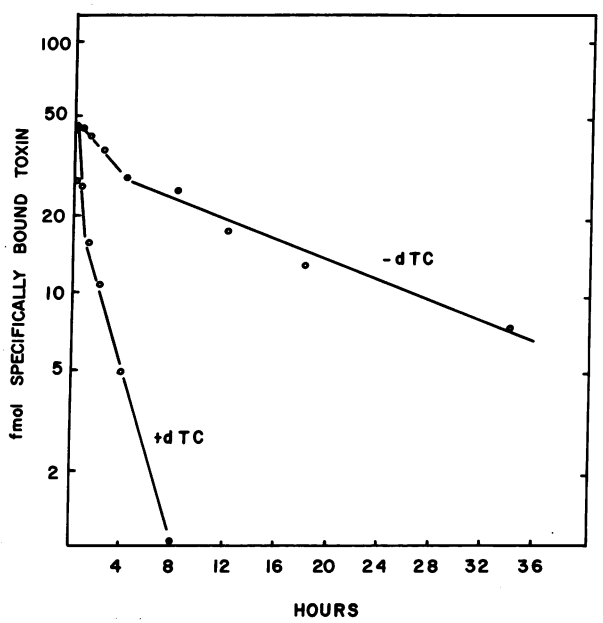

FIG. 4. Dissociation of the toxin–receptor complex. Cells were maximally labeled with toxin in the presence or absence of dTC, and the loss of toxin from the cells was followed in medium with or without dTC. The amount of specifically bound toxin is defined as that bound to cells labeled in the absence of dTC minus that remaining bound to cells labeled in the presence of dTC.

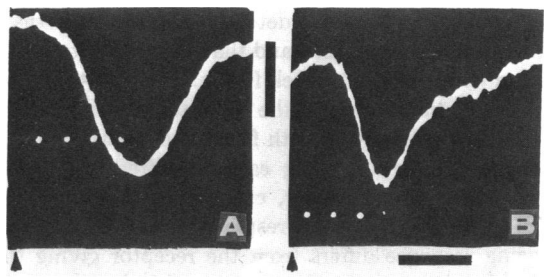

FIG. 5. Effect of toxin on the slow hyperpolarizing response to acetylcholine. At the time indicated by the *arrow*, the bias current on the acetylcholine pipette was turned off, and square pulses of 100-msec duration (10^{-9} coulombs of charge per pulse) were delivered at a frequency of 0.5 Hz. The resting potentials at the time of the recordings were 35 mV for the cell in *A*, and 32 mV for the cell in B. Medium containing 2.5 μg/ml neurotoxin was added 80 min before the record in *B* was taken. The culture was maintained at 35° for these records. *Calibration bars* are 5 mV and 5 sec; pulses were delivered at times indicated by the *dots*.

iontophoretically applied acetylcholine. It has been reported (13) that the myoblast responds to acetylcholine with a slow hyperpolarizing response (Fig. 5) while the application of acetylcholine to myotubules elicits a fast depolarizing response (Fig. 3). These different responses might be a consequence of two different acetylcholine receptors, one present in the myoblasts and another present in the myotubules, or one acetylcholine receptor possessing different functions in the two cells. We examined these possibilities first by determining whether toxin, which completely blocks the fast response, had any effect on the slow hyperpolarizing response. As seen in Fig. 5, concentrations of toxin 100-fold greater than those that block the fast response are without effect on the slow response. We then examined the ability of cells to bind toxin as a function of their state of differentiation. The results in Fig. 6 show

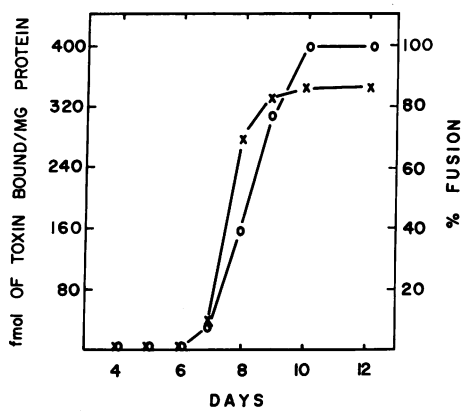

FIG. 6. Time course of development of toxin-binding sites. A large number of plates were seeded with myoblasts at a density of 5×10^4 per plate. At the indicated days after plating, several plates were removed for determination of specific toxin-binding sites, DNA content, protein content, and degree of fusion. Fusion was determined by counting nuclei in cells fixed and stained with crystal violet. Percent fusion is defined as the percent of nuclei in cells with more than one nucleus. No increase in the amount of DNA per plate was seen after the eighth day in culture. X——X, toxin binding capacity; O——O, percent fusion.

that no toxin binding was detectable in cultures that consisted entirely of myoblasts, and that the ability to bind toxin appeared with the onset of cell fusion. The results do not permit a distinction between the simultaneous appearance of toxin-binding capacity in both fused and unfused cells or the appearance of toxin-binding capacity after fusion of cells. Several points are, however, clear from these experiments. The acetylcholine receptor responsible for the slow hyperpolarizing response differs from the receptor giving the fast depolarizing response in that it is not blocked by toxin and does not appear to bind toxin. If the association of acetylcholine sensitivity with myotubules is a consequence of activating previously existing receptors, the activation process must include the generation of a toxin-binding site. The same requirement must hold for conversion of a slow hyperpolarizing receptor into a fast depolarizing receptor. Alternatively, the appearance of sensitivity may be due to *de nova* receptor synthesis or insertion into the membrane of previously hidden receptors.

DISCUSSION

A prerequisite to the use of a labeling reagent is demonstration of the specificity of the labeling and an understanding of the labeling reaction. In this paper, we have shown that a neurotoxin isolated from the venom of the Indian Cobra, *Naja naja*, binds with a high degree of specificity to acetylcholine receptors of a muscle cell line propagated in tissue culture. As many as 94% of the toxin-binding sites may be protected by acetylcholine receptor agonists and antagonists. That this binding is in fact to the acetylcholine receptor is demonstrated by the similarities between the toxin-binding site and the active site of the acetylcholine receptor as determined by protection experiments, and by the fact that the rate of specific binding of toxin is the same as the rate of inactivation of the cellular response to acetylcholine.

The interaction of toxin and receptor can be described as pseudo first-order since toxin concentration does not change over the course of the reaction. The second-order rate constant calculated from the dependence of the initial reaction rate on toxin concentration is $9 \times 10^4\ M^{-1}s^{-1}$. It is interesting to note that this is similar to that obtained for interaction of the same toxin preparation with membrane fragments prepared from eels (submitted for publication). It is also similar to the value obtained for the interaction of a related toxin with eel membrane fragments (18), although this toxin is thought to bind irreversibly.

The dissociation of toxin from receptor exhibits biphasic exponential kinetics. Since the forward reaction exhibits simple first-order kinetics, it seems unlikely that the biphasic back reaction is a consequence of two different classes of receptor molecules. One interpretation is that the receptor is mobile in the membrane, making the dissociation of toxin dependent on the characteristics of the toxin-binding site and on the orientation of the receptor in the cell membrane.

If we assume that acetylcholine receptors are distributed evenly over the surface of the myotubules and that there is only one toxin-binding site per receptor, we find about 100 receptors per μm^2. This value is considerably less than that estimated for receptor density at motor end plate regions or in denervated muscle (10, 11) and probably represents the receptor density seen in fetal muscle before innervation. This is consistent with the observation that the interaction of nerve cells with myotubules results in a large increase in the sensitivity to acetylcholine at the region of contact between the nerve and muscle (13).

It is clear that the myoblast receptor differs from the acetylcholine receptor found in the myotubules, not only in its function, but in its resistance to neurotoxin. It may thus represent a molecular entity distinct from the depolarizing acetylcholine receptor or it may be the same type of receptor performing a different function in the myoblast membrane.

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