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CONTROL OF ACETYLCHOLINE RECEPTOR MOBILITY AND DISTRIBUTION IN CULTURED MUSCLE MEMBRANES

A FLUORESCENCE STUDY

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Summary

The molecular control of the distribution and motion of acetylcholine receptors in the plasma membrane of developing rat myotubes in primary cell culture was investigated by fluorescence techniques. Acetylcholine receptors were marked with tetramethylrhodamine-labeled α-bungarotoxin and lateral molecular motion in the membrane was measured by the fluorescence photobleaching recovery technique. Three types of experiments are discussed: (I) The effect of enzymatic cleavages, drugs, cross-linkers, and physiological alterations on the lateral motion of acetylcholine receptors and on the characteristic distribution of acetylcholine receptors into patch and diffuse areas. (II) Observation of the distribution and/or motion of fluorescence-labeled concanavalin A receptors, lipid probes, cell surface protein, and stained cholinesterase in acetylcholine receptor patch and diffuse areas. (III) The effect of a protein synthesis inhibitor and electrical stimulation on membrane incorporation of new acetylcholine receptors.

Some of the main conclusions are: (a) acetylcholine receptor lateral motion is inhibited by concanavalin A plant lectin and by anti-α-bungarotoxin antibody, but marginally enhanced by treatment with a local anesthetic; (b) patches are stabilized by an immobile cellular structure consisting of molecules other than the acetylcholine receptors themselves; (c) this structure is highly selective for acetylcholine receptors and not for other cell membrane components; (d) acetylcholine receptor patch integrity and diffuse area motion are independent of direct metabolic energy requirements and are sensitive to

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Abbreviations: TMR, tetramethylrhodamine; IgG, immunoglobulin G; diI-C_{18}-(3), dioctadecylindocarbocyanine; LETS protein, large, external, transformation-sensitive protein; EGTA, ethyleneglycolbis-(β-aminoethylether)-N,N'-tetraacetic acid.

electrical excitation of myotube; (e) lipid molecules can move laterally in both acetylcholine receptor patches and diffuse areas; and (f) acetylcholine receptor lateral motion in diffuse areas and immobility in patch areas are not altered by specific agents which are known to affect extrinsic cell surface proteins, or cytoplasmic microfilaments and microtubules.

Introduction

Studies of the appearance, distribution, and lateral motion of acetylcholine receptors on fused embryonic muscle cells are important to understanding the molecular sequence of events during neuromuscular synapse formation. Such studies are also potentially significant for understanding membrane structure and biophysics, for several reasons. First, the acetylcholine receptor is a well-defined membrane protein whose chemistry in vitro and physiological function in vivo have been extensively studied [1]. Second, the distribution and lateral mobility of acetylcholine receptors on the surface of developing muscle cells in primary culture [2—7] is heterogeneous, coexisting in both uniform diffuse regions at low surface density with significant lateral mobility, and in densely packed, speckly patches of immobilized acetylcholine receptors. Third, acetylcholine receptors can be marked specifically and irreversibly by radioactivity or fluorescence-tagged α-bungarotoxin.

In this paper, we describe experiments designed to yield information on the cellular mechanisms that control acetylcholine receptor distribution and motion on myotubes. Our results reveal several structural and dynamic relationships between acetylcholine receptors and certain other cellular components, and also exclude several otherwise reasonable models of the interaction between acetylcholine receptors and the cell surface. Three types of experiments are described: (a) the effect upon acetylcholine receptor motion and distribution of chemical agents which alter the molecular structure or physiology of the cell surface; (b) study of the motion and distribution of other specific cell surface constituents relative to the characteristic acetylcholine receptor distribution; and (c) a limited study of treatments affecting cell surface acetylcholine receptor turnover rates.

We performed all of the experiments upon primary cultures of embryonic rat muscle. Cell surface components were visualized by fluorescence microscopy by marking them with fluorescent groups; in particular, acetylcholine receptors were marked by tetramethylrhodamine α-bungarotoxin (TMR-α-bungarotoxin) [8]. Lateral motion of fluorescent molecules on the cell surface was measured by the fluorescence photobleaching recovery technique [9,10]. Fluorescence photobleaching recovery previously has been applied to study the lateral motion of a wide variety of model membrane [11,12] and cell surface [13—19] constituents including acetylcholine receptors on rat and chick myotubes [4,10].

Experimental procedures

Culturing of rat myoblasts. The culturing procedure was performed as described previously [4]. Myoblasts usually began fusing with each other to

form myotubes on or about the third day after plating. The experiments on myotubes described herein were performed on 4—10-day-old cultures.

Fluorescence-labeled α-bungarotoxin. We purified α-bungarotoxin from snake venom (Sigma) by the method of Lee et al. [20]. We labeled the α-bungarotoxin with tetramethylrhodamine (TMR) as described previously [8]. Fluorescein α-bungarotoxin was prepared by exactly the same procedure as TMR-α-bungarotoxin. By this procedure, the final TMR-α-bungarotoxin preparation is monolabeled, whereas the fluorescein-α-bungarotoxin is a mixture of mono and higher degrees of labeling.

TMR-α-bungarotoxin is monovalent and is unlikely to cross-link or redistribute acetylcholine receptors. In addition, pre-fixing the myotubes with formaldehyde does not alter the TMR-α-bungarotoxin marking pattern, nor is the pattern qualitatively a function of TMR-α-bungarotoxin incubation time on the myotubes.

Other chemical agents. Concanavalin A was obtained from Miles-Yeda. Succinyl-concanavalin A, tetramethylrhodamine (TMR) concanavalin A and TMR-succinyl-concanavalin A were gifts of Dr. Kenneth Jacobson. Concanavalin A-bound blood platelets were a gift of Dr. Gerald Edelman.

Cytocholasin B was obtained from Imperial Chemical Co.; collagenase and trypsin from Worthington Biochemical; colchicine, vinblastine, dithiothreitol, neuraminidase, hyaluronidase, puromycin · HCl, α-mannosidase and dibucaine from Sigma; and A23187 for Eli Lilly.

Rabbit antibody to α-bungarotoxin was a gift of Dr. Mathew Daniels; TMR-conjugated IgG fraction goat antibody to rabbit IgG was obtained from Cappel Laboratories; dioctadecylindocarbocyanine (diI-C_{18}-(3)) was a gift of Dr. Alan Waggoner. Antibody to 'cell surface protein' (sometimes called 'large external transformation-sensitive' protein or LETS) was a gift of Dr. Ira Pastan. Anti-LETS protein was labeled with TMR by the method of Goldman [21].

Fluorescence measurements. Fluorescence of myotubes adhering to the tissue culture dish was observed by a photomultiplier mounted on a fluorescence microscope using vertical illumination through the objective. The 568.2 nm or the 530.9 nm line of a krypton laser was the excitation light source. We used a 40X, numerical aperture 0.75 microscope objective directly immersed in the Hank's balanced salt solution bathing the cells. The exact optical and electronic system is described elsewhere [10].

In applying the fluorescence photobleaching recovery technique to measure lateral molecular motion, the fluorescence of labeled molecules or probes in an approx. 3 μm^2 area on a cell surface is bleached by a 0.4 s pulse of intense (about 1 mW/μm^2) focused laser light. The subsequent lateral motion of un-bleached fluorophore into the bleached area is measured by the recovery of fluorescence excited by the same, although much attenuated ($\approx 10^4$ times) laser beam, flashed at 0.4 s pulse duration every 4 s. The lateral motion was quantitated in terms of a two-dimensional diffusion constant, calculated according to the method of Axelrod et al. [9] by measuring the time required for the fluorescence to recover halfway from its immediate postbleach level to its long-time asymptotic level. The completeness of the fluorescence recovery at long times was used as a measure of the fraction *f* of the total fluorophore which is mobile [9].

The bleaching laser pulse does not overheat the cell surface [22]. In addition, prolonged illumination by laser light of higher power than that employed here has been observed to not affect the viability of other cell types (Schlessinger, J., personal communication).

Results

We performed three classes of experiments designed to probe the molecular interaction between acetylcholine receptors and the cell surface: (A) fluorescence photobleaching recovery measurements and/or photographs of TMR-α-bungarotoxin-labeled myotubes before (as a control) and after treatment with various chemical agents; (B) fluorescence photobleaching recovery measurements on other fluorescence marked cell surface constituents in acetylcholine receptors patch vs. diffuse areas simultaneously visualized with fluorescein α-bungarotoxin by a fluorescence doubling labeling technique, and (C) fluorescence observation of the slow (on the order of hours) turnover of acetylcholine receptors in the presence and absence of electrical stimulation and protein synthesis inhibitor puromycin.

TMR-α-bungarotoxin-acetylcholine receptor motion and distribution

It has been shown in previous fluorescence photobleaching recovery work on rat myotubes [4] that acetylcholine receptors in high-density patches are immobile and the acetylcholine receptors in the remaining lower-density diffuse areas are mobile. We examined the effects of various chemical agents on the diffusion constant D of TMR-α-bungarotoxin-acetylcholine receptor in diffuse areas and the presence and shape of TMR-α-bungarotoxin-acetylcholine receptor patches as recorded in sequential photographs over periods of 2—7 h.

Table I lists the chemical agent, the treatment protocol, the fluorescence photobleaching recovery-measured diffusion constants D_T and D_C of the treated cultures and controls, respectively, and the corresponding fractions f_T and f_C of the total TMR-α-bungarotoxin-acetylcholine receptor which was mobile. Unless otherwise noted, the treatments were preceded by a standard protocol for TMR-α-bungarotoxin labeling: 10^{-7} M TMR-α-bungarotoxin in Hank's balanced salt solution for 1 h at 22°C followed by a 0.5—1 h wash (with several solution changes) in Hank's balanced salt solution containing 2 mg/ml bovine serum albumin. While we have not directly examined the effectiveness of the treatments employed on the expected sites of action, the conditions for each treatment were chosen as being effective in other cell systems.

Three treatments affected TMR-α-bungarotoxin mobility in diffuse areas: tetravalent concanavalin A, anti-α-bungarotoxin antibody (both decreasing the mobile acetylcholine receptor fraction), and dibucaine (marginally increasing the diffusion constant). Acetylcholine receptor visualization by TMR-α-bungarotoxin was not inhibited or altered by prior (rather than post) treatment with concanavalin A. TMR-α-bungarotoxin motion appeared insensitive to all the other treatments, at least to within the uncertainty (generally ±50%) of the results.

A significant fraction of the acetylcholine receptors in diffuse areas remained immobile despite the treatments administered to the cells. Using a high power

objective (numerical aperture 1.25), small clumps of acetylcholine receptors of diameter less than 1 μm are occasionally visible; these acetylcholine receptor clumps in otherwise diffusely labeled areas may represent the immobile fraction.

None of the treatments led to any dramatic redistribution of acetylcholine receptors noticeable upon observation several hours after the beginning of treatment. Although there was some internalization of fluorescence most likely due to acetylcholine receptor-TMR-α-bungarotoxin turnover [4], patches remained in approximately the same regions with the same highly speckled and granular internal structure after chemical treatment. If chemical treatment had altered the molecular mechanism confining acetylcholine receptors in patches, one would at least expect the fine structure of the patches to become blurred; this was not observed. In some instances, the treatment caused some myotubes to round up, a preliminary state before losing contact with the dish. In these cases, patches were occasionally still found on the rounded-up cell.

Motion and distribution of other membrane components

In order to probe acetylcholine receptor interactions with other membrane proteins and lipids, we optically examined the distribution and motion of certain other myotube surface components. In particular, we discuss the following experiments in this section: (a) comparison of the motion of TMR-succinyl-concanavalin A receptors in acetylcholine receptor diffuse regions vs. acetylcholine receptor patch regions as visualized by fluorescein-α-bungarotoxin; (b) a similar double-labeling experiment using fluorescent lipid probe diI-C_{18}-(3); (c) the sensitivity of succinyl-concanavalin A motion to cytocholasin B treatment; and (d) comparison of the characteristic acetylcholine receptor distribution with the distributions of cholinesterases using the stain procedure of Karnovsky and Roots [23], and of cell surface protein (LETS protein) using direct antibody fluorescence staining.

Unlike acetylcholine receptors, concanavalin A receptors were not grouped into large dense patches anywhere on the myotube surface, including in acetylcholine receptor patch regions. In fact, local quantitative measurement of TMR-succinyl-concanavalin A fluorescence suggested that concanavalin A receptor density in acetylcholine receptor patches is only about 60 ± 20% of that in acetylcholine receptor diffuse areas. The surface density of succinyl-concanavalin A in diffuse acetylcholine receptor areas was comparable to the local acetylcholine receptor density: 1000 molecules/μm^2. No minute high-density speckles or strands of concanavalin A receptors like those of acetylcholine receptor were visible in patches.

One might argue that a very high density of concanavalin A receptors might cause fluorescence self-quenching, thereby leading to a misinterpretation of low fluorescence. This situation seems unlikely here since the fluorescence of even very high density fluorescent concanavalin A receptors (10^5—10^7/cell) in ligand induced caps on lymphocytes and other cells show no evidence of such drastic self-quenching.

The results of fluorescence photobleaching recovery experiments the lateral motion of TMR-succinyl-concanavalin A are shown in Table II. The fractional mobility of concanavalin A receptors in acetylcholine receptor patches was

TABLE I

PROTOCOLS AND TMR-α-BUNGAROTOXIN ACETYLCHOLINE RECEPTOR MOBILITY RESULTS FOR CHEMICAL TREATMENT EXPERIMENTS

Subscripts "T" and "C" refer to treatment and control (i.e. without treatment), respectively. Each treatment is assigned a letter code, with special notes as follows: Q, The EGTA treatment caused much cell motion, making it impossible to make a good estimate of fluorescence at times long after bleaching. However, the initial slope of the recovery, which can also be used to estimate diffusion constants [9] indicates no great effect of EDTA on the diffusion constant D; S, the usual TMR-α-bungarotoxin protocol was performed after carbamyl choline treatment; U, a suspension of concanavalin A platelets was added at a concentration sufficient to cover 1—10% of the myotube surface with adhered platelets. "Near" refers to fluorescence photobleaching recovery observation at a spot within 3 μm of a group of platelets; "far" refers to observation at distances much greater than 3-μm platelets. Comparison of the treated cell data with its own control is a more precise estimate of the treatment efficacy than treatment to treatment comparisons, because the focused laser beam width and profile were somewhat variable over periods of weeks. This slowly-varying beam quality can affect the numerical results by as much as a factor of two. The uncertainty is the standard deviation of the data over the number of experimental runs shown in parentheses. Acetylcholine receptor distribution was not obviously affected by any of the treatments below (see text). Those treatments for which only acetylcholine receptor distribution, but not mobility, data are available are marked "N.D." HBSS, Hanks's balanced salt solution; BSA, bovine serum albumin.

Code	A	B	C	D	E	F	G	H	I	J	K
Treatment	Concanavalin A (tetravalent)			Vinblastine following concanavalin A treatment B		Colchicine	Cytocholasin B	Dithiothreitol	2-Deoxyglucose + sodium azide	Dibucaine	Trypsin
Concentration	10 μg/ml	100 μg/ml	1000 μg/ml	10^{-5} M	10^{-4} M	10^{-5} M	100 μg/ml	10^{-3} M	10^{-2} M of each	10^{-4} M	0.0025%
Duration	15 min	15 min	1 h	1 h	1 h	1 h	1 h	15 min	1 h	30 min	30 min
Temperature (°C)	22	22	22	37	37	22	22	37	22	37	37
Wash	1 h	1.5 h	1 h	rinse	rinse	rinse	rinse	rinse	No	No	rinse
D_T (10^{-10} cm^2/s)	0.8 ± 0.5 (7)	0.6 ± 0.3 (8)	<0.1 (3)	0.9 ± 0.4 (6)	1.0 ± 0.5 (6)	1.0 ± 0.3 (6)	0.9 ± 0.3 (6)	1.0 ± 0.3 (7)	1.3 ± 0.7 (6)	2.1 ± 0.4 (4)	0.7 ± 0.2 (8)
D_C (10^{-10} cm^2/s)	1.0 ± 0.3 (9)	1.0 ± 0.4 (10)	0.7 ± 0.3 (3)	N.D.	N.D.	1.0 ± 0.3 (10)	1.0 ± 0.3 (10)	1.3 ± 0.2 (2)	1.0 ± 0.4 (4)	1.2 ± 0.2 (5)	0.9 ± 0.2 (7)
f_T	0.3 ± 0.1 (7)	0.2 ± 0.1 (8)	—	0.1 ± 0.1 (6)	0.1 ± 0.1 (6)	0.7 ± 0.2 (6)	0.7 ± 0.2 (6)	0.6 ± 0.3 (7)	0.5 ± 0.1 (6)	0.6 ± 0.2 (5)	0.4 ± 0.1 (9)
f_C	0.6 ± 0.1 (9)	0.6 ± 0.1 (10)	0.6 ± 0.1 (3)	N.D.	N.D.	0.7 ± 0.1 (10)	0.7 ± 0.1 (10)	0.5 ± 0.3 (2)	0.4 ± 0.1 (4)	0.6 ± 0.2 (5)	0.5 ± 0.2 (7)

Table I (continued)

Code	L	M	N	O	P	Q	R	S	T	U	V
Treatment	Collagen-ase	Neura-minidase	Hyaluro-nidase	α-Methyl manno-sidase	Triton X-100	EGTA	High K$^+$	Carbamyl choline	A23187	Concana-valin A-platelets	Anti-α-bungaro-toxin
Concentration	300 μg/ml	100 μg/ml	70 μg/ml	10 μg/ml	0.1%	2 mM	Increase HBSS K$^+$ by 0.2 M	50 μM in medium	100 nM in medium	see legend	50 μg/ml in HBBS + 1 mg/ml BSA
Duration	3 h	15 min	90 min	2.5 h	up to 5 h	30—85 min	5—60 min	27 h	36 h	45 min	15 min
Temperature (°C)	22	37	37	37	22	22	22	37	37	37	37
Wash	rinse	rinse	rinse	rinse	No	No	No	see legend	No	(carefully) rinse	1 h in HBBS + 1 mg/ml BSA
D_T (10^{-10} cm^2/s)	N.D.	1.0 ± 0.2 (4)	0.8 ± 0.1 (3)	0.8 ± 0.1 (3)	N.D.	see legend	0.8 ± 0.2 (5)	N.D.	1.7 ± 0.5 (9)	1.0 ± 0.4 (5) (near)	1.3 ± 0.7 (4)
D_C (10^{-10} cm^2/s)	N.D.	0.9 ± 0.4 (5)	1.3 ± 0.5 (3)	N.D.	N.D.		0.8 ± 0.4 (3)	N.D.	1.4 ± 0.6 (7)	0.8 ± 0.5 (5) (far)	0.7 ± 0.2 (2)
f_T	N.D.	0.5 ± 0.2 (4)	0.5 ± 0.2 (3)	0.4 ± 0.1 (3)	N.D.		0.6 ± 0.2 (5)	N.D.	0.7 ± 0.1 (9)	0.4 ± 0.1 (5) (near)	0.3 ± 0.1 (4)
f_C	N.D.	0.8 ± 0.1 (5)	0.7 ± 0.1 (3)	N.D.	N.D.		0.6 ± 0.3 (3)	N.D.	0.4 ± 0.2 (2)	0.4 ± 0.1 (5) (far)	0.6 ± 0.1 (2)

TABLE II

FLUORESCENCE PHOTOBLEACHING RECOVERY RESULTS FOR LATERAL MOBILITY OF TMR-SUCCINYL-CONCANAVALIN A AND diI-C_{18}-(3) IN ACETYLCHOLINE RECEPTOR PATCH vs. DIFFUSE AREAS

These were double-labeling experiments: the below protocols were preceded by treatment of cells with 10^{-7} M fluorescein-bungarotoxin at 22°C for 1 h followed by 0.5 h wash. The diI-C_{18}-(3) was introduced by adding 10 μl of 0.3 mg/ml diI-C_{18}-(3) in ethanol to 1 ml of Hank's balanced salt solution bathing the cells. The cells for both the TMR-succinyl-concanavalin A and diI-C_{18}-(3) experiments were grown in medium containing 10^{-5} M cytosine arabinoside during days 2—4 after plating to prevent division and proliferation of overgrowing mononucleated cells.

Probe	TMR-succinyl-concanavalin A	diI-C_{18}-(3)
Concentration	10 μg/ml	see legend
Duration	15 min	3 min
Temperature (°C)	22	37
D patch (10^{-10} cm^2/s)	0.6 ± 0.6 (6)	12 ± 3 (4)
D diffuse (10^{-10} cm^2/s)	0.7 ± 0.3 (10)	15 ± 3 (7)
f patch	0.2 ± 0.1 (6)	0.7 ± 0.1
f diffuse	0.5 ± 0.1 (10)	0.6 ± 0.1

somewhat reduced relative to the concanavalin A fractional mobility in acetylcholine receptor diffuse areas. However, a significant portion of concanavalin A receptors in acetylcholine receptor patches remained mobile, with a diffusion constant similar to concanavalin A receptors elsewhere on the myotube surface.

Table II also shows fluorescence photobleaching recovery results on lateral mobility of lipids. We used dioctadecylindocarbocyanine (diI-C_{18}-(3) as a fluorescent probe of membrane lipid regions [10,11,13,24,25]. The mobility of diI-C_{18}-(3) was unimpaired by the high concentration of acetylcholine receptor molecules in a patch, relative to the diI-C_{18}-(3) mobility elsewhere on the surface. The surface densities of diI-C_{18}-(3) in acetylcholine receptor patch and diffuse areas were similar. The diI-C_{18}-(3) labeling was fairly uniform on the cell surface except for occasional labeled strands above the surface and occasional slight internalization (see Discussion).

Although acetylcholine receptor was unaffected by cytocholasin B treatment, concanavalin A receptor fractional mobility was substantially decreased by the identical treatment, as shown in Table III.

Since acetylcholinesterase and acetylcholine receptor are both localized at adult motor endplates, we stained TMR-bungarotoxin-labeled myotubes by the Karnovsky-Roots method to determine if cholinesterases were localized in

TABLE III

THE EFFECT OF CYTOCHOLASIN B TREATMENT ON TMR-SUCCINYL-CONCANAVALIN A MOBILITY

See Table I, experiment G for protocol. The cells were grown in medium containing 10^{-5} M cytosine arabinoside on days 2—4 after plating. "CB" subscript refers to cytocholasin B; "Cont" refers to control (without cytocholasin B).

Parameter	Value
D_{CB} (10^{-10} cm^2/s)	0.8 ± 0.2 (4)
D_{Cont} (10^{-10} cm^2/s)	0.6 ± 0.2 (5)
f_{CB}	0.27 ± 0.04 (4)
f_{Cont}	0.53 ± 0.13 (5)

acetylcholine receptor patches. Only very light cholinesterase staining was detected, with no special higher density at acetylcholine receptor patches.

We observed the distribution of cell surface protein, otherwise called "large external transformation-sensitive" protein [15,26], giving special attention to labeling pattern of TMR-anti-LETS protein antibody on a myotube in the vicinity of an acetylcholine receptor patch (which could be visualized with fluorescein-α-bungarotoxin). LETS protein labeled in a network of thin strands on the edge of cells in a manner entirely dissimilar to the typical patch-and-diffuse acetylcholine receptor distribution.

It is conceivable that the lack of high-density decoration of acetylcholine receptor patch regions by TMR-succinyl-concanavalin A, cholinesterase stain, and TMR-anti-LETS protein arises from an accessibility problem. For example, since most (but not all) acetylcholine receptor patches are on the bottom surface of the myotubes, perhaps large labeled proteins cannot "fit" in some areas between the dish and the cell. We tested this possibility by using indirect antibody fluorescent staining with fluorescein-α-bungarotoxin followed by rabbit anti-α-bungarotoxin antibody followed TMR-anti-rabbit IgG. Some acetylcholine receptor patches could be visualized by this technique, although the patch labeling was less intense, more speckled and more annular than the typical TMR-α-bungarotoxin patch labeling. We conclude that at least part of the patch is accessible to binding by large proteins such as antibodies (and also that fluorescein labeling does not destroy the antigenicity of anti-α-bungarotoxin.

Turnover

Acetylcholine receptors undergo a continual turnover, with half-times for membrane incorporation of new acetylcholine receptors and for internalization of old acetylcholine receptors on the order of 12—24 h [4,27]. When visualized by TMR-α-bungarotoxin, acetylcholine receptor internalization is manifested by the slow internalization of surface TMR fluorescence. New acetylcholine receptor incorporation is seen by appearance of new TMR-α-bungarotoxin binding sites after blockage of old acetylcholine receptor with unlabeled α-bungarotoxin [4].

In the present series of experiments, we have verified that these "new" acetylcholine receptors are not simply pre-existing membrane acetylcholine receptors from which α-bungarotoxin has become unbound. A 3.5 h treatment with the protein synthesis inhibitor puromycin · (HCl) (at 20 μg/ml in medium at 37°C) before and during a 1 h α-bungarotoxin acetylcholine receptor blockage treatment, followed by an additional 6 h in puromycin (at the same concentration) prevented the appearance of TMR-α-bungarotoxin visualized new acetylcholine receptors. A control dish not treated with puromycin but otherwise similarly treated did show labeling of newly incorporated acetylcholine receptors in the same time period. In like manner, we have demonstrated the irreversibility of TMR-α-bungarotoxin by assaying after several hours for the appearance of new acetylcholine receptor with fluorescein-α-bungarotoxin.

The lifetime of a myotube acetylcholine receptor patch is on the order of days [4], considerably longer than the surface lifetime of the individual acetyl-

choline receptor molecules of which it is comprised. This observation raises the question of where newly incorporated acetylcholine receptors first appear in an already existing patch. We visualized new acetylcholine receptor incorporation by labeling with TMR-α-bungarotoxin several hours after saturation blockage and visualization of pre-existing acetylcholine receptors by fluorescein-α-bungarotoxin. (The excitation and emission spectra of fluorescein-α-bungarotoxin and TMR-α-bungarotoxin are non-overlapping, so their labeling patterns are easily distinguishable). We found that newly incorporated acetylcholine receptors appear uniformly throughout the area of a patch, rather than selectively in, for example, the center of the patch or in an annular ring around its periphery.

The rate of new acetylcholine receptor incorporation may be somewhat variable from myotube to myotube, and even from patch to patch on the same myotube; the fluorescence intensity of TMR-α-bungarotoxin-acetylcholine receptor patches labeled several hours after α-bungarotoxin blockage ranged from very dim to quite bright.

General electrical stimulation of the myotubes in the dish had a profound effect on the presence of acetylcholine receptors. 38 h of 20 V pulses (groups of 20 pulses of 3 ms duration every 10 s) applied across agar-buffer electrodes spaced 2 cm apart immersed in the medium caused at least a 5-fold reduction in the number of patches/unit length of myotube, relative to unstimulated dishes. Stimulated myotubes whose action potentials were blocked with $2 \cdot 10^{-5}$ M tetrodotoxin did not show a reduction in number of acetylcholine receptor patches, so it appears that the action potential or the ensuing contraction is causally related to the acetylcholine receptor patch disappearance. In addition to the effect on patches, stimulation for greater than 20 h reduced the diffuse area acetylcholine receptor density by a factor of about 1/3 relative to the unstimulated or tetrodotoxin-blocked controls. Qualitatively similar effects of stimulation have been observed by Cohen and Fischbach [28] on chick primary myotubes. We do not yet know whether the stimulation effect results from enhanced removal or decreased new incorporation of patch acetylcholine receptors into the membrane.

Discussion

The localization and perhaps mobility of specific membrane proteins play a critical role in the development of multicellular systems. We discuss here the following questions about the molecular dynamics of acetylcholine receptors in the myotube membrane: (A) What maintains the acetylcholine receptor patch structure? (B) What controls the lateral motion of acetylcholine receptors in diffuse areas? (C) What is the relevance of myotube acetylcholine receptor patch and diffuse area distribution to adult muscle acetylcholine receptor distribution?

(A) Maintenance of acetylcholine receptor patch structure

Some inferences concerning the structure of acetylcholine receptor patches can be drawn from results presented previously [4]. Acetylcholine receptors are continually incorporated into and removed from a patch although the patch

as a structure is relatively stable. From where do newly incorporated patch acetylcholine receptors come? They do not come from neighboring diffuse areas: we have shown previously that patch and diffuse areas exchange acetylcholine receptors very slowly, if at all [4]. Therefore, the cell must possess an immobile, stable structure which assembles new acetylcholine receptors in the vicinity of a pre-existing patch, guides newly synthesized intracellular acetylcholine receptors to a pre-existing patch region, or perhaps lenghens acetylcholine receptor lifetime in a patch.

It seems unlikely that this immobile structure consists solely of the patch acetylcholine receptors already present in the membrane. If high densities of acetylcholine receptors could alone somehow attract new acetylcholine receptors or lengthen membrane lifetime of patch acetylcholine receptors, one would expect new patches to form frequently in previously diffuse areas where concentration fluctuations of mobile acetylcholine receptors create transient local high acetylcholine receptor densities. However, such ongoing formation of new patches in previously diffuse areas has never been observed on rat myotubes. Therefore, it seems probable that some immobile molecular or subcellular system other than acetylcholine receptors plays a role in maintaining the stability of a patch structure. The highly granular patch fine structure of relatively immobile speckles and continuous strands also suggests the existence of a fixed non-acetylcholine receptor structure, perhaps even a filamentous structure to which lines of acetylcholine receptors attach.

Although the long-term existence and stability of a patch appear to involve non-acetylcholine receptor structures, the immobility of acetylcholine receptors within a patch could still directly involve only acetylcholine receptor molecules. In analogy with a recent proposal [29] and results [30—32] concerning possible structural differences between classes of acetylcholine receptors from the same cell, it is possible that myotube patch acetylcholine receptors have a different molecular structure than diffuse acetylcholine receptors. Perhaps this difference allows only patch acetylcholine receptor molecules to "stick" to each other in patch areas, thereby immobilizing themselves. However, this hypothesis still requires some non-acetylcholine receptor patch structure to maintain particular membrane regions as patch-type acetylcholine receptor repositories.

The remarkable integrity of patches under attack by detergent (Table I, column P), protease (columns K, L), disulfide bond cleavage (column H), glycosidases (columns M, N, O) and divalent ion chelation (column Q), even to the point of removing cells from the dish, indicates that patches are not stabilized by cell surface extrinsic proteins or other externally accessible structures. This situation contrasts strongly with that of certain other cell surface proteins. Trypsin, for example, under the conditions used here nearly completely removes all the LETS proteins from the cells [26]. Yet no effect of trypsin on acetylcholine receptors was observed.

Although patch stability is maintained by cytoplasmic or inassessible intramembrane structures, the external cell surface does appear to be involved in the formation of a new patch, perhaps through some mechanism based on physical contact with outside objects. The evidence for this is the preferential occurrence of rat myotube patches on the surface of the myotube in closest

contact to the tissue culture plastic [4,7]. On L6 cloned rat myotubes (which normally do not show acetylcholine receptor patches), external treatment with nerve extract can induce patches [33]. Therefore, although patches are stabilized by a structure inaccessible to the exterior, treatments originating from the exterior can induce patch appearance.

The stability of patches during attack by colchicine or cytocholasin B (columns F and G) indicates that the targets of these drug (presumably including cytoplasmic microtubules and microfilaments) are not alone involved in patch structure.

The lack of similarity between the distribution pattern of acetylcholine receptors and that of concanavalin A, LETS protein, cholinesterases, and diI-C_{18}-(3) emphasizes how selective must be the cellular mechanisms that control acetylcholine receptor distribution. Specifically, such selectivity argues againt simple membrane folding (e.g. like that of adult postsynaptic membrane) or any other mechanism that non-specifically concentrates membrane molecules into a small lateral area. For chick myotube acetylcholine receptor clusters, the same conclusion was reached using thin section electron microscopy [34].

The diI-C_{18}-(3)/fluorescein-α-bungarotoxin double-labeling results show that the acetylcholine receptors in patches do not impede the lateral motion of lipid-like molecules over micron-scale distances. Acetylcholine receptors may be closely packed in individual small speckles in a patch region, but the membrane-imbedded portion of these speckles must be small enough ($\leqslant 1~\mu m^2$) and spaced widely enough to allow the motion of diI-C_{18}-(3) molecules around them.

A portion of the diI-C_{18}-(3) becomes noticeably internalized into the cytoplasm in about 1 h on rat primary myotubes at 22°C. This internalization is manifested by (a) fluorescence originating from planes of focus throughout the bulk of the myotube thickness rather than just the surfaces; (b) spatially inhomogeneous labeling of organelles; and (c) distinct fluorescent exclusion from the nucleii. Our experiments were performed well before optically noticeable internalization, but some possible diI-C_{18}-(3) labeling of amphipathic submembrane structures may have contributed to the fluorescent intensity and fluorescence photobleaching recovery mobility results. Perhaps the 1/3 immobility fraction of diI-C_{18}-(3) noticed in both acetylcholine receptor patch and diffuse areas represented diI-C_{18}-(3) bound to immobile structures immediately above or below the plasma membrane bilayer.

(B) Control of acetylcholine receptor motion in diffuse areas

Our results show the dynamic behavior of acetylcholine receptors in diffuse areas to be somewhat different than that of certain other membrane proteins. For example, we have shown here that cytocholasin B decreases the mobility of concanavalin A receptors on rat primary myotubes in a manner similar to cytocholasin B's effect on concanavalin A receptors [13], general surface proteins [14] and IgE-Fc receptors [17] on other cell types. Cytocholasin B, however, does not alter the motion of acetylcholine receptors in the diffuse areas. This experiment clearly shows that the concanavalin A receptors and acetylcholine receptors are different classes of proteins and that they differ in their interaction with cytoskeletal elements.

These two classes of proteins nevertheless can interact with each other. Concanavalin A receptors which have been immobilized by cross-linking through tetravalent concanavalin A can inhibit acetylcholine receptor motion. The molecular interpretation of this result is somewhat complex. Acetylcholine receptors in vitro bind concanavalin A [35]; if they do so in vivo, then concanavalin A receptor cross-linking by tetravalent concanavalin A would directly involve acetylcholine receptors, and hence concanavalin A would inhibit acetylcholine receptor motion, as observed. However, concanavalin A receptors and diffuse area acetylcholine receptors appear to be distinct, as explained above. Also, the lack of high concentration TMR-succinyl-concanavalin A labeling on acetylcholine receptor patches and on intrapatch speckles argues against concanavalin A binding to patch acetylcholine receptors. Indeed, the TMR-succinyl-concanavalin A concentration in acetylcholine receptor patches is lower than elsewhere on the myotube surface. Assuming, then, that membrane acetylcholine receptors do not bind concanavalin A significantly, then the concanavalin A-induced inhibition of TMR-α-bungaro-toxin-acetylcholine receptor mobility must be due to a more indirect effect. Perhaps acetylcholine receptors are "caged in" by surrounding cross-linked immobilized concanavalin A receptors. Or perhaps, the concanavalin A receptors and acetylcholine receptors are connected directly or indirectly via cytoskeletal filaments; certain interprotein filamentous connections are inferred from studies on lymphocytes [36]. If such connections exist here, they probably do not involve only microtubules, since vinblastine treatment (experiments D and E in Table I) will not release the concanavalin A-induced acetylcholine receptor mobility inhibition. The concanavalin A-platelet experiments (see also ref. 16) show that concanavalin A inhibition of TMR-α-bungarotoxin-acetylcholine receptor mobility is a local effect, confined to within less than 3 μm from locally bound concanavalin A.

Acetylcholine receptor motion can also be inhibited by treatment with α-bungarotoxin followed by anti-α-bungarotoxin antibody, probably through extensive cross-linking of acetylcholine receptors via antibody molecules. This cross-linking involves no optically observable acetylcholine receptor redistribution, in contrast to anti-antibody cross-linking-induced receptor redistributions seen on other cell types [37]. The acetylcholine receptor immobilization by concanavalin A and by anti-α-bungarotoxin observed here should become a useful future tool for inhibiting developmental or externally-induced acetylcholine receptor redistributions.

The possibility that acetylcholine receptor motion is modulated by lipid ordering or acetylcholine receptor-lipid interactions is supported by the effect of dibucaine (J) in marginally increasing the acetylcholine receptor diffusion constant. Dibucaine would be expected to disorder lipid [38] and perhaps alter protein-lipid interactions. On the other hand, alteration of phospholipid fatty acyl composition and saturation does not affect acetylcholine receptor mobility on chick myotubes [10].

Acetylcholine receptor lateral motion appears to be a passive process, inasmuch as it is not affected by blockage of the oxidative phosphorylation and/or glycolytic pathways (Column I, Table I). As with acetylcholine receptor patch

stability, acetylcholine receptor lateral motion in diffuse areas is not modulated by a variety of externally exposed surface constituents.

(C) Patches vs. endplates: acetylcholine receptor turnover

Despite the concentrated, immobilized nature of both myotube patch acylcholine receptors and the junctional acetylcholine receptors in adult motor endplates, the disappearance of patches upon prolonged electrical stimulation reported on chick myotubes [28] and on our rat myotubes, points to a major structural or biochemical difference between myotube acetylcholine receptor patches and the junctional acetylcholine receptor localization at adult motor endplates. The disappearance of myotube patches upon prolonged electrical stimulation is in closer analogy with denervated extrajunctional acetylcholine receptors (which decrease in concentration upon stimulation of the denervated muscle) than with junctional acetylcholine receptors. Myotube diffuse area acetylcholine receptors are also decreased in density, but not nearly as dramatically as the number of surface patches. This difference may indicate that different cellular mechanisms control patch and diffuse acetylcholine receptors presence on the membrane.

Ravdin [39] has shown that exposing myotubes to 0.5 mM dibutyryl cyclic AMP + 5 μM RO-20-1724 (a phosphodiesterase inhibitor) in the medium for 24—48 h prior to TMR-bungarotoxin labeling also leads to at least a 5-fold reduction of number of patches/unit length of myotube, although the diffuse acetylcholine receptor surface density appears unaffected. Dibutyryl cyclic AMP causes a marked increase in spontaneous twitching which might account for the similarity of effects of dibutyryl cyclic AMP and direct stimulation on acetylcholine receptor patch frequency. The regular spontaneous twitching which occurs in older myotube cultures may be responsible for the previously observed gradual disappearance of the patches [4] on such myotubes.

(D) Conclusions

On the basis of both the positive and negative results presented here, we postulate several conclusions concerning the mechanisms that control acetylcholine receptor motion and distribution. We believe that acetylcholine receptor patches are specifically stabilized by an immobile, intra- or submembrane filamentous structure, composed of molecules other than the acetylcholine receptors themselves. This structure is attached to either: (a) otherwise mobile acetylcholine receptors thereby forming a patch; or (b) only "special" acetylcholine receptors which are capable of immobilizing themselves by self-cross-linking. The attachment of acetylcholine receptors to such a structure is reversible during the normal course of acetylcholine receptor turnover. The points of attachment, or the filamentous structure itself, can be dismantled by the cell upon prolonged electrical stimulation. Acetylcholine receptor packing in patches is open enough to allow unretarded lateral motion of membrane lipids. Acetylcholine receptor motion in diffuse areas can be somewhat dependent on lipid interactions and can be inhibited by ligand-mediated cross-linking, either directly, or indirectly through lectin receptors. Finally, the cellular mechanisms determining myotube acetylcholine receptor motion and distribution appear to be different from those operational in adult muscle.

Acknowledgements

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