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Crosslinkage and visualization of acetylcholine receptors on myotubes with biotinylated α-bungarotoxin and fluorescent avidin

(receptor turnover/membranes/developing muscle/photobleaching recovery/patches)

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ABSTRACT **A biotinylated derivative of α-bungarotoxin and tetramethylrhodamine-labeled avidin were employed to fluorescence label the acetylcholine receptors (AcChoR) on the surface of rat myotubes in primary culture. Because of the multivalency of both the biotinylated bungarotoxin and the avidin, this treatment extensively crosslinks the AcChoR. AcChoR crosslinking immobilizes more than 90% of the normally laterally mobile AcChoR as verified by the fluorescence photobleaching recovery technique; it also redistributes the AcChoR into visible micropatches. Biotinylated α-bungarotoxin/avidin-induced AcChoR crosslinking greatly accelerates the rate of internalization of surface AcChoR; this rapid internalization affects both the normally mobile AcChoR in areas of diffuse distribution and the normally immobile AcChoR in preexisting patches. The peculiar pattern of fluorescent avidin binding to AcChoR patches previously bound with biotinylated bungarotoxin suggests that almost all large AcChoR patches are in very close contact (<70 Å) with the glass substrate. AcChoR immobilization leads to a partial immobilization of concanavalin A receptors in the myotube membrane.**

Much of the environmental sensing performed by a cell is mediated by protein or glycoprotein receptors in the plasma membrane. Influences that alter the organization, lateral mobility, surface concentration, or rate of turnover of cell surface receptors can be critically important in determining the cell's response to external signals. One such influence is the binding of ligands, which can affect the concentration of receptors on the surface. Ligand-induced receptor "modulation" (1) often appears to involve receptor crosslinking or redistribution into clusters or micropatches. For example, crosslinking of cell surface protein by multivalent antibodies (2–7) and clustering by lectins (8) often lead to loss of the protein from the cell surface, usually by internalization. Clustering of certain receptors by their nominally monovalent ligands precedes internalization of the complex at coated pits (9).

Both crosslinking and clustering greatly reduce the lateral mobility of individual receptor molecules (10, 11). But the state of immobility itself does not always lead to a loss of receptor from the cell surface. Concanavalin A (Con A), which immobilizes some acetylcholine receptors (AcChoR) on the surface of developing muscle cells in culture (12) also slows their degradation (7). Naturally occurring dense clusters of immobile AcChoR on rat primary myotubes ("patches") are as stable on the surface as the laterally mobile nonclustered ("diffuse area") AcChoR (13). At synapses, AcChoR are immobile and closely aggregated but they are much more stable than the nonclustered extrajunctional AcChoR (14). The diverse consequences of receptor immobilization raise questions about molecular mechanisms and possible biological roles (1).

The present study examines, by fluorescence techniques, the effect of specific external crosslinking of AcChoR on developing rat muscle cells in primary cultures. The method of crosslinking is nonimmunological. AcChoR are irreversibly bound with α-bungarotoxin (Bgt) covalently derivatized with several biotin groups (Bio-Bgt). These complexes are then crosslinked with tetramethylrhodamine-labeled avidin (R-Av), which is tetravalent with respect to biotin binding. Several conclusions can be drawn: (*i*) Bio-Bgt/R-Av redistributes and immobilizes those cell surface AcChoR in areas of diffuse AcChoR distribution; (*ii*) Bio-Bgt/R-Av crosslinking greatly reduces the net surface concentration of AcChoR by speeding their internalization; (*iii*) preexisting immobile AcChoR patches as well as diffuse area AcChoR are rapidly internalized; (*iv*) almost all preexisting AcChoR patches appear to be located in exceedingly close proximity to the glass substrate (i.e., <70 Å); (*v*) immobilization of AcChoR partially immobilizes Con A receptors.

MATERIALS AND METHODS

Bio-Bgt and Fluorescent Avidin (Av). Bgt (Miami Serpentarium) was biotinylated by Vector Laboratories (Burlingame, CA) so that biotin was covalently bound to exposed amino groups on approximately 95% of the bungarotoxin molecules. Assuming that each amino group reacts with equal probability, this amount of derivatization ensures that 80% of the Bgt molecules have at least two attached biotin groups and that the mean derivatization is 3.0 biotins per Bgt molecule. Some of the effects observed with the Bio-Bgt obtained from Vector Laboratories were qualitatively confirmed with a sample of Bio-Bgt obtained as a gift from Kate Barald (Department of Biological Sciences, Stanford University).

Av (Sigma) was fluorescence labeled by reaction with tetramethylrhodamine isothiocyanate (Research Organics, Cleveland, OH) at a molar ratio of 4 rhodamines per Av in 0.1 M $NaHCO_3$ for one hour at 22°C. Unreacted dye was separated from the labeled protein (R-Av) on a Sephadex G-25 column equilibrated with Dulbecco's phosphate-buffered saline, followed by dialysis against this buffer for 24 hr at 4°C. The final labeling ratio was 2–3 rhodamines per Av molecule.

Rat Myotube Primary Cultures. Muscle tissue from hindlimbs of 20-day-old Sprague–Dawley rat embryos was dissociated by a 1-hr treatment with 0.25% trypsin in calcium-free Hanks' balanced salt solution (HBSS). After centrifugation, complete medium consisting of Dulbecco's modified Eagle's medium plus 10% fetal calf serum plus 0.5% penicillin/streptomycin (each at 10,000 units/ml in stock solution) was added to the pellet, followed by trituration. (All solutions were obtained from GIBCO.) The nonmyoblast population was reduced

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Abbreviations: AcChoR, acetylcholine receptor(s); Bgt, α-bungarotoxin; Bio-Bgt, biotinylated α-bungarotoxin; Av, avidin; Con A, concanavalin A; HBSS, Hanks' balanced salt solution; R-, tetramethylrhodamine-labeled; Suc-, succinoylated; FPR, fluorescence photobleaching recovery.

by preplating these cells onto plastic petri dishes for 20 min at 37°C. The nonadherent cells were filtered through two sheets of lens paper and plated in 2 ml of medium in 35-mm dishes at a density of 5×10^5 cells per dish. The dishes were specially prepared by drilling out a large hole in the plastic bottom and covering the hole with a circular glass coverslip (no. 1 ½ thickness) and sealing it in with encapsulating resin (Dow Corning Sylgard no. 182). No collagen coating was used.

Medium was subsequently replaced every 3–4 days with fresh medium. Myoblast fusion into myotubes generally began on day 3; experiments were performed on myotubes in 7- to 10-day-old cultures.

Treatment of Cells with Bio-Bgt and R-Av. Cells were treated with Bio-Bgt at 4 μg/ml in medium for at least 10 min at 37°C. After several washings with HBSS containing bovine serum albumin (albumin, Miles) at 5 mg/ml, cells were treated with R-Av at 4 μg/ml in HBSS/albumin for at least 5 min at 22°C. After several more washings with HBSS/albumin, cells were bathed in HBSS/albumin for fluorescence microscopy.

This treatment produced a fluorescence labeling pattern on the cell surfaces that could be completely blocked by pretreatment of the cells with underivatized Bgt at 1 μg/ml in medium for 30 min at 37°C. Therefore, Bio-Bgt bound specifically only to the same sites as underivatized Bgt. Treatment of cells by underivatized Bgt followed by R-Av produced no specific labeling. Therefore, R-Av bound specifically only to Bio-Bgt.

The Bio-Bgt/R-Av protocol described above produced near saturation binding of both Bio-Bgt to AcChoR and R-Av to Bio-Bgt. The concentrations and treatment durations for either Bio-Bgt or R-Av could be reduced by more than 1:4 before the fluorescence labeling intensity became noticeably dimmer. Increases of concentration and duration further than above had no noticeable effect.

Fluorescent Bgt and Succinoylated Con A (Suc-Con A). Tetramethylrhodamine bungarotoxin (R-Bgt) was prepared as described (15). Cells were treated with R-Bgt at 1 μg/ml in medium for 1 hr at 37°C, followed by several washings in HBSS/albumin. Con A (Sigma) was fluorescence labeled with either dichlorotriazinylaminofluorescein (Research Organics) or tetramethylrhodamine isothiocyanate by a protocol similar to that for the rhodamine labeling of Av, except that the Sephadex G-25 column and dialysis was in a pH 6.5 buffer containing 150 mM NaCl, 10 mM sodium phosphate, and 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$. Succinoylation was then performed according to the method of Gunther *et al.* (16).

Cells were incubated with fluorescent Suc-Con A at 50 μg/ml in HBSS for 10 min at 22°C, followed by several washes in HBSS/albumin. The binding of fluorescent Suc-Con A to cell surface sugar residues was specific: fluorescent labeling could be blocked by simultaneous incubation with α-methyl D-mannoside at 10 mg/ml but could not be blocked by α-methyl D-galactoside at 10 mg/ml (both sugars from Sigma). Cells pretreated with unlabeled Suc-Con A did not bind detectable amounts of R-Av in the absence of Bio-Bgt.

Fluorescence Measurement. Fluorescence of cells was viewed with an inverted microscope (Leitz Diavert) by epi-illumination through a ×50, numerical aperture = 1.00 water immersion objective. The light source was the 514-nm line of an argon laser (Lexel 95-3).

Lateral motion of fluorescence-marked receptors was measured by the fluorescence photobleaching recovery (FPR) technique (17) on an apparatus that has been described (18). In these experiments, the focused spot had a radius of 0.6 μm (measured as described in ref. 18). The photobleaching flash duration was 50 ms at a power of 2 mW, and the power during observation was 1 μW.

The intensity of fluorescence from the cell surface was measured with the photometer (Leitz MPV-1) and photon counting system of the FPR apparatus. Surface fluorescence is distinguished by its apparent brightness at the edges of the cell due to the tangential direction of view. Internalized fluorescence, on the other hand, is very dim at the edge but is brightest at the center of the myotube width, where the cell is the thickest. AcChoR surface concentration was measured by illuminating the whole cell but masking out fluorescence from all but a very small region (≈0.8 μm × 0.8 μm) at the edge of a myotube with the aid of a centerable image plane aperture in the MPV-1 photometer.

RESULTS

Redistribution into Micropatches. Bio-Bgt/R-Av brightly labeled the myotube surface, while leaving other cells virtually unlabeled. Fig. 1*A* shows a high-magnification view of the edge of a myotube in a diffuse (i.e., non-AcChoR patch) area. Compared to the labeling pattern of R-Bgt (Fig. 1*B*), fluorescence from Bio-Bgt/R-Av is highly granular with "micropatches" of ≲1 μm typical size. Av is tetravalent for biotin, and most Bio-Bgt molecules contain at least 2 biotins. It is reasonably assumed that extensive crosslinking of AcChoR occurs because of this multivalency, and that the crosslinking leads to a microscopic redistribution of uniformly distributed AcChoR into micropatches.

FPR measurements showed that previously mobile AcChoR were virtually immobilized by Bio-Bgt/R-Av. The fraction of AcChoR that are mobile in areas of diffuse AcChoR distribution decreased from 0.61 ± 0.09 (SEM) in R-Bgt/R-Av controls to only 0.08 ± 0.03 (SEM) in cultures treated by Bio-Bgt/R-Av. Obvious micropatches were avoided as sites for lateral mobility measurements. Apparently the great majority of AcChoR are immobilized by Bio-Bgt/R-Av, whether or not they become part of an obvious micropatch.

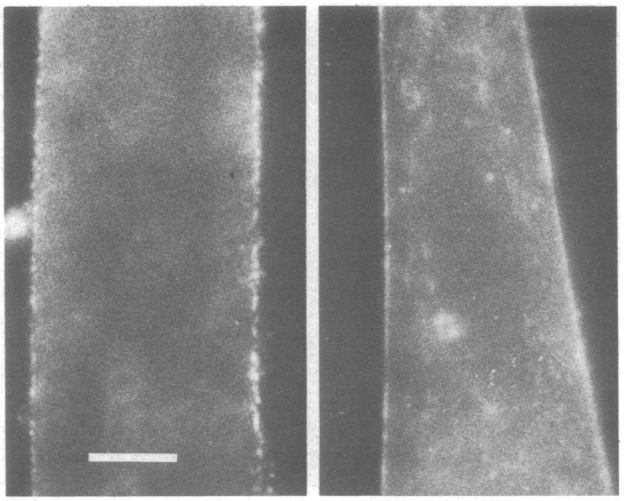

FIG. 1. Fluorescence micrographs of rat myotubes immediately after treatment with Bio-Bgt/R-Av (*A*) or R-Bgt/Av (*B*). These are areas of diffuse AcChoR distribution, with the focus on the edges of the myotubes. Easily visible cell surface micropatches of AcChoR are induced by Bio-Bgt/R-Av but not by R-Bgt/Av. In regions of somewhat higher diffuse AcChoR density than depicted here, the images of edge speckles begin to overlap and appear somewhat contiguous; in these cases, micropatching is best observed by focusing on the upper or lower surface of the myotube. The R-Bgt/Av labeling pattern shows small (<1 μm) inhomogeneities (even in areas of "diffuse" AcChoR distribution) when viewed at the upper or lower surface, but these inhomogeneities are less discrete and of lower contrast than the Bio-Bgt/R-Av pattern. Bar = 10 μm.

Accelerated Internalization. The time course of AcChoR internalization was observed after a "pulse" of either Bio-Bgt/R-Av or R-Bgt/Av (as a noncrosslinking monovalent control) at time zero. Immediately after time zero, photographs and quantitative photometer measurements at myotube edges (as a measure of AcChoR concentration on the surface) were taken with the cells in HBSS/albumin at 22°C. This photography and measurement procedure was repeated on companion cultures at 3 hr and again at 8 hr. Fig. 2 shows the fluorescence micrographs and Fig. 3 shows the average fluorescence intensity measured at the myotube edges during the time course. AcChoR that are crosslinked by Bio-Bgt/R-Av are clearly internalized much more quickly than AcChoR tagged with monovalent R-Bgt. The internalized fluorescence is highly concentrated in bright speckles.

Long-term immobilization of AcChoR by Bio-Bgt/R-Av was found to decrease the net AcChoR concentration on the surface. Continuous AcChoR immobilization for 14 hr in the incubator was achieved by alternating 3-min exposures of a culture of Bio-Bgt and R-Av (each exposure separated by a 2-min wash in culture medium) with a system of peristaltic pumps controlled by a digital electronic timing circuit. Fluorescence photographs taken both before and after this 14-hr period are shown in Fig. 4. Continuous AcChoR immobilization led to a virtually complete loss of detectable surface fluorescence; all the fluorescence arises from internalized speckles. Control experiments in which R-Bgt was alternated with unlabeled Av show easily detectable surface fluorescence as judged by edge brightening (not illustrated). It can be concluded that the surface concentration of AcChoR greatly decreases upon continuous AcChoR immobilization, probably due to the accelerated internalization confirmed by the pulse experiments above. This experiment also confirms that the loss of surface fluorescence within 3.5 hr after a pulse immobilization should not be ascribed solely to detachment of Bio-Bgt/R-Av from surface AcChoR.

Both the pulse and the continuous AcChoR immobilization treatments greatly reduced the number of AcChoR patches.

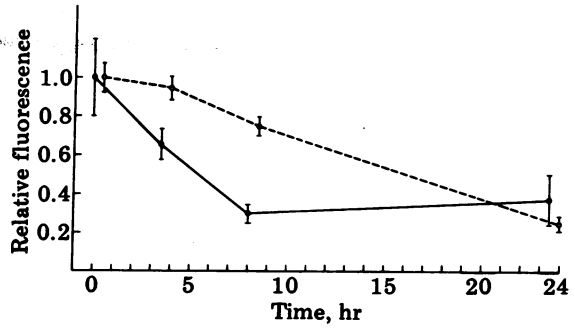

FIG. 3. Fluorescence intensity (with background subtracted) at edges of myotubes treated with a pulse of either Bio-Bgt/R-Av (●) or R-Bgt/Av (○), as a function of time after treatment. Cells were kept at 37°C until fluorescence observation. Each data point is based on an average of 20–48 fluorescence intensity measurements on different randomly selected myotubes in the dish; the error bars represent the SEM. The 0.8 μm × 0.8 μm observation region from which fluorescence was collected was defined by an adjustable square aperture in an image plane of the microscope and positioned at the edge of the myotube image. Because the observation region necessarily includes some submembrane cytoplasm, the measured fluorescence represents an upper bound estimate of AcChoR concentration on the surface. The upper bound time for 50% loss of surface AcChoR is $\lesssim$ 5 hr for Bio-Bgt/R-Av and $\lesssim$ 15 hr for R-Bgt/Av. The latter time is only slightly shorter than that measured on chick myotubes by Devreotes and Fambrough (19) from the release of ^{125}I-labeled Bgt degradation products.

Immediately after a noncrosslinking pulse of R-Bgt/Av, a total of 259 patches could be counted in several fields of view; 8 hr later, 237 of these patches were still present in the same fields (i.e., 92% patch survival). Immediately after a crosslinking pulse of Bio-Bgt/R-Av in a different dish, 133 patches were counted. But 8 hr later only 59 patches were still visible in the same fields (i.e., 44% survival), and most of these were quite dim. Essentially no patches were visible after a 14-hr long-term alternating exposure to Bio-Bgt and R-Av as described above.

Proximity of AcChoR Patches to Substrate. As noted previously (12), the great majority of large ($\gtrsim$25 μm^2) AcChoR

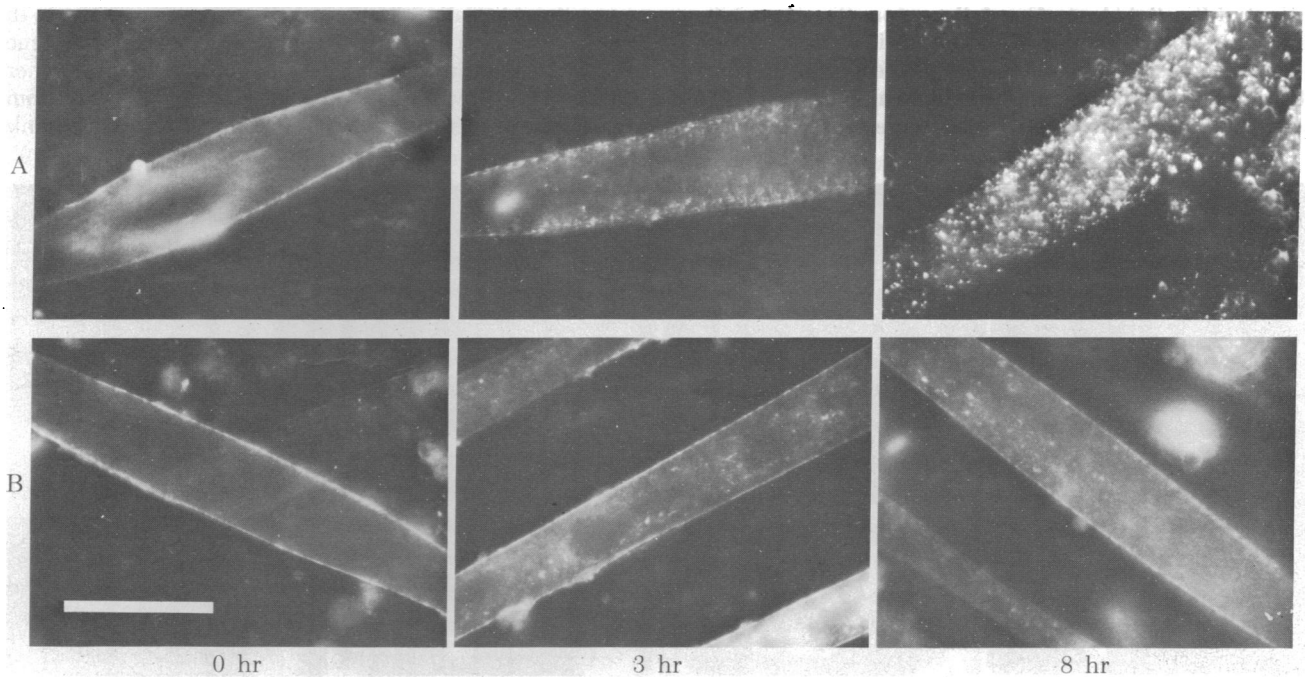

FIG. 2. Fluorescence micrographs taken at 0 hr, 3 hr, and 8 hr after a pulse treatment of Bio-Bgt/R-Av (*A*) or R-Bgt/Av (*B*). Comparison of *A* and *B* shows the relatively rapid internalization of fluorescence into small discrete cytoplasmic aggregations after Bio-Bgt/R-Av treatment. To avoid photobleaching artifacts during photography, a different myotube was selected for each exposure. Bar = 40 μm.

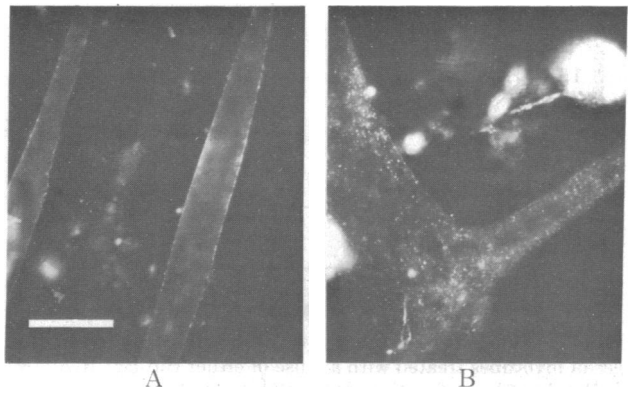

FIG. 4. Fluorescence micrographs of myotubes at the beginning (*A*) and 14 hr after the beginning (*B*) of a continuous immobilization of AcChoR induced by alternating exposures to Bio-Bgt and R-Av. The loss of edge labeling apparent in the 14-hr photograph reflects a loss of surface AcChoR resulting from this protocol. Different myotubes in the same dish are depicted. Bar = 40 μm.

patches are on the bottom surface of the myotubes, facing the glass (or plastic) substrate. Bio-Bgt/R-Av labeling provides a vivid demonstration that virtually all of these patch regions are in intimate contact with the substrate. A culture was first treated with Bio-Bgt; the subsequent exposure to R-Av in HBSS/albumin at 22°C was continuous. At various times, fluorescence photomicrographs of an AcChoR patch were taken, as displayed in Fig. 5. More than 90% of the patches were labeled by R-Av in annular patterns. These fluorescent annuli became progressively thicker as exposure to R-Av continued. (By contrast, R-Bgt-labeled patches are as bright in their centers as at their peripheries.) The diffusional motion of R-Av toward the center of a Bio-Bgt-tagged AcChoR patch is apparently slowed by a tight "squeeze" of R-Av between the cell and the dish. The longest dimension of an Av molecule (M_r 68,000) is around 55 Å (20). Taking into account a possible charge double layer around the molecule, one can derive that the spacing between the patch and the dish is probably no larger than about 70 Å.

Effect of Bio-Bgt/Av on Con A Receptors. The immobilization of AcChoR by Bio-Bgt/R-Av is specific in the sense that Bio-Bgt binds specifically to AcChoR. FPR experiments show that AcChoR immobilization leads also to an immobilization of some Con A receptors tagged with fluorescent Suc-Con A. [Suc-Con A is claimed to be divalent rather than tetravalent, as is native Con A, but it retains the same chemical specificity for binding to glucose and mannose residues of glycoproteins and glycolipids (17). Suc-Con A appears to crosslink its own receptors less extensively than native Con A does and thereby better preserves receptor mobility (21).] In the present experiments, myotube cultures were treated with dichlorotriazinylaminofluorescein-labeled Suc-Con A followed by Bio-Bgt/R-Av, or with tetramethylrhodamine Suc-Con A followed by Bio-Bgt/unlabeled Av. Control cells, in which AcChoR was not immobilized, were treated similarly except that underivatized Bgt was substituted for Bio-Bgt. The fraction of Suc-Con A/Con A receptor complexes that are mobile on the upper surface of the myotubes decreased from 0.33 ± 0.03 (SEM) for controls to 0.19 ± 0.03 (SEM) for cells upon which AcChoR were immobilized. The average diffusion constant of the mobile Con A receptors, $(4 \pm 1) \times 10^{-11}$ cm^2/s, did not change significantly upon immobilization of AcChoR.

DISCUSSION

The present results demonstrate directly by fluorescence techniques that apparent crosslinking of AcChoR through Bio-Bgt and R-Av induces an accelerated internalization of AcChoR and a net loss of AcChoR from the surface. Previous studies on a variety of muscle systems have demonstrated indirectly by release of radioactive degradation products of Bgt that divalent anti-AcChoR antibodies (5–7) but not monovalent anti-AcChoR Fab fragments (5, 7) accelerate the degradation of cell surface AcChoR. Both the Bio-Bgt/R-Av and anti-AcChoR effects are examples of receptor population modulation by external crosslinking. This phenomenon may be relevant to autoimmune diseases such as myasthenia gravis (22, 23) and perhaps to cell–cell recognition during development.

It is not clear what the molecular mechanisms of the crosslinking-induced internalization are. One might guess that receptor redistribution, immobilization, or both are the triggers. In this regard, it is interesting that Bio-Bgt/R-Av accelerates the internalization both of previously immobile (13) AcChoR in preexisting patches and of laterally mobile AcChoR in diffuse areas. Receptor internalization rate thus appears sensitive to the manner in which the receptors are immobilized. An inference is that AcChoR in myotube patches (and in synapses, where AcChoR turn over very slowly) are immobilized by some mechanism other than direct AcChoR-AcChoR crosslinks,

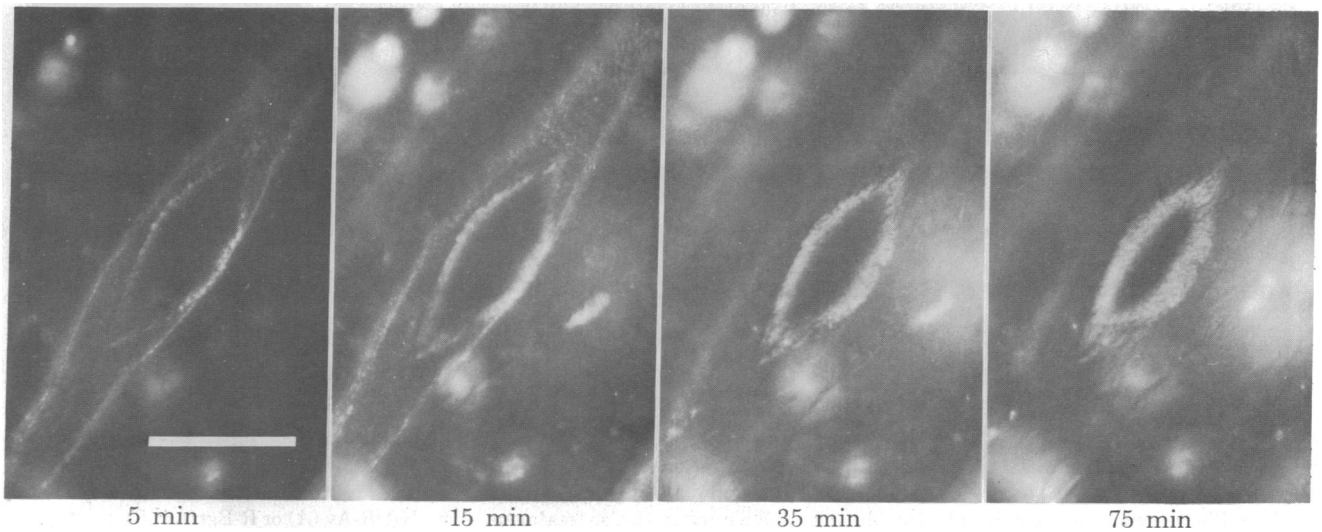

FIG. 5. Fluorescence micrographs of R-Av penetrating toward the central region of a Bio-Bgt-treated patch on the bottom of a myotube, as a function of the indicated duration of continuous exposure to R-Av. Bar = 40 μm.

perhaps by cytoplasmic structures (24). [This conclusion is consistent with Bloch's observation (25) that AcChoR patches can be dispersed by some metabolic inhibitors.] Direct crosslinking of AcChoR within a patch by Bio-Bgt/R-Av therefore must induce a submicroscopic AcChoR redistribution that triggers internalization.

In contrast with the trend of the present results, treatment of chicken myotubes with Con A reduces the internalization rate of AcChoR (7) while also reducing AcChoR mobility (12). However, Con A binding is considerably less specific than Bio-Bgt binding. Although direct crosslinkage of AcChoR by Con A might tend to enhance AcChoR internalization, concomitant Con A binding to other cellular components perhaps simultaneously depresses the process of membrane turnover.

Lennon (4) infers from indirect immunofluorescence studies either that anti-AcChoR redistributes diffusely distributed AcChoR into large patches (see also ref. 7) or that preexisting patches are resistant to internalization by anti-AcChoR crosslinking. Because Bio-Bgt/R-Av shows neither of these effects on rat myotubes, there may be a difference between Bio-Bgt and anti-AcChoR with respect to the detailed structure or kinetics of AcChoR crosslinkage.

The finding that virtually all preexisting patches on the bottom surface of the myotubes are probably within 70 Å of the substrate is supported by recent reflection contrast microscopic observations (26) that patches are within 300 Å of the substrate. Alternatively, the lack of central labeling in AcChoR patches by Bio-Bgt/R-Av could result, in principle, from extracellular material filling the space between the membrane and the substrate and forming a contiguous barrier to lateral diffusion of R-Av. This alternative hypothesis, although somewhat unlikely, still requires patch regions to be in intimate contact with a relatively solid material. The coincidence of intimate contact and AcChoR patches suggest either that physical contact can induce the formation of AcChoR patches or that physical contact occurs where AcChoR are predisposed to aggregate. Either way, the correlation between physical contact and AcChoR receptor aggregation may be relevant to the formation of synapses during development.

The fraction of Suc-Con A/Con A receptor complexes that are mobile on primary myotubes (33%) is relatively lower than that on L6 myoblasts (55%, ref. 21). The partial mobility may reflect either an actual heterogeneity of Con A receptors or a partial crosslinkng by the presumed divalent Suc-Con A. The low fractional mobility on primary myotubes measured here is even further reduced to about 19% when AcChoR are almost completely immobilized by Bio-Bgt and Av. The reduction of Con A receptor mobile fraction can be ascribed to any of the following: (*i*) direct immobilization of those Con A receptors that are also acetylcholine receptors (27); (*ii*) entrapment of Con A receptors in a cage of surrounding crosslinked AcChoR; or (*iii*) a submembrane filamentous connection between Con A receptors and crosslinked AcChoR. At least a partial specificity of the Bio-Bgt/R-Av effect is revealed by those Con A receptors that remain laterally mobile despite virtually complete AcChoR immobilization.

Several of the experimental approaches developed here may find application to other studies in cell biology. The time course of fluorescence measured selectively from edge regions of cells by quantitative photometry assays the loss of fluorescence-marked surface receptors by internalization or exocytosis. The slow penetration of large surface-binding fluorescent molecules into the interstices between a cell and the substrate visualizes these intimate contact points by characteristic fluorescence–dark regions. Treatment of cells by Bio-Bgt and Av brightly and specifically marks AcChoR for fluorescence observation. Finally, Bio-Bgt/R-Av treatment may serve as a tool for studying the biological consequences of specific AcChoR immobilization and induced internalization during the formation of nerve–muscle contacts.

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