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α-Bungarotoxin Binding Sites (Acetylcholine Receptors) in Denervated Mammalian Sarcolemma

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The nonsynaptic sarcolemma of denervated skeletal muscle of rat shows an abundance of ~15 nm intramembranous particles on the P face. These particles are either singly distributed or are in clusters, and they are essentially lacking from the comparable freeze-fractures of the innervated sarcolemma. Autoradiographic studies using ^{125}I-α-bungarotoxin (BGT) on 1 μ-thick sections, and freeze-etch studies using ferritin-α-BGT conjugates on membrane fractions, show that the distribution of the label corresponds to the distribution of the 15-nm particles in the nonsynaptic sarcolemma. On the basis of these results and existing physiologic and biochemical data, it is suggested that the 15-nm intramembranous particles are components of the α-BGT binding sites, ie, acetylcholine (Ach) receptors, in the nonsynaptic sarcolemma of denervated muscle and that the two types of distributions represent two spatial manifestations of Ach receptor molecules. The significance of these findings in relation to synapse formation in denervated muscle is discussed.

Key words: denervated sarcolemma, nonsynaptic acetylcholine receptors, ^{125}I-α-bungarotoxin, ferritin-α-bungarotoxin, electron microscopy, freeze-fracture, freeze-etching, autoradiography

Denervation of the adult mammalian skeletal muscle is being extensively applied to understanding the nerve-muscle interaction [1–6]. Physiologic and biochemical changes occurring in the sarcolemma of denervated muscle include a fall in the resting membrane potential and an increase in membrane resistance [7, 8] and a decrease in acetylcholinesterase activity (AchE), mainly the 16S form of AchE [6, 9]. Among the three known molecular forms of AchE, namely 4S, 10S, and 16S in mammalian muscle, the induction of the 16S form is under neural control [10]. Denervation also results in an increased sensitivity to acetylcholine (Ach) in nonsynaptic sarcolemma with a concomitant increase in the number of Ach receptors [11]. Earlier, we reported changes in the sarcolemma of denervated rat muscle examined by freeze-fracturing technique [4] and described the

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appearance of intramembranous particles (~15 nm) on the cytoplasmic fracture of the membrane (P face) in the nonsynaptic sarcolemma. These intramembranous particles were either singly distributed or in clusters. It was hypothesized that these ~15 nm particles which appear in the nonsynaptic region following denervation are the Ach receptors. The present paper describes the work primarily directed towards testing the above hypothesis by use of marker, α-bungarotoxin (α-BGT), which binds to the Ach receptors in a specific and irreversible manner [12]. α-bungarotoxin, when conjugated to ferritin, can be visualized by electron microscopy or, when conjugated to ^{125}I, can be visualized by autoradiography at light or electron microscope level. The results of such an investigation are included in this paper.

It should be pointed out that in mammalian muscle the size of intramembranous particles thought to correspond to the Ach receptor complex is 11–15 nm [6, 37]. In Xenopus embryonic muscle and cultured myotubes of chick, such particles are 10–19 nm [38, 39]. These sizes differ from the 7 nm given for the Ach receptor complex in the electroplaques [40]. Though the significance of these differences remains to be investigated, the intramembranous particles in the segments of cultured myotubes of chick containing identifiable regions of high acetylcholine sensitivity measure 10–19 nm [39].

MATERIALS AND METHODS

Denervation Procedure

The lumbricals and extensor digitorum longus (EDL) of rats of the Sprague-Dawley strain weighing 100–120 g were used in this study. The animals were anesthetized and denervated by transection of the sciatic nerve in the upper thigh region and sacrificed by cervical decapitation 2 weeks after denervation. The rationale for using such a period of denervation was based upon our previous morphologic and histochemical studies, which showed marked changes in the nonsynaptic sarcolemma after 2 weeks of denervation [4, 6]. Also, the incorporation of Ach receptors in the nonsynaptic sarcolemma is optimum 2 weeks after denervation [11]. The muscles of normal innervated rats were used as controls.

Preparation of Muscle Membrane Fraction

The muscles were homogenized and crude membrane fractions were isolated from control, as well as denervated animals, according to the procedure of Boegman et al [13].

Preparation of α-Bungarotoxin Conjugates

α-BGT supplied by Miami Serpentarium, Florida, was used for the following conjugates.

Iodination of α-BGT. α-BGT was iodinated with 1 mCi ^{125}I using the method of Greenwood and Hunter [14]. The iodination was carried out by Radiopharmacy Centre, University of Alberta. The specific activity and protein concentration of iodinated protein were 2.192×10^5 Ci/mole and 8.4 μg/ml, respectively.

Preparation of ferritin-α-bungarotoxin (Ft-α-BGT). Ferritin (6X and cadmium-free) obtained from Polysciences was conjugated to α-BGT by using glutaraldehyde, according to the method of Hourani et al [15].

Incubation of Muscles With ^{125}I-α-BGT

The EDL muscles from denervated and innervated rats were tied at both ends to a wooden stick and immediately transferred to an oxygenated Kreb's ringer containing ^{125}I-α-BGT (2×10^{-7} M). The muscles were incubated for 2 h. For determining the

specific binding of α-BGT, another set of EDL from each of the innervated and denervated rats was incubated initially in Kreb's ringer containing d-tubocurarine (10^{-4} M) for 1 h, followed by incubation in ^{125}I-α-BGT for 2 h. During incubation, the oxygen was continuously bubbled through the medium maintained at 37°C in water bath. After incubation, the muscles were thoroughly washed by repeated changes of buffer for 1 h and fixed in 2% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2). The tissue was then given several changes of buffer and gently dried on filter paper and weighed. The radioactive counting of these tissues was done in a gamma counter (Baird-Atomic). The tissue was then postfixed in OsO_4 and routinely processed for preparation of 1 μ-thick Araldite sections for autoradiography (see below).

Incubation of Membranes in ^{125}I-α-BGT

The membranes were assayed for their Ach receptor activity by the filter assay procedure suggested by Klett et al [16]: A 1 ml portion of Kreb's ringer contained 50 μg of membrane protein, ^{125}I-α-BGT (2×10^{-9} M), and 1% (w/v) Tween 80. Control samples were incubated with α-BGT (10^{-4} M) for 1 h prior to incubation in ^{125}I-α-BGT. The mixture was incubated at room temperature (20–22°C) for varying periods and then filtered through DE81 cellulose anion exchange filter disk (Whatman). The dried filter disks were counted for the radioactivity in a gamma counter.

Preparative Procedures for Microscopy

Light microscope autoradiography. Araldite sections 1 μ thick were cut from blocks of innervated and denervated muscles which had previously been incubated in ^{125}I-α-BGT as described above. The sections were coated with 1:1 diluted Ilford L_4 emulsion on gelatinized slides. After exposure for one week, the slides were developed and fixed in 25% sodium thiosulphate. The sections were stained with 2% phenylenediamine and examined under phase contrast microscope.

Incubation of muscle membranes with Ft-α-BGT. The muscle homogenate (1000 g) and the crude membrane fractions were incubated with Ft-α-BGT (0.6 μg/ml) for 1 h. For determination of specific binding, the fractions were first incubated in α-BGT (10^{-4} M) or d-tubocurarine (10^{-4} M), followed by incubation with the conjugate. The suspension of crude membranes was washed by filtration through millipore as described by Karlin et al [17]. Suspension of the homogenate was centrifuged at 1,000 g for 10 min. The supernatant was discarded and the pellet was washed several times with buffer. The samples were then fixed and processed for electron microscopy as described above. Thin sections were examined, unstained or stained with uranyl acetate and lead citrate, in Phillips EM 300 electron microscope.

Freeze-fracture and freeze-etch preparations. A portion of the homogenate incubated in Ft-α-BGT was used for freeze-etching. The pelleted samples were fixed in 2% glutaraldehyde buffered with 0.1 M phosphate (pH 7.2). The material was rinsed with buffer and finally with distilled water. Freeze-fracturing and etching were done in Balzers BA 360M and the etching period lasted up to 2 min. The replicas were washed in 40% chromic acid and rinsed in several changes of water and examined in Phillips EM 300 electron microscope.

RESULTS

Freeze-Fracture Studies of Innervated and Denervated Sarcolemma

A comprehensive account of the alterations in the structure of sarcolemma visualized in the freeze-fractured replicas of denervated muscle is given elsewhere [4–6]; therefore, only those features which are pertinent to the present investigation are mentioned below.

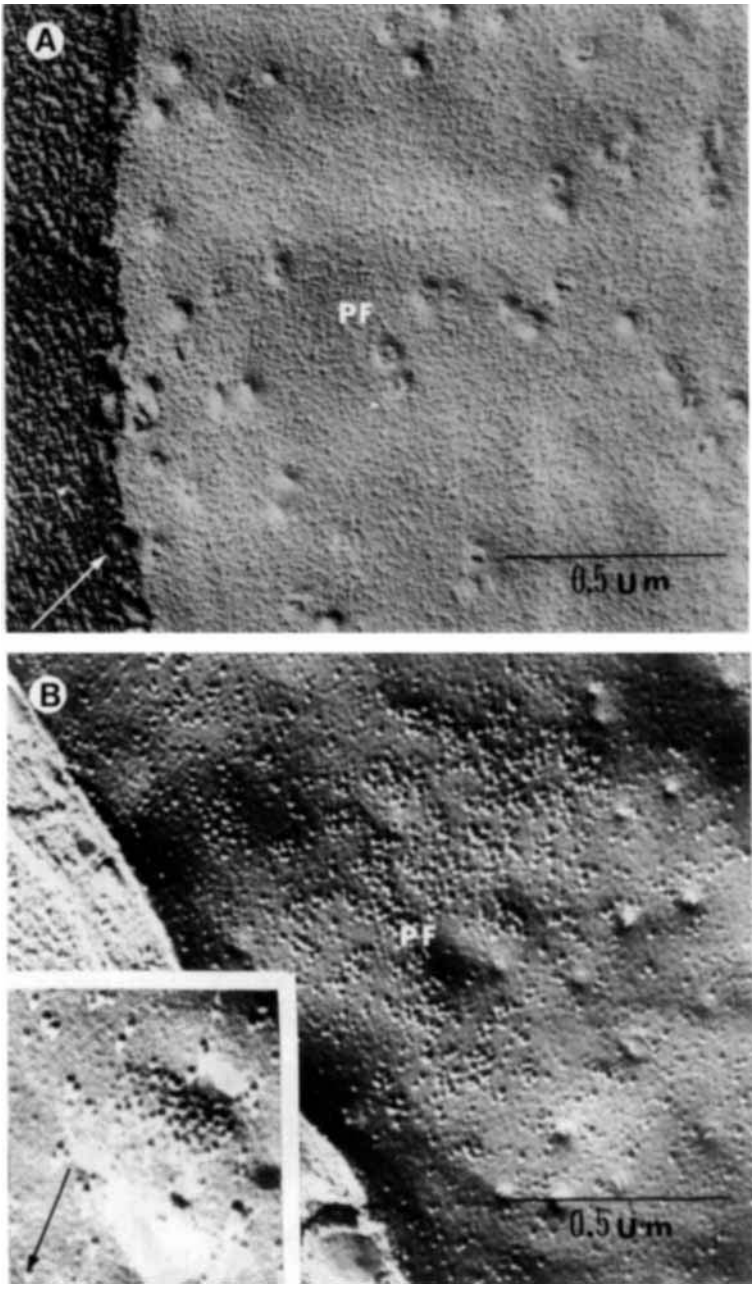

Fig. 1. A. Freeze-fractured preparation of a normal lumbrical muscle, showing the convex fractured face (PF) of nonjunctional sarcolemma. The intramembranous particles (~8 nm) are distributed uniformly over the entire fractured face. B. Fractured face (PF) of the sarcolemma from a lumbrical muscle of rat denervated for 2 weeks. A large number of particles are apparent in the denervated muscle which are not discerned in the normal (innervated) muscle. These particles are bigger (~15 nm) than those seen on this face in the normal muscle and it is likely that they represent extrajunctional acetylcholine receptors in denervated muscle. The inset is a PF of the denervated EDL muscle showing an aggregate with a number of 15 nm particles. Arrow in lower left corner indicates direction of shadowing. From Malhotra [22], with permission of Plenum Publishing Corporation, New York.

In innervated sarcolemma, the P face (PF) shows randomly dispersed intramembranous particles which are approximately 8 nm in diameter (~2,000/μ^2) (Fig. 1A). In contrast, the corresponding face of the denervated sarcolemma shows an abundance of ~15 nm (15–18 nm) intramembranous particles. These particles are dispersed singly or in aggregates. These aggregates may be small, with as few as 4–10 particles, or large, with approximately 25–100 particles (Fig. 1B). The average density of the particles on the PF (convex fracture) is ~400 particles/μ^2 and is predemoninantly made up by the 15-nm particles. A precise correlation between the intramembranous particles and the Ach receptors remains to be determined. It is of interest, however, that there are ~1150 α-BGT binding sites/μ^2 in the denervated extrajunctional sarcolemma. This estimate is based upon the assumption that each receptor binds one ^{125}I-α-BGT molecule (Tipnis and Malhotra, unpublished data), yet there may be more than one binding site per receptor molecule [43]. On the basis of existing physiological and biochemical evidence [11, 18], it has been hypothesized that these particles are components of the α-BGT binding sites (Ach receptors) [4, 5] and the two types of distributions of particles noted in these freeze-fractured replicas represent two distinct topographic distributions of receptors in the nonsynaptic sarcolemma of denervated muscle.

The following results refer to the experiments designed to test the above hypothesis by localization of Ach receptors through the use of ^{125}I-α-BGT and Ft-α-BGT conjugates.

Incorporation of ^{125}I-α-BGT Into Muscle

Incubation of innervated and denervated muscles in media containing ^{125}I-α-BGT shows a marked increase in the binding of toxin by denervated muscle over the innervated muscle (Table I). Preincubation of the muscles with d-tubocurarine leads to a marked sup-

TABLE I. Specific Binding of EDL Muscles of Innervated and Denervated Rats (^{125}I-α-BGT counts/min/mg muscle)

Experiment	^{125}I-α-BGT	d-tubocurarine and ^{125}I-α-BGT	Specific labeling
Innervated	604	292	312
Denervated	2394	1186	1208

The EDL muscles from innervated and denervated rats were incubated in oxygenated Kreb's ringer with 2×10^{-7} moles of ^{125}I-α-BGT. For determining the specificity of binding, another set of muscles was incubated with d-tubocurarine (10^{-4} moles) for 1 h and subsequently incubated with ^{125}I-α-BGT.

TABLE II. Distribution of Autoradiographic Grains in Nonsynaptic Region over 1-μ-Thick Sections of Innervated and Denervated Muscles Labeled With ^{125}I-α-BGT (number of silver grains /μ^2)

Experiment	^{125}I-α-BGT	d-Tubocurarine and ^{125}I-α-BGT
Innervated	0.6	0.5
Denervated	5	1.4

Control in both innervated and denervated muscles represents the preincubation of muscles in d-tubocurarine followed by incubation in ^{125}I-α-BGT. The muscles were incubated under the same conditions as mentioned in Table I.

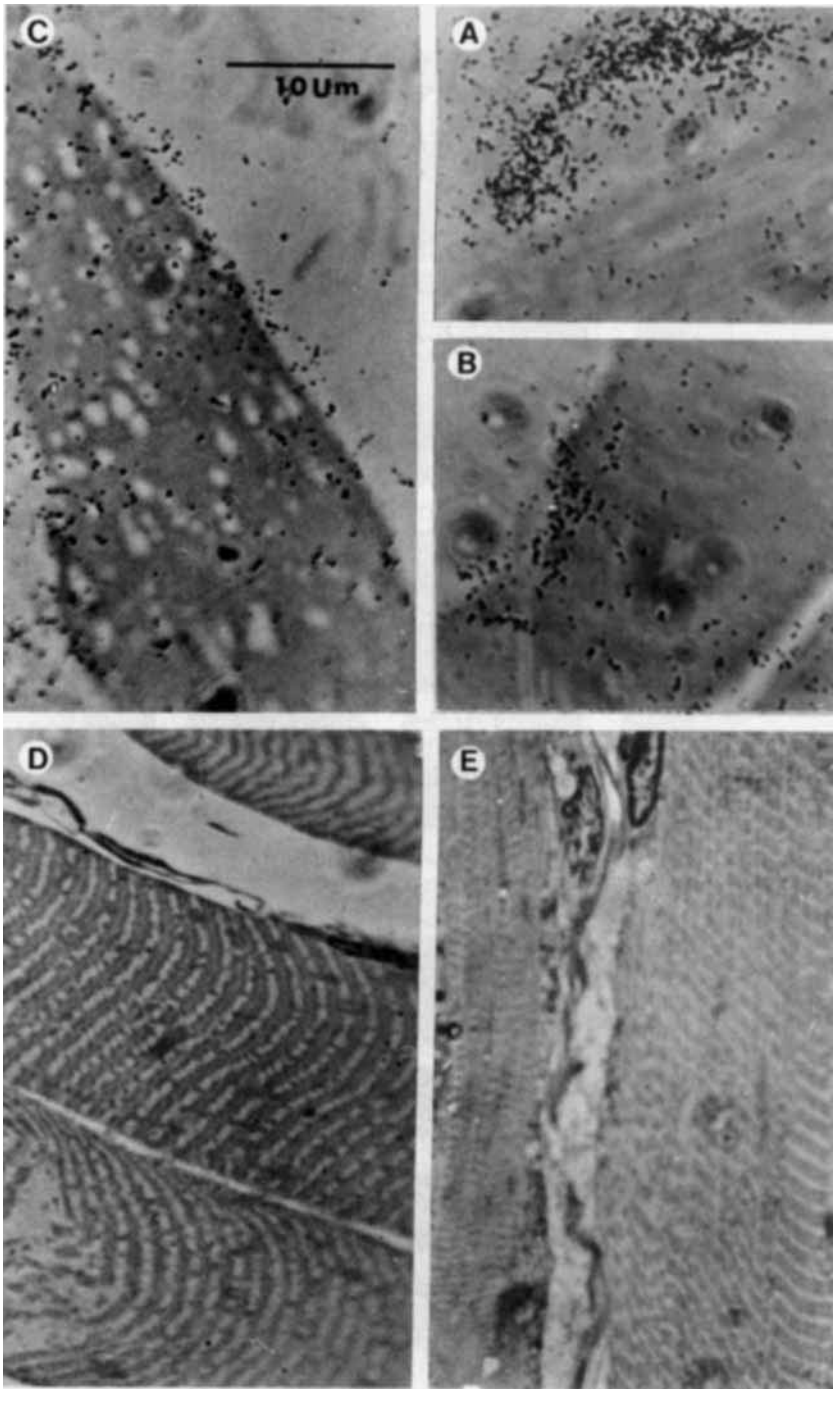

Fig. 2. Comparison of the specific incorporation of the labeled toxin in denervated vs innervated muscle. Sections of denervated muscle (1 μ thick) showing synaptic region with high density of grains (A) and nonsynaptic regions with uniformly dispersed grains (C) and clusters of grains (B). Sections of innervated muscle (D) or d-tubocurarine-treated denervated muscle (E) do not show grains. Calibration line in C applies to all illustrations in this figure.

pression in the binding of the toxin (Table I). Since d-tubocurarine is known to be a specific ligand for Ach receptors [19] and binds to the same site as the α-BGT, the data on the specific incorporation of ^{125}I-α-BGT indicate the labeling of the Ach receptors. As seen in Table I, the specific incorporation of ^{125}I-α-BGT in denervated muscle is approximately four times more than that in the innervated muscle. In both innervated and denervated muscle, d-tubocurarine inhibits toxin labeling by about 50%, which is in agreement with previous findings [41, 42].

Light Microscope Autoradiography

Table II shows the extent of silver grains seen in the nonsynaptic sarcolemma of 1 μ-thick sections. Silver grain counts given are after subtraction of the background grains. In each experiment, the background grains have been counted in areas located approximately 5 μ away from the tissue. The background counts in sections of ^{125}I-α-BGT labeled muscle are comparable to the background grains encountered in sections prepared from muscles incubated in cold α-BGT and processed for autoradiography.

The distribution of silver grains in 1-μ-thick sections from innervated and denervated muscle is displayed in Figure 2. The silver grains in the nonsynaptic sarcolemma of denervated muscle appear either dispersed singly (Fig. 2C) or clustered (Fig. 2B). The number of silver grains per square micrometer is approximately 5–10 times higher in the clusters than outside such regions. Such areas of higher density do not represent the synaptic regions where the density of silver grains is far more in innervated as well as denervated muscle (Fig. 2A).

In contrast, the nonsynaptic regions of the innervated muscle have very few grains and their number is close to the background density (Fig 2D). Also, sections of denervated muscle treated with d-tubocurarine prior to incubation in ^{125}I-α-BGT are generally lacking in silver grains (Fig. 2E). It is concluded from the above data that the silver grains in the nonsynaptic sarcolemma of denervated muscles are far more numerous and represent much more binding of ^{125}I-α-BGT to specific sites on denervated muscle than in the corresponding regions of the innervated muscle.

Incorporation of ^{125}I-α-BGT Into Crude Membrane Fractions

The presence of sarcolemma in the crude membrane fraction was ascertained by assaying for Ach receptor activity. (Though the Ach receptors have been reported to reside in the Golgi apparatus during synthesis [20], it is assumed that in intact cells the receptors are exposed at the surface only in the plasma membrane.) In the membrane fractions from both innervated and denervated muscle, the binding of the toxin is linear during the first 10 min, after which saturation occurs (Fig. 3A). The specific activity in denervated membranes is 2.99×10^{-2} pmoles/μg as compared to 9.2×10^{-4} pmoles/μg in innervated preparation. Figure 3B shows that the binding of ^{125}I-α-BGT to denervated membranes is specific, as it is greatly inhibited by preincubation with cold α-BGT.

Transmission Electron Microscopy of Membranes Incubated With Ft-α-BGT

Crude membrane fractions show membrane vesicles ranging from 0.2 to 2 μ in diameter. Many of these vesicles from preparations incubated in Ft-α-BGT conjugate and when filtered through millipore get trapped in the filter along with nonspecifically bound ferritin as reported by Karlin et al [17]. In the present study, however, vesicles trapped in the filter were not considered and only vesicles lying above the filter were examined. Denervated crude membranes incubated in ferritin conjugate show ferritin associated with the membrane of the vesicle. Ferritin particles may be situated slightly removed

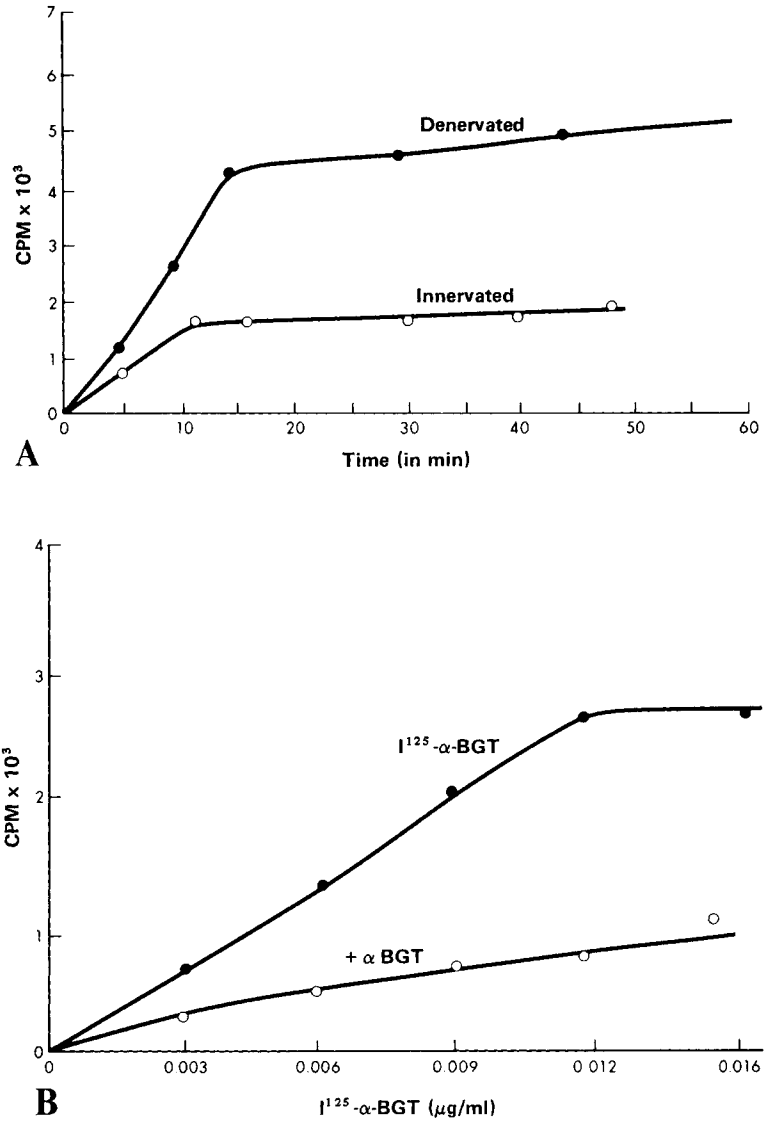

Fig. 3. A. Time-course binding of ^{125}I-α-BGT to crude sarcolemmal preparations from innervated and denervated muscles. The membranes from innervated and denervated muscles were incubated in ^{125}I-α-BGT. The incubation medium contained oxygenated Kreb's ringer, 50 μg/ml membrane protein, and ^{125}I-α-BGT (2×10^{-9} moles). The membranes were filtered through DE81 cellulose according to the method of Klett et al [16]. The filter paper was dried and the radioactivity was counted in a gamma counter. The specific activity of ^{125}I-α-BGT was 2.192×10^5 Ci/mole. B. Specific binding of ^{125}I-α-BGT to denervated crude sarcolemmal preparation. The membrane preparation (50 μg/ml of protein) was incubated in oxygenated Kreb's ringer containing ^{125}I-α-BGT (2×10^{-9} moles) for 1 hour at room temperature. The membranes were filtered through DE81 cellulose according to the method of Klett et al [16]. The filter was dried and the radioactivity counted in a gamma counter. For nonspecific binding, the aliquots were incubated in medium containing α-BGT (0.1 mg/ml) for 1 h. The specific activity of ^{125}I-α-BGT was 2.192×10^5 Ci/mole.

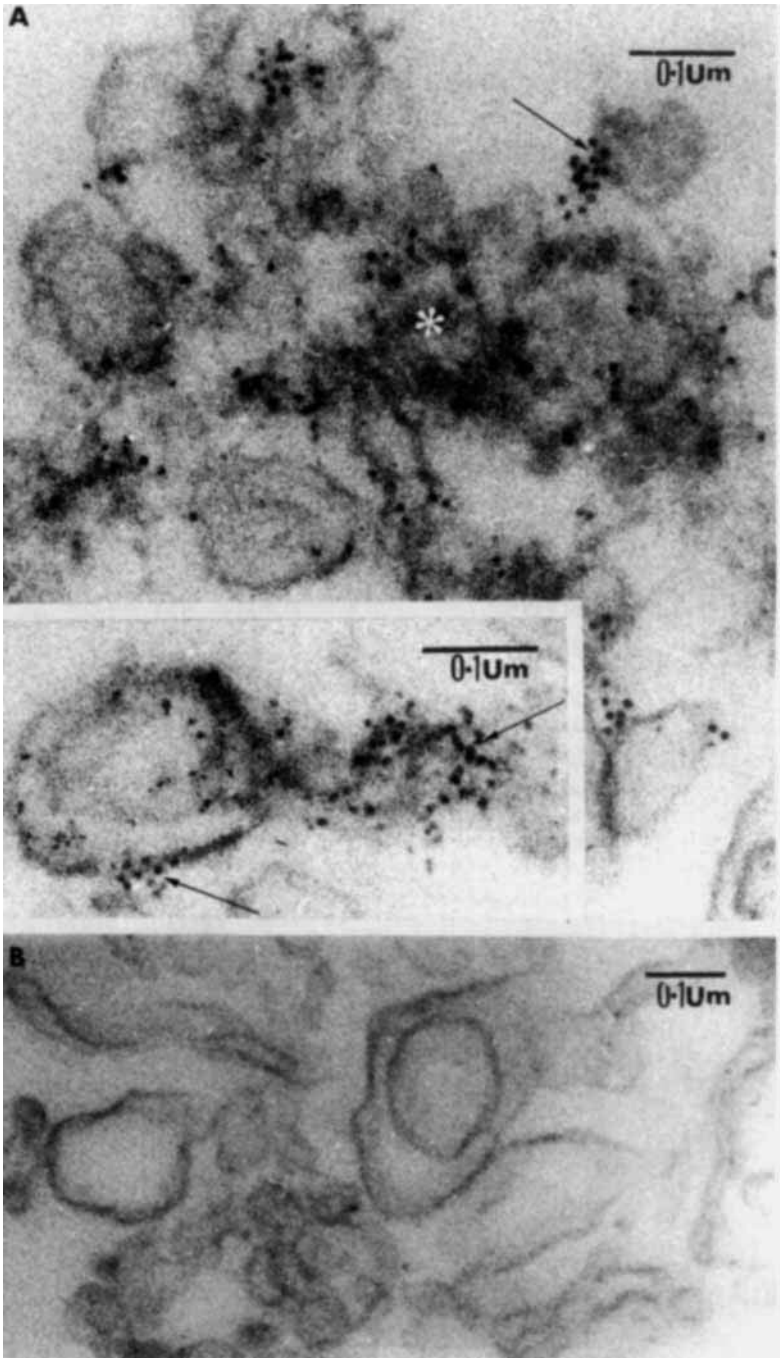

Fig. 4. A. Thin sections from a crude membrane fraction of denervated skeletal muscle incubated in Kreb's ringer containing 0.6 μg/ml of Ft-α-BGT conjugate showing ferritin binding. Several membrane vesicles are seen labeled. Arrows indicate clusters of ferritin particles. *Some unidentifiable material that could be cell debris. Such regions have not been considered in the estimation of Ft-α-BGT binding sites. B. Control from d-tubocurarine-incubated material showing paucity of ferritin binding.

from the surface of the membrane (~5–7 nm). The particles are seen as single molecules bound to the membrane or in small clusters (Fig. 4A). Several vesicles without the associated ferritin molecules are also encountered in electron micrographs. Estimation of the vesicles from four experiments indicates that 60% of the vesicles are labeled (Table III) whereas, in d-tubocurarine-treated controls, the number of vesicles showing associated ferritin is reduced to 11% of the total vesicles (Table IV). Small, dense particles are sometimes encountered inside the vesicles, both in the experimental and the d-tubocurarine-treated material. These are generally smaller than the ferritin molecules and their nature is not known. It appears that these particles do not result from the preparative fixation procedure, as membrane vesicles which have not been incubated in medium containing Ft-α-BGT conjugate do not show these particles. It is therefore assumed for the present that they represent degraded ferritin molecules, and the membranes are leaky to these particles.

TABLE III. Counts of Membrane Vesicles (denervated) Labeled with Ft-α-BGT

Experiment No.	Total vesicles counted	Labeled vesicles	Unlabeled vesicles
1	31	25	6
2	146	82	64
3	115	70	45
4	99	52	47
Total	391	229	162

Crude membrane fractions (~50 μg protein) were incubated in Kreb's ringer containing Ft-α-BGT conjugate (0.6 μg/ml). The membranes were filtered through millipore and, subsequent to washing, were processed for electron microscopy and embedded in Araldite. The vesicles lying above the filter were randomly counted in thin sections. It is apparent that nearly 60% of the vesicles were labeled. The number of ferritin molecules on the labeled vesicles may vary from 2 or more. The presence of ferritin molecules in the background is rare as the millipore filters were soaked in 2% albumin to minimize nonspecific binding [17].

TABLE IV. Counts of Membrane Vesicles (denervated) Incubated With d-Tubocurarine Followed by Incubation in Ft-α-BGT

Experiment No.	Total vesicles counted	Labeled vesicles	Unlabeled vesicles
1	95	15	80
2	153	18	135
3	252	11	241
4	116	23	93
Total	616	67	549

The procedure in these experiments was similar to the one outlined in Table III except in these experiments, the membrane fractions were incubated in d-tubocurarine (10^{-4} moles prior to incubation in Ft-α-BGT conjugate. The counting of more than 600 vesicles indicate that only 11% of these were labeled.

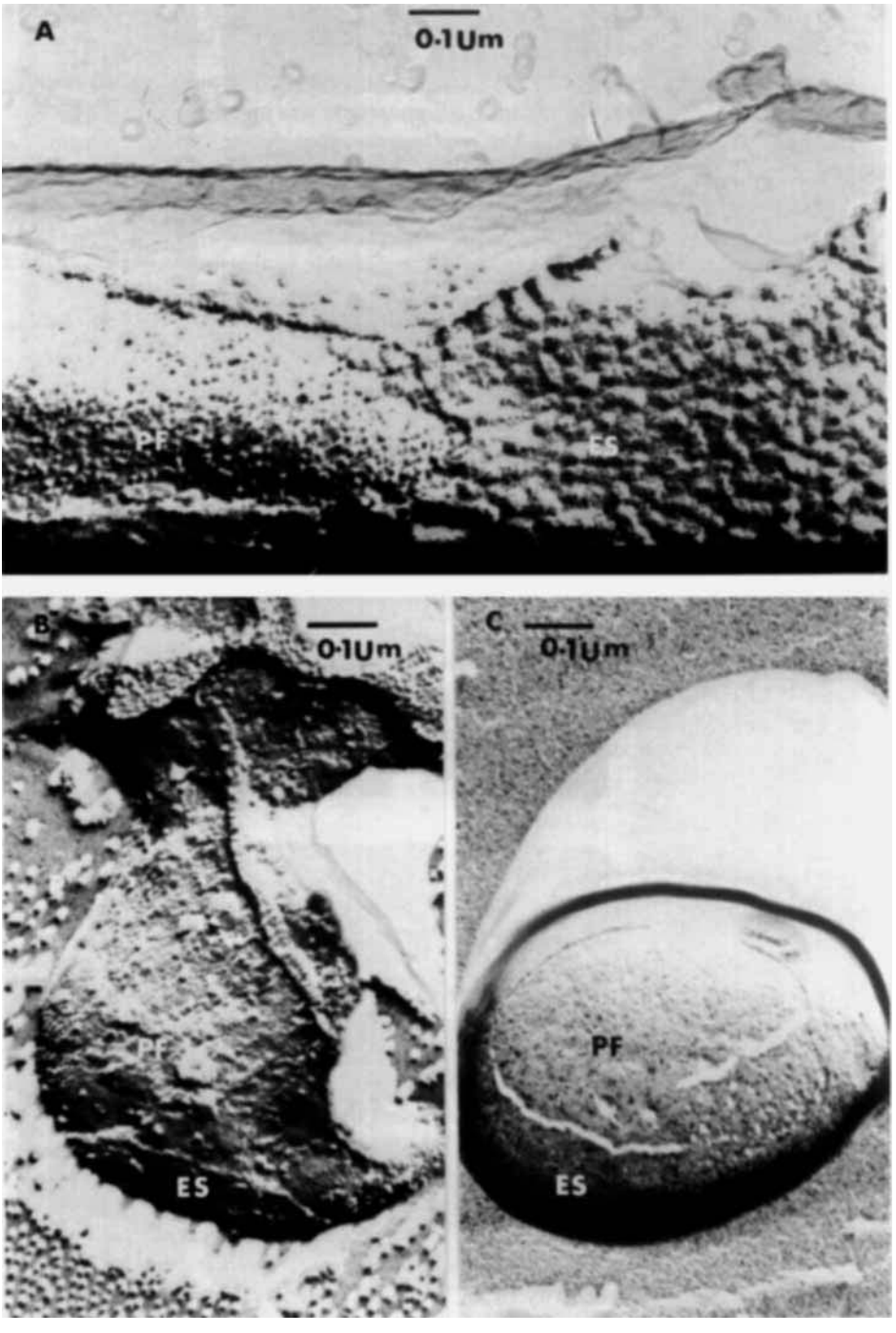

Fig. 5. A. Freeze-etch replica of homogenate from denervated muscle incubated in Kreb's ringer containing 0.6 μg/ml of Ft-α-BGT conjugate showing bumpy appearance of the etched face (ES), presumably due to binding to the conjugate. B, C. Controls without incubation in conjugate showing relatively smooth etched face (ES). Arrow in lower left corner indicates direction of shadowing.

Freeze-Etching of Ft-α-BGT-Labelled and Unlabelled Homogenate of Denervated Muscle

Initially, experiments were conducted on Ft-α-BGT-labeled crude membrane preparation, but despite several (40–50) attempts, satisfactory fractures of such membranes were not obtained. Therefore, muscle homogenate was investigated as a source of Ach receptor containing membranes. The results reported in this section are based on studies on Ft-α-BGT-labeled homogenate.

Replicas of freeze-etched membranes in muscle homogenate incubated with Ft-α-BGT show vesicles with a fractured face (PF) and an etched face (ES). The etched face often displays densely packed bumps (Fig. 5A) comparable to the size of ferritin molecules discernible in metallic replicas [21]. Some of these bumps are larger than individual ferritin molecules and may represent clusters of ferritin. It is emphasized that only some of the vesicles show bumpy etched face, whereas others have relatively smooth etched face. Also, comparable membrane faces in replicas of the homogenate without incubation in the Ft-α-BGT show relatively smooth etched faces (Figs. 5B, 5C). It is therefore concluded that the bumps on the etched face of the membranes displayed in Figure 5A represent ferritin particles presumably bound to the Ach receptors. A comparison of the number of labeled and etched vesicles with the number of labeled vesicles in thin sections would be valuable, but the etched vesicles are not of as frequent occurrence as the labeled vesicles in thin sections. This discrepancy is presumably due to difficulties in getting large areas of etched vesicles in replicas.

It should be remarked that membrane vesicles with scanty intramembranous particles on the fractured face are seen in the replicas. It is conceivable that such fractured faces represent regions of the membrane that are deficient in particles. Alternatively, they may represent inside-out vesicles and the two fractured faces show an asymmetric distribution of intramembranous particles, there being a few on one half [22].

DISCUSSION

The results from autoradiography of ^{125}I-α-BGT and labeling with Ft-α-BGT lead to the conclusion that the Ach receptors are either distributed singly or clustered in the nonsynaptic sarcolemma of denervated muscle.

The experiments on ^{125}I-α-BGT binding were primarily undertaken to ascertain the specificity of incorporated radioactivity in both muscle and crude membrane fractions (Figs. 3A, 3B; Tables I and II). The filter assay of Klett et al [16], employed in the present study, is based on the use of anion exchange cellulose filters for filtering the Ach receptor preparation. The filter binds anionic molecules while ensuring the elimination of cationic substances like unbound α-BGT. In both muscle and the crude membrane fraction from denervated animals, there is a marked increase in incorporated radioactivity. This binding, which is inhibited both by d-tubocurarine and cold α-BGT, is considered specific. The qualitative data based on light microscope autoradiography demonstrates the increased labeling in extrajunctional regions and supports the quantitative data reported in Table I. These findings, therefore, are in agreement with several physiologic studies that have demonstrated the extrajunctional sensitivity to acetylcholine in denervated muscle [18, 11, 23]. This extrajunctional sensitivity results from the incorporation of newly synthesized receptors [24–26].

The findings on localization of Ach receptors, obtained by using ^{125}I-α-BGT and Ft-α-BGT at the level of light and electron microscopy, respectively, indicate that there are two distinct populations of Ach receptors, viz., singly dispersed and clustered. The labeling

of receptors with Ft-α-BGT is never as much as that observed in Ach receptor-rich preparations from *Torpedo* electroplaque [17]. This difference, however, is likely, as there is an extremely high concentration of Ach receptors in *Torpedo* electroplaque (40,000–50,000/μ^2) [27] compared to the relatively low concentration (1,695 receptors/μ^2) in the nonsynaptic region of denervated sarcolemma [11]. The membrane vesicles from synaptic sarcolemma, which would be expected to show dense labeling of Ft-α-BGT, may not have been encountered and possible explanations for the difficulty in finding such vesicles are 1) that membrane vesicles representing the synaptic sarcolemma are few and are lost during isolation; and 2) that the membrane preparation is filtered through millipore which traps unbound ferritin as well as vesicles.

The ferritin molecules may appear to be slightly removed from the surface of the membrane in thin sections (Fig. 4A), and this is consistent with the recent studies on Ach receptor-rich membranes from *Torpedo* in which it has been reported that the receptor molecules traverse the membrane and project approximately 5.5 nm outside the membrane bilayer [28].

The presence of Ach receptors in the nonsynaptic sarcolemma is a property of adult denervated muscle, as well as that of developing muscle cell [29, 30]. The nonuniformity of Ach receptor distribution reported in the present study has also been demonstrated in uninnervated developing muscles from chick and *Xenopus laevis* [31–33]. The presence of Ach receptor clusters in denervated muscle suggests that in relation to sarcolemma and Ach receptors, the muscle cell reverts to its embryonic state. Ach receptor clusters were thought to be the site of synapse formation [31]. It is pertinent to mention in this regard that the formation of synapse beside the original endplates is known to occur either by muscle injury or by removal of a portion of the muscle with the original nerve [34, 35]. However, recently Anderson and Cohen [33] have followed the distribution of Ach receptors (labeled with α-BGT) during synapse formation formation on myocytes cultured from *Xenopus laevis*. Their studies indicate that the clusters of Ach receptors are not the site of synapse formation.

The significance of the clustered Ach receptors is not yet clear. Axelrod et al [36] found that the receptors in the clusters are not free to diffuse into the surrounding areas. It is not as yet clear whether cluster formation occurs by movement and aggregation of individual particles or if there are distinct sites on the membrane where bulk incorporation of Ach receptor molecules takes place. Studies are in progress to further elucidate the role of the clustered Ach receptors in mammalian skeletal muscle.

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