Distribution of Extrajunctional Acetylcholine Receptors on a Vertebrate Muscle: Evaluated by Using a Scanning Electron Microscope Autoradiographic Procedure

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Abstract. A scanning electron microscope (SEM) autoradiographic technique was calibrated and used to determine the site density of acetylcholine receptors within 250 μm of the neuromuscular junction in innervated as well as 3- and 10-d denervated sternomastoid muscle of the mouse. In all these groups sharp gradients of receptor site density are seen around the endplates in the first 2–7 μm, continuing less sharply to between 25 and 50 μm. Beyond 50 μm (to 250 μm) a spatial density gradient is present 3 d after denervation, but none exist by 10 d. These results suggest that the postdenervation steady-state extrajunctional receptor site density is reached sooner near the junction than away from the junction.

The usefulness of SEM autoradiography to study the expression and distribution of membrane molecules at high resolution is demonstrated.

INFORMATION on the quantitative distribution of cell surface receptors is usually obtained using light microscope or transmission electron microscope (TEM),[1] autoradiography, biochemical-binding studies, and physiological tests. These procedures are limited either by resolution or by an inability to assess variations in receptor distribution over large areas in a morphologically intact cell. In the present study we demonstrate the value of using scanning electron microscope (SEM) autoradiography for that purpose. A SEM technique, modified from that of Junger and Bachmann (1980) is described. The procedure had already been illustrated in a nonquantitative manner to study acetylcholine receptors (AChRs) on striated muscle cells in culture (Neugebauer et al., 1985; Salpeter, 1986). In the present study, we calibrated the procedure for sensitivity and chemography and used it to demonstrate gradients of AChRs around the neuromuscular junction (nmj) on innervated and denervated mouse sternomastoid muscle. The results are consistent with, and extend those previously derived using other techniques.

We conclude that SEM autoradiography can be a reproducible quantitative procedure, with sensitivities comparable to those of TEM and light microscope autoradiography and with the potential of answering questions regarding the distribution of surface receptors on a fine structural scale.

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; nmj, neuromuscular junction; SEM, scanning electron microscope; TEM, transmission electron microscope.

Materials and Methods

Labeling the Receptors

The acetylcholine receptors in the mouse sternomastoid muscle were saturated in vivo by topically applying ^{125}I-α-bungarotoxin onto the surgically exposed muscle in anesthetized mice (10% Nembutal in 10% ethanol, 0.1 cc/10 g body wt) as previously described (Fertuck et al., 1975). Innervated muscles, or muscles denervated either 3 or 10 d previously from adult female mice (25–35 g) were used. Once the receptors on a muscle were saturated, the wound was sutured and the animal allowed to recover for ∼3 h. (We have found that nonspecific binding is more effectively removed in active animals than by washing in vitro after the muscle is removed from the animals.) Earlier studies have shown that these procedures affect neither receptor site density nor the morphology of the junction (Loring and Salpeter, 1980; Matthews-Bellinger and Salpeter, 1983).

Preparing Muscle Fibers for SEM Autoradiography

After the 3-h recovery period, the animals were again anesthetized and the muscle prefixed by intracardial perfusion with 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature. The muscle was then removed and fixation continued by immersion in the same glutaraldehyde solution for 1 h followed by several washes overnight in phosphate buffer. Subsequent treatment was the HCl-OsO_4 method of Desaki and Uehara (1981). Basically the tissue was postfixed at room temperature in 2% OsO_4 in distilled water for 30 min, washed in distilled water for at least 10 min, and placed in 8 N HCl at 60°C for ∼1 h until fibers separate. The HCl was diluted to 50% by adding distilled water, and the fibers filtered over a 47-mm, 8-μm pore size, nuclepore filter (polycarbonate film; cat. No. 28158-840; VWR Scientific Div., Univar, San Francisco, CA) in a nuclepore filter funnel [cat. No. 4626-R10; Thomas Scientific, Philadelphia, PA]). The fibers were then washed with distilled water for 10 min, dehydrated in 50, 70, and several changes of 100% ethanol for an additional 2 h. Cells can be stored on the nuclepore filter in 100% ethanol in petri dishes at room temperature until needed. Fibers were individually picked up, touched to Kimiwipe (Kimberly-Clark Corp., Roswell, GA) to drain excess alcohol, and placed onto double-sided stick tape on SEM stubs. Once on the stub, the cells were air

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dried for at least 1 h. Critical point drying of these fibers did not increase their morphological integrity for SEM autoradiographic purposes. For other cells critical point or freeze drying may be necessary.

The cells were then coated in a vacuum evaporator with four thin layers of carbon (70–75Å each, pale gray interference color). The total carbon layer was found to be reproducibly ~300 Å thick as measured using an interferometer (see Figs. 6–9 in Salpeter and Bachmann, 1972). The carbon layer prevented charging of the specimen in the SEM and was also intended to minimize chemography, i.e., the chemical interaction between tissue and emulsion (for a description of chemography, see Bachmann and Salpeter, 1965; Rogers, 1973; Salpeter, 1981).

Emulsion Coating and Preparation of Autoradiograms

Before emulsion coating, the stubs were examined in the SEM to determine whether a good yield of endplates was obtained. Good stubs were then coated with emulsion. We could not use liquid emulsions or even the looped emulsions proposed by Caro and Van Tubergen (1962), since even looped layers are not dry (Harris and Salpeter, 1983) and do not dry uniformly over the cells (Junger and Bachman, 1980). We therefore chose a stripping film previously calibrated for light autoradiography when using cells on tissue culture dishes (Land et al., 1977; Lane et al., 1977). The emulsion was prepared and applied as follows. Monolayers of Ilford L4 emulsion (Polysciences, Inc., Warrington, Inc., PA) purple-blue interference color; Bachman and Salpeter, 1967) were prepared on 0.3% collodion-coated slides. (For making collodion coated slides see page 8 of Salpeter, 1981.) Once the emulsion had dried, the collodion layer with the emulsion was stripped onto a water surface, and picked up from underneath over a hole in a filter paper. The filter paper was allowed to dry slightly and was then placed emulsion side down onto the cells, leaving the collodion support film on top (see page 22 of Salpeter, 1981). During the exposure time, the stubs containing the coated cells were kept in the refrigerator in black boxes with dessicant. Appropriate exposure times were estimated to give between 0.2 and 0.6 grains/μm^2 using the equation from Matthews-Bellinger and Salpeter (1978), based on the expected receptor site density, the desired grain density, the specific activity of the ^{125}I-α-bungarotoxin, and the calibrated sensitivity of the technique (see below). The final exposure time was adjusted as specimens were developed and the correct site density determined. In these studies specific activities of ^{125}I-α-bungarotoxin ranged from 50 to 150 Ci/mmol and exposure times from 10 to 30 d.

Developing and Viewing of SEM Autoradiograms

When the stripping film emulsion is placed over the cells, the collodion support film used for stripping the emulsion off the slide stays on top (i.e., the specimen sandwich [from bottom up] consists of SEM stub, cells, carbon layers, emulsion, and collodion support film) (Fig. 1 *A*). Before the emulsion can be developed, the collodion has to be removed. This was done by three 5-min washes in 100% alcohol, followed by three 1-min washes in 100% alcohol. The emulsion was air dried and developed for 4 min in D-19 (Kodak Laboratory and Specialty Chemicals, Rochester, NY) at 20°C, stopped by two 30 s washes in distilled water, and fixed in nonhardening fixer for 2 min, followed by four 1-min washes in distilled water, and again air dried. After the developed emulsion was dry, the specimen was again coated with 300 Å of carbon. (We use carbon since we found that gold coating obstructs the viewing of the silver grains in the SEM.) (For a schematic of developed specimen see Fig. 1 *B*.) Silver paint was painted around the edge of the stub to avoid charging in the microscope, and the specimen was viewed in the SEM (Philips 505) with specimen stage at zero angle tilt and using secondary or backscatter electrons. Secondary emission was quite acceptable if viewed with low energy (~10 KeV) and high bias gain, saturating the filament at 120 mA. The KeV and bias was adjusted for best image. Pictures were taken at 2,000–3,000× magnification and grains counted either from the negative or positive prints.

Calibration of Sensitivity

The sensitivity (defined as grains/decays) was calibrated using a modification of the procedure of Bachmann and Salpeter (1967), and Fertuck and Salpeter (1974). A radioactive layer of albumin was formed on a glass slide. The thickness of this test layer was measured interferometrically and the specified measured areas marked. We used albumin layers which were either 1,000 Å thick to mimic TEM sections, or 300 Å thick. Since self-absorption with ^{125}I increases beyond 1,000 Å (Fertuck and Salpeter, 1974) the 300-Å specimen would give results more comparable to that from label on the surface of a cell. The sensitivity of an emulsion layer, formed on a collodion coated slide, was tested by pressing the emulsion, face to face, against the sensitivity test specimen. After an appropriate exposure time, the emulsion-coated slide was separated from the test specimen and the emulsion developed. The grains in the developed emulsion were expressed either as sensitivity (grains/decays) or as the inverse of sensitivity (d), i.e., the number of decays to give one developed grain.

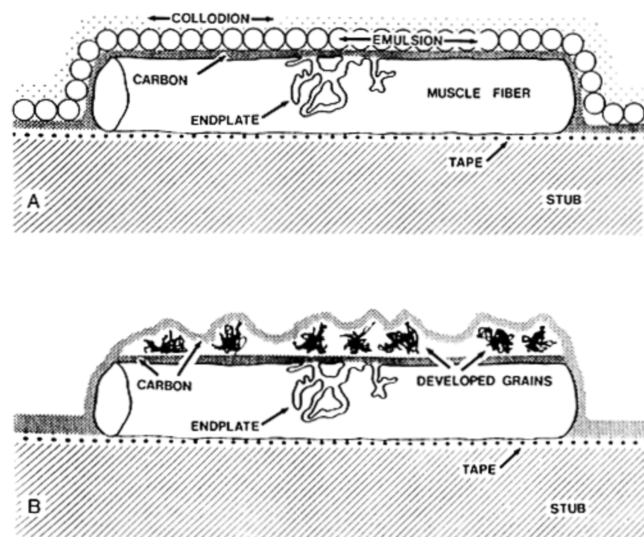

Figure 1. Schematic diagram of SEM autoradiographic specimen (*A*) before and (*B*) after developing the emulsion. (Diagram is not to scale.) (*A*) The muscle fiber is attached to the SEM specimen stub by double-sided sticky tape, and is coated with a 300-Å layer of carbon. The emulsion is placed over the carbon-coated fiber so that the collodion backing remains on top. Before the emulsion can be developed the collodion is removed by ethanol. (*B*) After the collodion is removed and the emulsion developed, a second 300-Å carbon layer is evaporated over the developed grains.

Test for Effect of the Carbon Layer between the Cells and the Emulsion

The 300-Å carbon layer that is evaporated over the cells, and thus interposed between the labeled material and the emulsion, could affect self absorption and thereby the final sensitivity value. This effect was determined by putting an equivalent layer of carbon over the ^{125}I-labeled sensitivity test specimen. When the test specimen was pressed against the emulsion, this carbon layer became interposed between the radioactive source and the emulsion just as it was in the SEM preparation. The grain density obtained with and without the carbon layer was then compared. Data was collected from SEM photographs at 2,000–7,000× and for TEM negatives at 7,000× for comparison.

Test for Effect of Chemography (Chemical Interaction) between the Cells and the Emulsion

Chemical interaction between tissue and emulsion can affect the latent image and thus the final developed grain yield. This effect is called chemography and can be both positive (increasing tissue background) or negative (giving a lower grain yield in the emulsion in contact with the tissue) (see Bachmann and Salpeter, 1965; Rogers, 1973; Salpeter, 1981). To test whether our tissue had a chemography effect on the emulsion, we used the test specimen to irradiate an emulsion while the emulsion was in contact with nonradioactive tissue. The chemography test was performed as follows. Muscle fibers prepared as for SEM autoradiography, but which were unlabeled, were placed on TEM grids held on a glass slide by double-sided sticky tape (We placed the cells on the grids instead of directly on an SEM stub to provide an easier surface for pressing against the sensitivity test specimen.) The unlabeled cells were carbon coated with the usual ~300-Å-thick carbon layer which should prevent chemography, and then coated with the

stripping film emulsion using our standard technique. The sensitivity test specimen was then pressed against the emulsion from the top; thus irradiating the emulsion. (Before this was done, however, the collodion support film over the emulsion was removed with ethanol as described above to avoid the collodion being interposed between the radioactive test specimen and the emulsion.)

After an exposure time which had been calculated to give ~0.2–0.5 grains per μm^2 of emulsion, the sensitivity test specimen was removed. The irradiated emulsion was then either developed immediately or after ~2 wk, a period equal to a typical exposure time for our SEM autoradiographic specimen. After development, the grids with the cells and developed emulsion were stuck onto SEM stubs, carbon coated, silver painted, and viewed in the SEM. By irradiating the emulsion from above, a uniform exposure is given to the entire emulsion layer, thus providing areas of irradiated emulsion both over and away from the tissue. Grain densities in the emulsion developed immediately or after 2 wk in contact with the muscle fibers indicated the extent of chemography. If developed immediately, a comparison between grain yield in the emulsion over the tissue and that in the emulsion away from the tissue also yielded information on any effect the muscle fibers may have on our ability to see and tabulate developed grains.

Results

Sensitivity in SEM Autoradiography

Our sensitivity studies were designed to compare the sensitivity in SEM autoradiography with that in TEM autoradiography. Factors of importance were: (*a*) the possible damage to grains in the SEM relative to the TEM; (*b*) the 300-Å-thick carbon layer over the cells and thus between the radioactive source and the emulsion. (This was expected to be most important when ^{125}I labeling was used, since the low energy electrons from ^{125}I are easily subject to self absorption [Fertuck and Salpeter, 1974; Salpeter et al., 1977]); and (*c*) the chemographic effect of the cells in contact with the emulsion.

We found the following. (*a*) Grain counting from the SEM autoradiograms shows grain densities equal to that from TEM autoradiograms. In 10 comparisons the ratio SEM/TEM grain counts were 1.03 ± 0.14. The grains were, however, very subject to beam damage in the SEM. For accurate counts of grains in SEM autoradiograms the tissue to be photographed should, therefore, not be in the beam during focusing or lengthy examination.

(*b*) The 300-Å carbon layer between the 1,000-Å ^{125}I-test specimen and the emulsion caused a loss in grain yield by a factor of $1.30 \pm .09$. The same factor was seen whether the effect of the 300-Å interposed layer was evaluated in the TEM or the SEM, since it was due to the self absorption of the low energy ^{125}I radiation and not a function of the SEM procedure. No similar effect was seen with tritium, which is as expected since with tritium no significant self absorptions should occur in a 1,000 + 300-Å layer (Salpeter, 1973). Since many specimens using surface-labeled cells would have a radioactive source considerably thinner than the 1,000-Å test specimen used above, we evaluated the effect of the 300-Å carbon layer when interposed between the 300-Å ^{125}I-test specimen and the emulsion. In this case the effect of the carbon layer was to decrease the grain yield by only a factor of $1.06 \pm .01$. This result is as expected from the earlier data on self absorption of ^{125}I as function of tissue thickness given in Fertuck and Salpeter (1974), and the findings of Junger and Bachmann (1980). Thus less than a 10% loss in sensitivity is expected when an ^{125}I-labeled cell surface is evaluated by the SEM autoradiographic procedure used here.

(*c*) There was no significant difference in grain counts between the irradiated emulsion in contact with the tissue and that not over the tissue when the emulsion was developed immediately after irradiation. In a few specimens we saw at most a 15% decrease in grain yield after 2 wk exposure to the tissue. We thus found that the 300-Å carbon layer was sufficient to prevent chemography during a 2-wk exposure time. In actual autoradiographic experiments we saw no increased chemographic effect with exposures up to 1 mo, which was our longest exposure time used. Without a carbon layer over the tissue negative chemography (loss of grains over the tissue) was extensive.

In summary, with ^{125}I, and the SEM procedure described here, the sensitivity is lower (*d* value higher) than with our standard TEM procedure by less than a factor of 1.3. This factor applies provided care is taken not to lose grains from the SEM specimen by excess beam exposure of the tissue.

Distribution of AChR around nmj

The sternomastoid muscle fibers are ~6–9 mm long and each endplate is ~40 × 20 μm (judged by esterase staining). The endplates form a relatively compact endplate band around the middle of the muscle, and endplate bands <2 mm wide can be easily disected freehand. The appearance of the isolated fibers is seen in Fig. 2. We see that the postjunctional area is somewhat smaller than usually measured after AChR staining.

In Fig. 3 a typical SEM autoradiogram, as was analyzed for this study, is illustrated. The emulsion over the nmj was allowed to become overexposed to give better statistics for the extrajunctional receptors, and grain counting was begun 2 μm beyond the edge of the endplate (defined by the extent of the folded region of the junction) to avoid the radiation spread for the heavily labeled endplate. We counted grains up to 250 μm away from the endplate, thus restricting this study to perijunctional and extrajunctional label well within the endplate band.

When autoradiograms were exposed for short periods (~6 h) to give a countable grain density over three endplates, we obtained an average site density of 20,817 sites/μm^2. This value is close to the average of 18,000 sites/μm^2 obtained by TEM autoradiography for both innervated and denervated sternomastoid muscles (Loring and Salpeter, 1980; Levitt-Gilmour and Salpeter, 1986). It is less accurate, however, due to the nonuniform surface at the top of the endplate and the unknown contribution from labeled receptors dipping down into the folds. These receptors are expected to increase the value obtained from the SEM autoradiograms, which is what we indeed obtained.

The AChR-binding site density distribution is given in Fig. 4. We see that between 2 and 7 μm from the edge of the nmj the site density in all three groups (innervated, 3-d denervated, and 10-d denervated) has already dropped considerably below the value of the 18,000 sites/μm^2 used for the postjunctional membrane in both innervated and denervated sternomastoid muscle (see above). Within the 7 μm from the endplate, the initial gradient is steepest for the innervated muscle in which the site density drops to an average of 150 sites/μm^2 (a decrease of almost 150-fold). After denervation the extrajunctional site density at 7 μm from the junction rises to ~300 sites/μm^2 in the 3-day denervate (50-fold

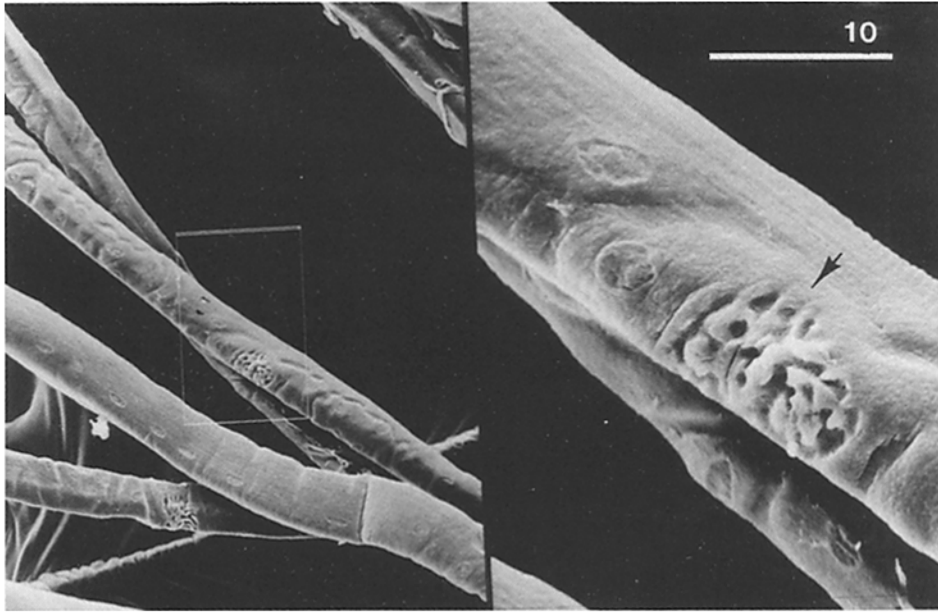

Figure 2. Isolated fibers similar to those used for autoradiography. Right frame is enlargement of marked rectangle in left frame. Arrow points to postjunctional area. Bar value is in micrometers.

lower than at the junction), and to ~625 sites/μm^2 in the 10-d denervate (30-fold lower than at the junction). With continued distance from the junction the gradients are much more shallow but the site densities decrease another factor of 2 or 3 by 25–50 μm for the endplate. Beyond that distance the shapes of the distributions differ significantly in the two denervated groups. In the 10-d denervated muscles, the site density essentially levels out beyond 50 μm to ~150–200 sites/μm^2. In the 3-d denervate, however, the extrajunctional site density is not constant even beyond 50 μm, decreasing significantly with distance. In the innervated muscle, the site density is almost level at <50 sites/μm^2, and drops to undetectable levels beyond 250 μm. The term perijunctional region is often used without a clear definition. Based on the shapes of the distribution in all three groups we suggest designating the first 25–50 μm from the endplate as perijunctional.

Since the statistical sampling with the SEM autoradiography is so good, we could compare individual fibers with each other. As reported previously (Loring and Salpeter, 1978) there is a considerable fiber to fiber variation in extrajunctional site density. Fig. 5 shows the range in fiber to fiber variation for the different groups by illustrating the site density distrubition seen in the fiber with the highest and lowest site density. We note that the overall range is greatest 3 d after denervation, overlapping both the control and the 10-d denervated values as if different fibers begin to respond at different times after denervation.

Discussion

Earlier studies have proposed SEM autoradiographic procedures (see for example, Paul et al., 1970; Hodges et al., 1974; Weiss, 1980; Junger and Bachmann, 1980). The only study of the above to apply the procedure in a quantitative manner is that of Junger and Bachmann (1980). The present study extends the calibration of a SEM autoradiographic technique and shows that it is applicable to describing the distribution of a cell surface receptor in relation to an identified morphological structure, namely the neuromuscular junction.

Sensitivity

In the present study, we used Ilford L4 emulsion developed with D-19. The Ilford emulsion is recommended because: (*a*) its high silver halide to gelatin ratio allows it to form good monolayers (or multiple layers) with tightly packed silver halide crystals, easily evaluated by its interference colors (Salpeter and Bachmann, 1964; Salpeter, 1981); (*b*) its low sensitivity to gamma radiation (Fertuck and Salpeter, 1974; Junger and Bachmann, 1980) makes it ideally suited to optimize the resolution when used with ^{125}I (Salpeter et al., 1977).

Development with D-19 is recommended because: (*a*) the developed grains are large enough (~0.25–0.5 μm) to be seen easily and counted at 2,000–3,000× magnification in the SEM; (*b*) the sensitivity with D-19 shows little dose dependence and is as good as that obtained with gold latensification Elon ascorbic acid. (Salpeter and Szabo, 1972).

The sensitivity obtained with the SEM procedure is at most ~1.3 times poorer than that with the TEM technique when using ^{125}I, and is the same as with the TEM technique when using ^{3}H. Higher energy isotopes are expected to have the same relation to the TEM value as is seen with tritium.

Protecting against chemography is important. Unembedded tissues and tissues heavily impregnated with OsO_4 have strong chemographic effects (Salpeter, M., unpublished observations). However, earlier studies have shown that carbon layers considerably thinner than 300 Å easily eliminate chemography in the standard TEM technique with embedded tissue (Bachmann and Salpeter, 1965; Salpeter, 1981). In the present study 300 Å essentially eliminates chemography in the SEM tissue. Since 300 Å of carbon was necessary over the tissue to prevent specimen charging in the beam, we did

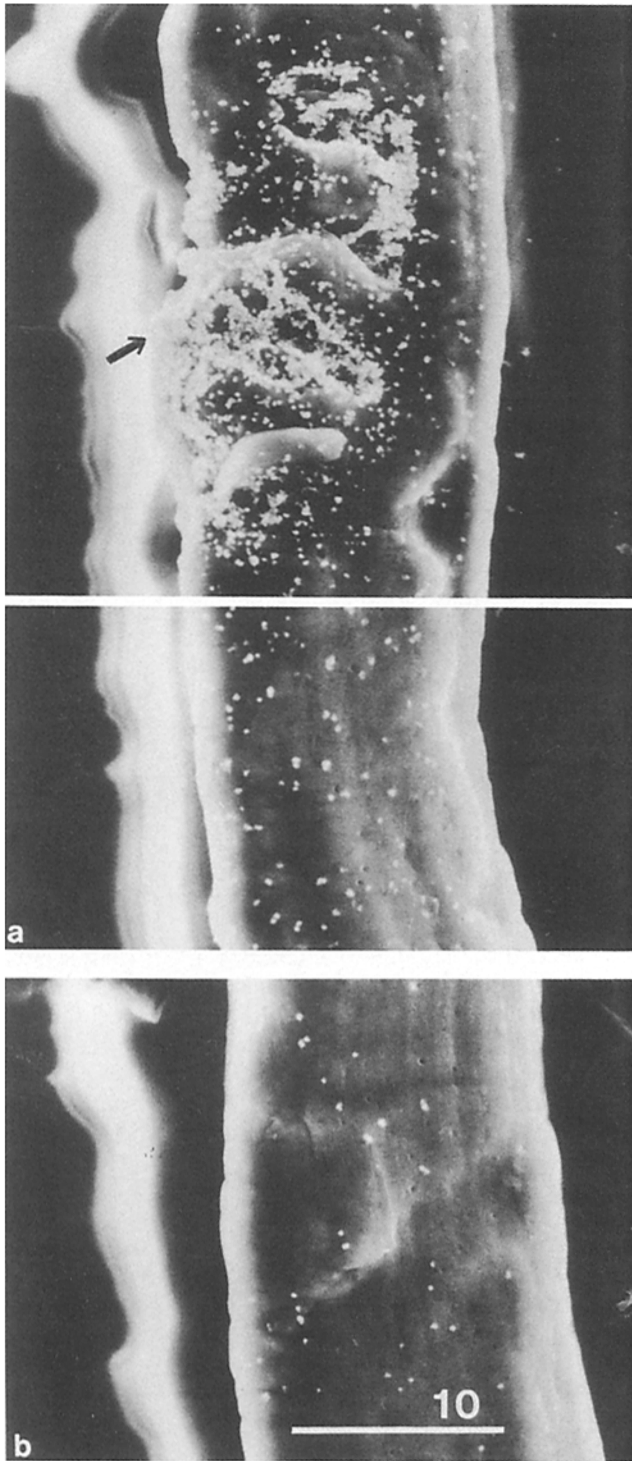

Figure 3. SEM autoradiogram of muscle fiber. (*a*) Overexposed endplate (*arrow*) and extrajunctional membrane up to 30 μm from edge of junction. (*b*) The same fiber between 80 and 120 μm from the junction. Bar value is in micrometers.

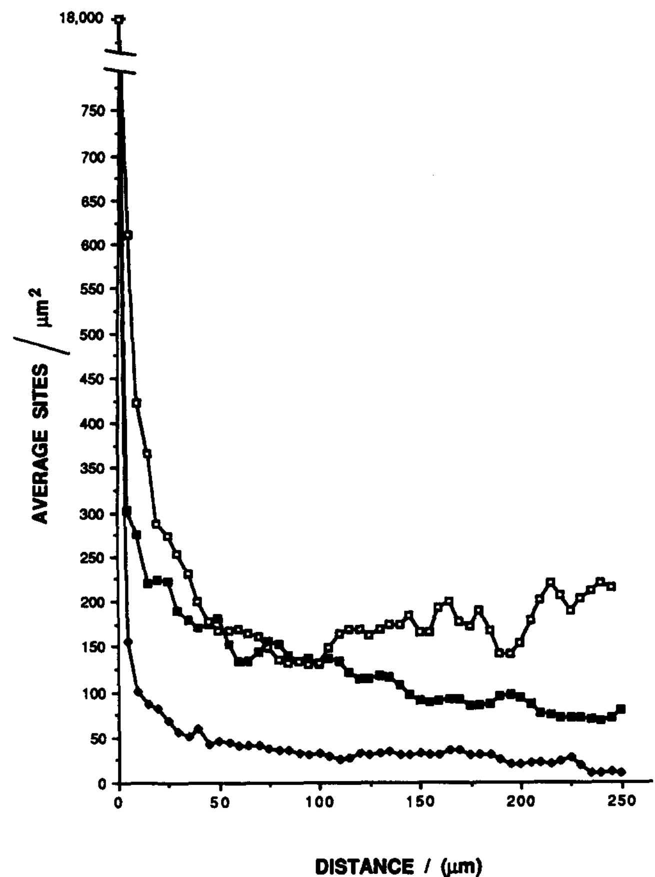

Figure 4. Histogram of grain density seen for three groups. Grains were counted only over the flat top surface of the muscle going in either direction from the endplate. The two sides were subsequently pooled. AChR-binding site density was calculated from the grain densities using the equation as previously described (Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1978). (●) Control; (■) 3-d denervates; (□) 10-d denervates. Data was averaged over 5-μm distance steps and then smoothed using the Hanning formula: $(\frac{n}{2}) + (\frac{n-1}{4}) + (\frac{n+1}{4})$. The site density of 18,000 sites/μm² at the edge of the endplate (0 distance) was derived from TEM autoradiography and combined in low exposure SEM autoradiograms. Extrajunctional values start between 2 and 7 μm from the endplate edge. Data based on 7 control, 13 3-d, and 4 10-d fibers.

not test to see if chemography would also be controlled by thinner carbon, as it may well be. If chemography is found to be a problem even with 300 Å of carbon under different conditions, it could be further controlled by thicker carbon layers or possibly a collodion layer between the cell and emulsion. This would however be at the expense of sensitivity due to greater self absorption, especially with ^{125}I, which is the most common label for cell-surface receptors.

Procedure for Routine Calibration of Sensitivity

In our laboratory, the standard procedure for determining *d* values (see Materials and Methods) in any quantitative studies is to have a tritium test specimen. Due to the long half-life >12 yr, only small corrections for decay need to be introduced with repeated use over several years. Each new batch of emulsion is tested by TEM using that tritium test specimen, and a test is performed before each experiment to be sure nothing has gone wrong. A correction factor for the *d* value labeled with tritium can then be used to apply to different conditions or isotopes. For example, Fertuck and Salpeter (1974) have shown that the TEM *d* value for ^{125}I is

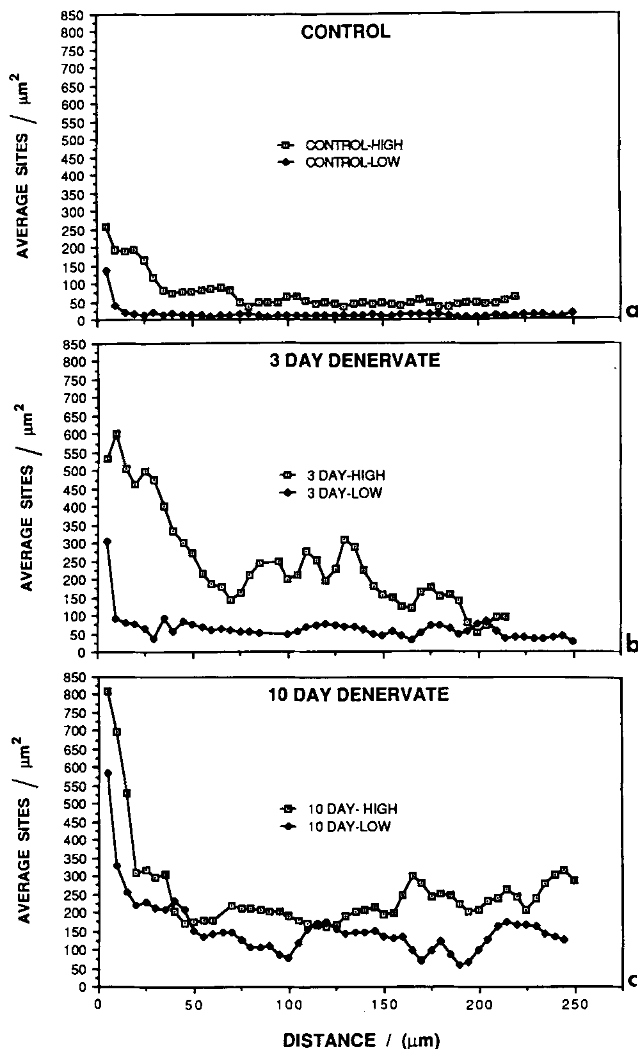

Figure 5. Three frames showing the AChR site density distributions found on the individual fibers with the highest and lowest average values for the control (*top*), 3-d denervated (*center*), and 10-d denervated (*bottom*) muscles. Note 3 d after denervation (*center*) the fiber to fiber spread in site densities is greatest, overlapping both the control and 10-d denervated values, as if there is an asynchronous response to denervation. The 3-d denervate also illustrates the developing gradient in site density within 200 μm from the nmj.

consistently about half that for tritium, even though there can be variations between batches of emulsion. A 0.5 times correction factor can then be used to give the *d* value needed for quantitation with the ^{125}I-labeled sample and TEM autoradiography. In the present study we obtained the correction value applicable for the SEM specimen. We multiply the TEM tritium value by 0.65 to be applicable to the SEM procedure using ^{125}I. For other isotopes or other conditions expected to effect sensitivity, such a correction factor needs to be determined only once to be generally applicable.

Resolution

Previous studies show that with monolayers of Ilford L4 emulsion. TEM autoradiographic resolution values using ^{3}H and ^{14}C are applicable to the light microscope autoradiographic specimen (Salpeter et al., 1974). They must, therefore, also apply to the SEM specimen, as must the published values of ^{125}I (Salpeter et al., 1977). The fine structural resolution will of course be better with the TEM. Since geometric factors are the main cause for poor resolution when higher energy isotopes are used (Salpeter et al., 1977, 1988), resolution should be excellent with surface receptors labeled with any isotope because they represent a very thin specimen. The resolution would then be limited only by the emulsion thickness, the size of the silver halide crystal of the emulsion, and the size of the developed grain which have all been established for L4 (Ilford) and D-19 development (Salpeter et al., 1977).

ACh Receptor Distribution Relative to the nmj

By calibrating the procedure for absolute sensitivity the AChR site density was calculated. Our basic finding is that the general shape (if not the absolute site densities) of the AChR distribution around the endplate is similar in innervated and denervated muscle after a steady state has been established. The shapes of the distributions, 3 and 10 d after denervation, between 25 and 250 μm from the endplate, suggest that the increase in extrajunctional receptors after denervation develops initially near the junction. Thus although it has been unequivocably established that extrajunctional receptors result from de novo synthesis, and that there is no physical spread of receptors from the junction (see review in Fambrough, 1979), a spread of sensitivity (defined physiologically) was reported by Axelson and Thesheff (1959) could occur if soon after denervation the postdenervation newly synthesized receptors are preferentially inserted or retained near the junction.

Whenever the absolute site densities in our study can be compared with that reported in the literature, we find that the data are similar both in innervated and denervated muscle. In innervated muscle the observation that there is a very steep gradient in the first few micrometers was described by Peper and McMahan (1972), Kuffler and Yoshikami (1975), Fertuck and Salpeter (1976), and Bekoff and Betz (1977). The low level of extrajunctional receptors up to 100 or 200 μm away from the endplate, beyond which no receptors are detectable, was also described either on physiological or autoradiographic grounds by Miledi (1960), Albuquerque and McIsaac (1970), Bekoff and Betz (1977), and Steinbach (1981).

In denervated muscle extrajunctional AChR distributions reported in the literature are more variable and may depend on the muscle and the technique used. The claim that an extrajunctional gradient does not exist after denervation (see for example, Fambrough, 1974; Steinbach, 1981; Hartzell and Fambrough, 1972) may be due to the time after denervation and the region of muscle sampled. The actual receptor site densities given here are however in line with those reported by others. Our values obtained beyond 50 μm from the endplate, range, on the average, from ~100 sites/μm² for 3-d denervates to ~200 sites/μm² 10 d after denervation. These values are consistent with those given by different techniques in studies using rodent extensor digitorium longus, soleus, or sternomastoid muscles (Pestronk et al., 1976; Change et al., 1975; Levitt-Gilmour and Salpeter, 1986; Loring and Salpeter, 1978). These above mentioned studies report 150–180 sites/μm² 4–5 d after denervation,

and 200–370 sites/μm^2 7 and 8 d after denervation. Higher values (~500 sites/μm^2) given by Fambrough (1974) for rat diaphragm 14 d after denervation may reflect differences in technique or a continued rise in site density with time after denervation.

The advantage of SEM autoradiography is that it overcomes many problems inherent in other techniques for assessing the distribution of surface receptors. It has better resolution than the physiological, biochemical, or the light autoradiography techniques, and better statistical sampling than the TEM autoradiographic procedures since we can easily count several thousand grains per muscle fiber. With the SEM technique it is also possible to visualize the intact structure over long distances and relate it to a morphologically identifiable landmark. The SEM procedure could thus assess the site densities within the perijunctional region (defined as the region just beyond the ridges characteristic of the junctional folds) to ~25 or 50 μm from the junction, as well as identify the continuous shallow gradient seen early (3 d) after denervation. Such shallow gradients are difficult to demonstrate by the other procedures even when suspected (see for example, Levitt-Gilmour and Salpeter, 1986, for an attempt using TEM, an effort that was very time consuming and, nevertheless, incomplete). Modifications such as dry emulsion (Harris and Salpeter, 1983; Salpeter et al., 1987), labeling tissue after dissociation, and rapid fixing (cross-linking of probe or freezing) should make the SEM technique applicable to reversible probes.

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