Acetylcholine Receptors in Extrajunctional Regions of Innervated Muscle Have a Slow Degradation Rate

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Scanning EM autoradiography was used to determine the degradation rate of extrajunctional ACh receptors (AChRs) in innervated sternomastoid muscles of the mouse. We report that in innervated muscles, extrajunctional AChRs have a slow degradation rate ($t_{1/2}$, ~8 d), similar to that seen at the neuromuscular junction. We conclude that slowly degrading AChRs (R_s) need not be localized at the specialized structure of the nerve–muscle junction. Degradation of extrajunctional as well as junctional AChRs may depend primarily on the state of innervation of the muscle.

It has been suggested that the degradation rate of ACh receptor (AChR) molecules at the neuromuscular junction (nmj) of adult muscle depends in part on whether the AChRs were synthesized in innervated or denervated muscle (Shyng and Salpeter, 1990). Whereas junctional AChRs synthesized in innervated muscle have a degradation $t_{1/2}$ of ~8–10 d (which accelerates to one of ~2–4 d after denervation) (Levitt and Salpeter, 1981; Stanley and Drachman, 1981; Brett et al., 1982; Bevan and Steinbach, 1983), AChRs synthesized after denervation degrade with a $t_{1/2}$ of ~1 d not only at extrajunctional regions (literature reviewed by Fambrough, 1979), but also at the nmj (Levitt and Salpeter, 1981; Shyng and Salpeter, 1989, 1990; Shyng et al., 1991). AChR degradation may thus depend more on when the AChR is synthesized than on where it is localized. To test the extent of this conclusion, we examined the degradation rate of extrajunctional AChRs in innervated muscle.

We report that in mouse innervated sternomastoid muscle, extrajunctional AChRs degrade with an average measured half-life of 7–9 d, similar to that of the AChRs at the innervated junction. These results show that AChRs with a slow degradation rate (called R_s AChRs) can be located throughout the extrajunctional muscle membrane even where the AChR binding site density has dropped to <8 sites/μm^2 (<4 AChR molecules/μm^2). It was previously shown that R_s AChRs are not necessarily localized at a high AChR site density (Salpeter and Harris, 1983). The present study extends this finding to even lower site densities and also shows that R_s AChRs need not be restricted to the morphologically specialized region of the nmj. These results add to the mounting evidence derived also from studies on AChR channel kinetics (Brehm and Kullberg, 1987; Henderson et al., 1987; Brehm and Henderson, 1988) indicating that the distinction between junctional and extrajunctional AChRs must be reconsidered.

Materials and Methods

Specific labeling of AChRs. AChRs in innervated mouse sternomastoid muscles were labeled with ^{125}I-α-bungarotoxin (^{125}I-α-BGT) (3 μM) by topical application for 1.5 hr, as previously described (Fertuck et al., 1975). Atropine was administered (1.8 mg/kg) as needed during the operation to eliminate respiratory distress. The extent of saturation during a 1.5 hr incubation period was previously judged to be >95% (Loring and Salpeter, 1980; Shyng and Salpeter, 1989). Nonspecific binding, obtained by preincubating muscles with nonradioactive BGT for 1.5 hr followed by ^{125}I-α-BGT for the same time, was subtracted from total counts to get specific binding.

Determination of distribution and degradation of extrajunctional AChRs. AChR site densities and degradation rates were assessed using scanning EM (ScEM) autoradiography, since the more usual procedure of gamma counting (e.g., Berg and Hall, 1975; Levitt and Salpeter, 1981) could not unequivocally distinguish extrajunctional from junctional AChRs in innervated muscles, nor accurately detect very low levels of extrajunctional AChRs. For ScEM autoradiography, the procedure of Salpeter et al. (1988) was followed. At various times (3 hr, 1, 8, and 16 d) after labeling with ^{125}I-α-BGT, animals were anesthetized and killed by intracardial perfusion with 2.5% glutaraldehyde in phosphate buffer (0.067 M, pH 7.4). The labeled muscles were dissected from the animals, postfixed in glutaraldehyde (2.5% in phosphate buffer for 1 hr), and dissociated with the HCl-OsO$_4$ method of Desaki and Uehara (1981). The dissociated fibers were placed onto ScEM stubs over double-sided scotch tape, carbon coated, and coated with stripped preformed monolayers of emulsion (Ilford L4). After exposure times ranging from 30 to 60 d, they were developed for 2 min with D19 at room temperature. To avoid overexposing the emulsion and yet have sufficient grains for adequate statistical sampling, the exposure times were determined to give 0.1–0.8 grain per μm^2 of surface area in extrajunctional regions. Grain counts, obtained as described below, were used to calculate AChR density as previously described (Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1978). Values were expressed either as AChR site density (defined as the number of α-BGT or ACh binding sites/μm^2) or as AChR molecules, which is half the site density.

Extrajunctional AChR values were obtained using two different methods. In the first method, fibers from all time points were fully scanned and neuromuscular junctions (nmjs) were located by their unique morphology. The muscle fibers were then photographed (at 2000× magnification) and contiguous montages were made up to 130 μm from each junction. For each time point, micrographs were obtained from two to five fibers from each of two to four animals. Grains were counted from prints, starting 4.5 μm from the visually identified morphological edge of an nmj. [When averaging site densities over large distances along the muscle fiber, the effect of the density gradient from the nmj (Salpeter et al., 1988) was prevented from biasing the standard error of the mean (SEM) values as follows: average site densities were first obtained for each consecutive 5 μm distance bin, and the SEM value for that distance bin was expressed as a percentage of the average. All the individual bins and their percentage SEM values were then averaged over the full distance, and the averaged SEM expressed in absolute units of site density.]
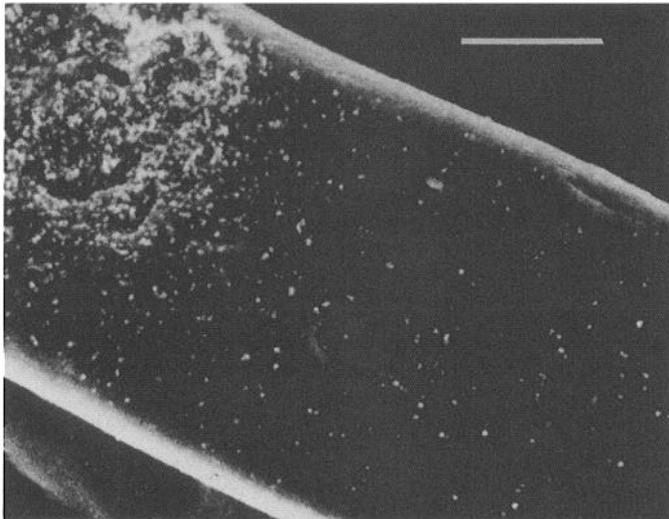
Figure 1. Sample ScEM autoradiogram with highly overexposed nmj (*upper left*) gives easy grain counting over nonjunctional regions. (The specific activity of BGT was 78 C/mmol on day of emulsion coating, and the exposure time was 58 d.) Note the very steep gradient in the vicinity (especially within 10 μm) of the edge of the nmj. Magnification, 1900×; scale bar, 10 μm.
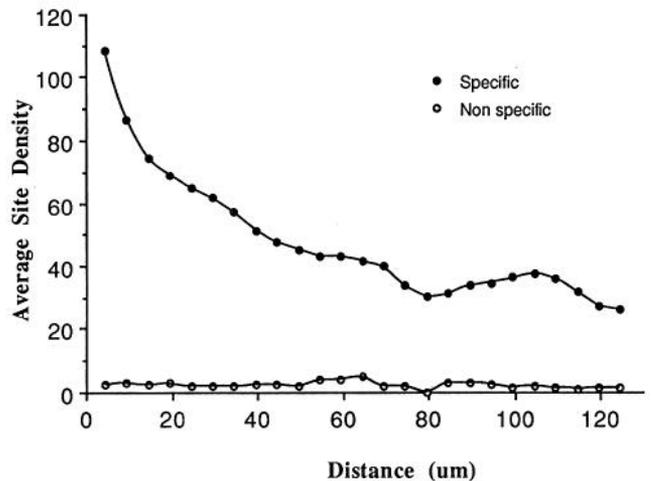

Figure 2. AChR binding site density with distance from edge of nmj. Experimental data were taken from muscles fixed 3 hr after labeling (to be called day 0). Nonspecific binding (O) (obtained as described in Materials and Methods) is given by the *lower curve* and is subtracted from the total counts to give the *upper curve* of specific label (●). Grains were counted in bins (of 5 × 5 μm) starting 4.5 μm from the edge of the nmj. Data were smoothed as previously described (Salpeter et al., 1988). Beyond the 120 μm shown here (where the site density is ~30 sites/μm²), the average random extrajunctional site density obtained from photographs of muscle segments without nmjs is ~7 sites/μm².

In the second method, AChRs of random extrajunctional regions were obtained by photographing muscle fiber fragments without nmjs. Ten fibers from each of two animals were analyzed for 1 and 16 d after labeling. To avoid needing a correction for muscle fiber curvature, grain counts in both methods were restricted to the top surface of a muscle fiber using an overlay counting grid with grid squares of 5 × 5 μm. Micrographs were also taken of emulsion areas near muscle fibers for background corrections.

The measured degradation rate of the AChRs was determined by the rate of decrease in the labeled AChR site density assuming first-order exponential decays. Degradation was measured from day 1 to day 16 after labeling. The first day was omitted to avoid the frequently reported effect of a rapidly degrading component seen during this early period even at the innervated neuromuscular junction (Stanley and Drachman, 1981; Bevan and Steinbach, 1983).

Results

Figure 1 shows a sample ScEM autoradiogram used to determine the extrajunctional AChR degradation rate. Figure 2 shows that very little nonspecific binding is present and that the specific AChR sites (defined as the number of α-BGT or ACh binding sites/μm²) have a distribution as previously described (Salpeter et al., 1988), with a sharp drop-off away from the nmj, followed by a very gradual decrease in density. The general shape of the grain density distribution, however, remained the same with time after labeling as the AChRs decayed (data not shown). We define the region up to ~50 μm from the edge of the nmj as perijunctional, since this distance delineates the region that has a very steep site-density gradient. Within 50 μm from the nmj, the AChR site density drops from the 18,000 sites/μm², characteristic for the nmj, to ~40 sites/μm² (see also Salpeter et al., 1988). As can be seen in Figures 1 and 2, most of this drop occurs in a rim of ~10 μm from the nmj. Beyond 50 μm from the nmj, the site density decreases more slowly from 40 to <30 sites/μm² by 120 μm. In the area between 54 and 130 μm, the average AChR site density is about 33 sites/μm², which allowed for easy analysis of degradation rates (see Fig. 3). Beyond 200 μm, the AChR site density drops to very low levels (Salpeter et al., 1988). To establish how characteristic our degradation re-

sults from the extrajunctional AChRs within 54–130 μm were, we also determined the site density and degradation of randomly photographed fiber segments and found this random extrajunctional AChR density to be ~7 sites (or 4 AChR molecules)/μm². In a mouse sternomastoid muscle fiber of ~0.8 mm length and 20–30 μm diameter, these randomly distributed extrajunctional AChRs represent ~2.2 × 10⁵ AChR molecules compared to about 10⁵ extrajunctional AChR molecules within 54–130 μm from the nmj. Since there are ~2.6 × 10⁷ AChR molecules at the nmj of that muscle [obtained by multiplying 2900 μm² of dense postjunctional membrane (Matthews-Bellinger and Salpeter, 1978) by 9000 AChR molecules/μm²], total extrajunctional AChRs constitute just over 1% of AChRs in innervated sternomastoid muscle, most of which are at very low density distributed randomly over the muscle fiber.

The degradation half-life for extrajunctional AChRs averaged over the area from 54 to 130 μm from the junction measured 9 d (Fig. 3), and that for the randomly distributed extrajunctional AChRs measured 7 d. Both degradation $t_{1/2}$ values are within the range previously reported for the R_s AChRs at innervated nmjs (for reviews, see Fambrough, 1979; Salpeter 1987b) and are not significantly different by F test (performed as previously described; Shyng and Salpeter, 1990).

Discussion

ScEM autoradiograms of innervated sternomastoid muscles labeled with ^{125}I-α-BGT show developed grains in significant numbers (giving >20 sites/μm²) up to ~130 μm from the nmj. The distances from the nmj over which these developed grains are seen is much larger than the 0.1 μm resolution half-distance characteristic for ^{125}I under our experimental conditions (Salpeter et al., 1977) and could therefore not be due to radiation spread from the nmj. Furthermore, the fact that the shape of

the grain distribution did not change with time after labeling signifies that the extrajunctional developed grains could not be due to AChR diffusion from the nmj. The extrajunctional grains, even those in the gradient around the nmj, must therefore reflect AChRs inserted within the extrajunctional membrane.

This study shows that in innervated mouse sternomastoid muscle, extrajunctional AChRs degrade with an average $t_{1/2}$ of ~8 d like junctional AChRs do. Since between 54 and 130 μm from the nmj the average AChR site density was about 33 sites/μm^2 and dropped to an average of ~7 sites/μm^2 over randomly selected extrajunctional areas, we conclude that, as is the case for AChRs at the lower regions of the junctional folds (Salpeter and Harris, 1983), slow degradation does not require high AChR site density. Primarily, however, this study shows that slowly degrading AChRs are not restricted to the postsynaptic membrane of the nerve–muscle contact area. Thus, unlike the conclusion of Rotzler and Brenner (1990) for rat soleus muscle, AChRs do not necessarily require some feature unique to the morphologically defined nmj to acquire or maintain a stable degradation rate. The degradation of extrajunctional as well as junctional AChRs may indeed depend on the state of innervation of the muscle, and not necessarily on either their location or site density.

Our study does not address the question of whether any rapidly degrading (R_r) AChRs are also expressed at some low concentration in innervated muscles. Such a population has been reported at the innervated nmj (Bevan and Steinbach, 1983; Stanley and Drachman, 1983) and was also seen in the present study, as can be seen by comparing Figures 2 and 3. The labeled site density within 54–130 μm from the nmj decays from about 33 to about 22 sites/μm^2 between day 0 and day 1 after labeling. It is also possible that a small percentage of R_r AChRs may account for the slightly shorter measured half-life for random extrajunctional AChRs than for those closer to the nmj. These aspects need further investigation. They do not detract, however, from the finding that in innervated mouse sternomastoid muscle the majority of extrajunctional as well as junctional AChRs are slowly degrading (R_s) AChRs.

The mechanism whereby innervation causes a switch to occur from R_r to R_s AChRs is not yet known, nor whether it can involve a stabilization of the R_r AChRs. Shyng and Salpeter (1990) suggest that the switch from R_r to R_s occurs after innervation by a downregulation of R_r synthesis and a replacement of the R_r AChRs by R_s AChRs. The possibility that there are two populations of AChRs with different degradation properties, whose synthesis depends on the state of innervation, is reminiscent of the presence of fetal (γ-containing) and adult (ϵ-containing) AChRs differing in channel conductance (Michina et al., 1986; Witzemann et al., 1987; Gu and Hall, 1988). It is of interest that the adult or high-conductance AChRs are also not restricted to the nmj (Brehm and Kullberg, 1987; Henderson et al., 1987; Brehm and Henderson, 1988), similarly arguing for the need to redefine the concept of junctional versus extrajunctional AChRs. It seems that the terms junctional and extrajunctional AChRs need to be restricted to morphological entities, as they do not appear to include inherent functional distinctions.

Although under steady state conditions [as in the present study for innervated muscle and in Shyng and Salpeter (1989, 1990) for chronically denervated muscles] AChRs may have the same degradation rate in the extrajunctional as in the junctional regions, this may not be the case immediately after denervation of adult
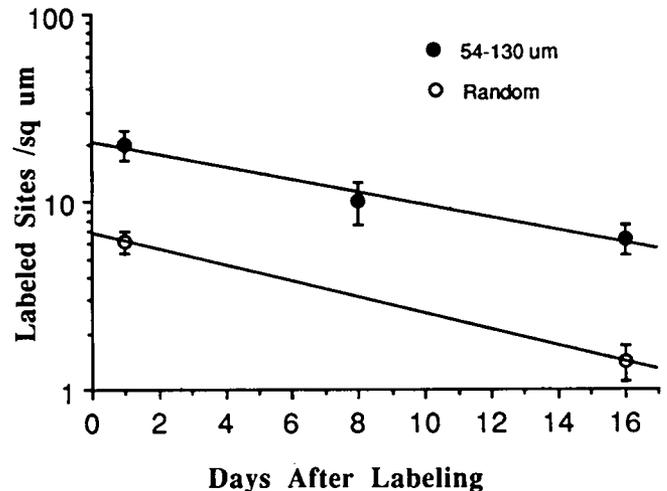

Figure 3. AChR degradation obtained for extrajunctional AChRs averaged over a distance from about 54–130 μm from the edge of the nmj (*top curve*, ●) and at random distances from the nmj (*bottom curve*, O). AChRs were labeled on day 0, and the number of labeled AChR sites/μm^2 of muscle surface remaining between 1 d and 16 d thereafter was tabulated. The data were fit by linear regression to a single exponential. The $t_{1/2}$ values were calculated from the slopes and were 9 d for the AChRs within 54–130 μm from the nmj and 7 d for the random AChRs on fiber segments without nmjs.

muscle or after innervation during development. For instance, in mouse sternomastoid muscle, R_r AChRs begin to accumulate in extrajunctional regions within 3–4 d after denervation (see review by Salpeter, 1987b), but the degradation rate of the junctional AChRs at the denervated nmj has not yet increased (Levitt et al., 1980). Since the absolute AChR site density at the nmj remains relatively constant after denervation (Frank et al., 1975; Loring and Salpeter, 1980; Bader, 1981; Levitt-Gilmour and Salpeter, 1986; Salpeter, 1987a), this delay after denervation could depend on how quickly the R_s population of AChR can degrade and be replaced by the R_r AChRs. Of greater interest is a second situation, illustrated in the report by Steinbach et al. (1979), who show that during development the junctional AChRs begin to degrade slowly while extrajunctional AChRs are still degrading rapidly. This delay in the downregulation of R_r AChRs at extrajunctional regions while R_s AChRs already predominate at the nmj suggests that these two populations of AChRs can be maintained simultaneously in different regions of the muscle. The periods of synaptogenesis or denervation and reinnervation thus provide windows of time for studying the regulation of both R_r and R_s AChRs. Similarly, the fact that in innervated muscle there are extrajunctional R_s AChRs at densities of >30 sites/μm^2 for at least 120 μm beyond the nmj (present study) provides a nonjunctional region of muscle in which many slowly degrading AChRs are either synthesized or stabilized and anchored in the membrane. These windows in time and space provide useful constraints in the study of the mechanisms determining AChR turnover and should allow us to determine the characteristics that are unique for the R_s and R_r AChRs.

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