Degradation Rate of Acetylcholine Receptors Inserted into Denervated Vertebrate Neuromuscular Junctions

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Abstract. Many studies exist on the effect of denervation on the degradation of acetylcholine receptors (AChRs) at the vertebrate neuromuscular junction (nmj). These studies have described the behavior of either the total population of junctional receptors at different times after denervation, or of the receptors present at the time of denervation (referred to as original receptors). No experimental studies yet exist on the degradation rate of the receptors newly inserted into denervated junctions. In the previous studies, the original receptors of mouse sternomastoid muscles were found to retain the slow degradation ($t_{1/2}$) of ~8-10 d of innervated junctional receptors for up to 10 d after denervation before accelerating to a $t_{1/2}$ of ~3 d. The total junctional receptors, on the other hand, showed a progressive increase in degradation rate from a $t_{1/2}$ of 8-10 d to a $t_{1/2}$ of 1 d.

To reconcile these earlier observations, the present study examines the degradation of new receptors inserted into the nmj after denervation. To avoid possible contamination of the data with postdenervation extrajunctional receptors, we used transmission electron microscope autoradiography to study only receptors located at the postjunctional folds of the nmj. We established that the new receptors inserted into denervated junctions have a $t_{1/2}$ of ~1 d, considerably faster than that of the original receptors and equivalent to that of postdenervation extrajunctional receptors. Both original and new receptors are interspersed at the top of the junctional folds. Thus, until all the original receptors are degraded, the postjunctional membrane contains two populations of AChRs that maintain a total steady-state site density but degrade at different rates. The progressive increase in turnover rate of total AChRs therefore reflects the combined rates of the original and new receptors, as earlier postulated by Levitt and Salpeter (1981).
Garotoxin and the degradation rate was measured directly. Receptors were saturated by topical application of 125I-α-bungarotoxin and innervated junctional AChRs after intraperitoneal injection of the nonradioactive α-BGT, causing a negligible effect (Bevan and Steinbach, 1983; Salpeter et al., 1986).

Materials and Methods

Determination of Degradation Rates

To eliminate contamination of the data with postdenervation extrajunctional AChRs, all analyses were done using TEM autoradiography to study only receptors located at the postjunctional folds. Deeply anesthetized animals were killed by intracardial perfusion with 4% paraformaldehyde in phosphate buffer (0.067 M, pH 7.4) at various times after radioactive labeling of new receptors. The denervated sternomastoid muscles were removed and stained for acetylcholinesterase (Karnovsky and Roots, 1964) to identify the endplate band.

Endplate band tissue was then dissected out and postfixed in OsO4, stained with uranyl acetate, and processed for TEM autoradiography using the flat substrate procedure of Salpeter and Bachmann (1964; see Fertuck and Salpeter, 1976; Salpeter, 1981). The α-BGT binding site density was determined specifically at the postjunctional membrane of the nmjs (as described by Fertuck and Salpeter, 1978; or Matthews-Bellinger and Salpeter, 1978) and the degradation rate assessed by the rate of decrease of this site density. Loss of radioactivity after labeling with 125I-α-BGT has been shown to reflect degradation of receptors (see review by Famorphou, 1979) and the degradation rate assessed by the rate of decrease of this site density. Loss of radioactivity after labeling with 125I-α-BGT has been shown to reflect degradation of receptors (see reviews by Famorphou, 1979).

Results

Control experiments for cold saturation showed that ~98% of the junctional receptors were inactivated with the nonradioactive α-BGT (Table I) as was also seen in previous studies using this procedure (Loring and Salpeter, 1980). Furthermore, fine structure studies established that the ligation of the nerve successfully prevented reinnervation during the time course of the degradation curves. Of a total of 250 randomly chosen endplates from 22 animals at both 6 and 14 d after denervation not one showed any preterminal nerve fibers. By Poisson statistics, one can calculate that the probability of even a 2% innervation is less than 0.01.

TEM autoradiographs (Fig. 1) showed that the labeled new receptors are distributed throughout the top of the folds. This relatively uniform distribution is maintained throughout the degradation period. A similar distribution during degradation has been seen for the original receptors (data not shown). These results indicate that no preferential localization for the new receptors relative to the original receptors can be discerned at the level of resolution of the electron microscope, these distortions do not affect TEM autoradiographic results.

Fig. 2 shows the degradation curve of new receptors. We chose to determine the degradation rate for new receptors at 6 and 14 d after denervation, because at 6 d the original receptors in the sternomastoid endplate are degrading with a t1/2 of ~8 d, and at 14 d the t1/2 has decreased to ~3 d (Levitt...
Figure 1. EM autoradiograph showing the relatively uniform distribution of newly inserted AChRs at the top of the junctional folds in a denervated endplate. Original receptors were saturated with nonradioactive α-BGT at the time that the nerve was cut. 6 d later, new receptors were labeled with ¹²⁵I-α-BGT. Since a similar distribution is seen for the localization of original receptors at all times during degradation (data not shown), this distribution profile indicates that a preferential localization for the new receptors is not discernible within the resolution of the TEM autoradiographic technique. Sch, Schwann cells; JF, junctional folds; and M, muscle. Bar, 1 μm.

Figure 2. Insertion and degradation of new AChRs at denervated nmj. Original receptors were saturated with nonradioactive α-BGT. New receptors were labeled with ¹²⁵I-α-BGT 6 or 14 d after nerve cut. Residual label is plotted as binding site density (α-BGT binding sites/μm² of thickened postsynaptic membrane) measured by TEM autoradiography at different times after labeling. Three curves are shown. a gives calculated number of new receptors expected to be accumulated at the nmj at different times after denervation. (Calculation assumes that the site density at the nmj is 18,000 sites/μm² at the time of denervation and that the original receptors degrade with a t₁/₂ of 8 d during the first 9 d of denervation and with a t₁/₂ of 2.5 d thereafter.) b gives the degradation of new receptors labeled at 6 d after denervation. c gives the degradation of new receptors labeled at 14 d after denervation. Each point is the averaged site density from two to five animals, and curves are fitted by linear regression. Error bars are standard error of the mean when the sample size is greater than two. In cases where the sample size is equal to two, the error bar represents the range of the two values. Degradation t₁/₂ of new receptors was calculated to be ≈1 d (see text).

Discussion

It has been established by several studies that metabolically stable AChRs, present at the nmj before denervation (original receptors), retain their stability for some time after denervation but then their degradation accelerates to a t₁/₂ of ≈3 d (see reviews Salpeter and Loring, 1985; Salpeter, 1987). We therefore asked whether the new receptors also showed a different behavior at these two time periods.

Interestingly, there often is little or no degradation of receptors during the first day (i.e., between 2 h and 1 d) after labeling (see also Loring and Salpeter, 1980; Salpeter and Harris, 1983). The reason for this phenomenon is unclear, but may represent a period of shock after the operating procedure involved in saturating the receptors with α-BGT. In assigning the half-life of the receptors we therefore report two values both obtained by linear regression: the first based on all the time points and the second only on those beginning 1 d after labeling. By both tallies, newly inserted receptors labeled either 6 or 14 d after denervation degrade with a t₁/₂ of ≈1 d, similar to the t₁/₂ of embryonic and denervation-induced extrajunctional receptors (see Salpeter, 1987 for review). With all time points included, the t₁/₂ values are 1.07 ± 0.25 d and 1.57 ± 0.31 d for 6 and 14 d, respectively. If only times after day 1 are included, as we believe to be more valid, they are 0.94 ± 0.27 and 1.1 ± 0.16 d, respectively.
The present study establishes (a) that the new receptors inserted into denervated nms are located at the top of the junctional folds (as are innervated receptors) and thus interspersed with the original receptors; and (b) that these new receptors degrade with a $t_{1/2}$ of 1 d, equal to embryonic receptors and to extrajunctional receptors that develop after denervation.

The degradation rate of new receptors was earlier postulated by Levitt and Salpeter (1981) based on data obtained by a gamma counting technique. However, after the study of Levitt and Salpeter (1981) was published, we found two sets of potential complications which could have affected their calculations. The first complication could arise from the use of the gamma counting technique to determine the specific junctional label. In this technique, the muscle is divided into three pieces, one containing the endplate band. The radioactivity bound to pieces without endplates is subtracted from the radioactivity of the piece containing endplates on a per weight basis. This subtraction assumes a uniform distribution of extrajunctional label. Thus any extrajunctional receptors in a gradient around the neuromuscular junctions within the endplate band will be included in the specific junctional label. In innervated muscles this is not a problem since the extrajunctional receptors which are distributed in a very steep perijunctional gradient (Fertuck and Salpeter, 1976; Salpeter et al., 1988) have a turnover rate equal to that of the junctional receptors (Salpeter, M. M., manuscript in preparation). In denervated muscles however, the level of extrajunctional receptors increases and, especially early after denervation, distribute over a long distance in a shallow gradient (Salpeter et al., 1988) with higher density in the tissue containing the endplate band than in that without the endplate band (Levitt-Gilmour and Salpeter, 1986). The endplate-specific counts by gamma counting will therefore include counts from the elevated extrajunctional receptors in the endplate band-containing tissue. This could distort the value obtained for the degradation rate of junctional receptors if the true degradation rate of the junctional receptors is different from that of the extrajunctional receptors.

The second complication could arise from the receptor labeling procedure. In the study by Levitt and Salpeter (1981), receptors were labeled by injecting $^{125}$I-$\alpha$-BGT intraperitoneally. In preliminary experiments, while preparing for the present study, we found by TEM autoradiography that this procedure results in a two-to-threefold higher label at innervated than at denervated junctions. However, when receptors were labeled to saturation with topical application of $^{125}$I-$\alpha$-BGT, as used in the present study, the junctional site density ratio of denervated to innervated muscle was $\sim 1$. Thus the AChR site density does not decrease but stays relatively constant after denervation (as also reported earlier by Frank et al., 1975; Porter and Barnard, 1975; Bader, 1981; Loring and Salpeter, 1980). Yet by intraperitoneal injection, which represents a short, nonsaturating pulse label, there is a preferential labeling of the innervated junctional AChRs. This preferential labeling was not seen in the study by Levitt and Salpeter using the gamma counting procedure, presumably because the increased extrajunctional label in the denervated muscle, discussed above, masked this effect.

The reason for the preferential label of innervated junctional receptors is not known. To our knowledge no reports exist that denervated receptors have a lower affinity for $\alpha$-BGT. In fact Almon et al., (1974) found the opposite to be true. Diffusion barriers, due to scar tissue or damaged blood supply, may have developed in the denervated muscle, decreasing the access of $\alpha$-BGT to receptors during the short pulse labeling of an intraperitoneal injection. Whatever the reason, this phenomenon would accentuate the extent of contamination of the endplate-specific label by extrajunctional receptors in denervated muscles labeled by $\alpha$-BGT injection and assessed by the gamma counting procedure.

It was therefore important to establish the turnover rate of new receptors in denervated junctions directly. For that, the specific junctional label had to be assessed by TEM autoradiography to exclude extrajunctional contamination. Fortunately, as the TEM autoradiographic results in this study show, the degradation rate of the new junctional receptors does indeed have a $t_{1/2}$ of $\sim 1$ d and thus is the same as that of the postdenervation extrajunctional receptors.

Preliminary results from studies in which new receptors were labeled 12 d after cold saturation and the degradation curve extended to $>16$ d, indicate that there is a small ($\sim 20\%$) component of slowly degrading receptors in the labeled pool. These slowly degrading receptors could be due in part to unbinding or destruction of the nonradioactive blocking toxin after cold saturation, which would cause some original receptors to be labeled together with the new ones. In addition, a delay in the full degeneration of the nerve after being cut, could cause a delay in the appearance of the rapidly degrading new receptors. This would mean that some receptors inserted after cold saturation would still have a slow degradation rate. Finally, there may be some slowly degrading receptors even in the absence of nerve. We are currently investigating the possible source(s) and extent of such slowly degrading receptors. Whatever the source however, the presence of these slowly degrading receptors would cause the $t_{1/2}$ measured for the new receptors in this study to be a slight overestimate and the new receptors would be degrading even faster than given here.

One can estimate the percentage by which a measured $t_{1/2}$ value ($t_{1/2}$ obs) is an overestimate since

$$\frac{1}{t_{1/2} \text{ obs}} = \frac{f_i}{t_{1/2} \text{ slow}} + \frac{(1 - f_i)}{t_{1/2} \text{ fast}},$$

where $f_i$ is the small fraction of slowly degrading receptors measured for the new receptors in this study to be a slight overestimate during the period that the degradation rate is being measured.

If $t_{1/2}$ slow $\gg t_{1/2}$ fast, then $t_{1/2}$ fast $\sim (1 - f_i)t_{1/2}$ obs.

Thus, the percentage by which $t_{1/2}$ obs is an overestimate is approximately equivalent to the percentage of the total pool that is degrading slowly.

The mechanism whereby the nerve regulates degradation is not known. From the present study we can say that the new receptors behaved as do extrajunctional receptors both at 6 and 14 d after cutting the nerve, when the original receptors have very different degradation half-lives. Thus the postdenervation degradation rates of junctional receptors seem to be related to whether the receptors had ever been stabilized by innervation or not. Since TEM autoradiography shows that the new and original receptors are interspersed in the postjunctional membrane, the control of their degradation is likely to be exerted in a microdomain, which could include the individual receptors, its surrounding membrane, and associated cytoskeleton or basal lamina.
In summary, this study established that new AChRs, inserted into a nmn after denervation, have a turnover half-life of ~1 d and are therefore in this respect equal to that of embryonic or postdenervation extrajunctional receptors. Thus, until all the original receptors are degraded, two metabolically distinct receptor populations (original and new) coexist at denervated nmj’s. These two receptor populations are interspersed within the postjunctional membrane and degrade at different rates. The results confirm the “dual population” hypothesis proposed by Levitt and Salpeter (1981). Any model to explain neural control of degradation must account for this coexistence of receptors differing in degradation rate.

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