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Degradation of junctional and extrajunctional acetylcholine receptors by developing rat skeletal muscle

(synapse development)

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ABSTRACT We have examined the rate of degradation of the total acetylcholine receptor content of diaphragm muscles of young rats and have found that even in muscles from 1-day-old rats some receptors are metabolically more stable than adult extrajunctional receptors. Further experiments have shown that acetylcholine receptors at junctional regions from young rats are degraded slowly, whereas those in extrajunctional regions are degraded rapidly. The results demonstrate that junctional acetylcholine receptors in rat diaphragm are degraded at a slow rate characteristic of adult junctional receptors at all ages after birth.

The neuromuscular junction is well suited for studies of the development of chemically transmitting synapses. Only three cell types—motor neuron, Schwann cell, and skeletal muscle—contribute to its structure, and its function is well understood both physiologically and biochemically. Techniques are also available for labeling various elements involved in neuromuscular transmission. In particular, use of the snake venom α-neurotoxins has greatly facilitated studies of the muscle membrane protein, the acetylcholine receptor (AcChoR).

The metabolism of AcChoR and its distribution on the sarcolemma are intimately linked with the state of nerve–muscle interaction (for review, see ref. 1). Before mammalian skeletal muscle is innervated, AcChoRs are distributed rather uniformly over the myofiber surface. After innervation, a high density of AcChoRs forms at the junctional region and the density of extrajunctional receptors diminishes to undetectable levels (2, 3). Upon denervation of adult muscle, however, extrajunctional AcChoRs again appear (4).

The junctional and extrajunctional AcChoRs of denervated adult skeletal muscle differ in many ways. In addition to being located in distinct areas of the muscle membrane, these two classes of AcChoR have different drug sensitivities (5), isoelectric points (6), and mean channel open times (7). They also have widely different metabolic stabilities: extrajunctional AcChoR is degraded with a half-time of about 20 hr, whereas degradation of junctional AcChoR is at one-seventh this rate (8).

We have addressed the question of whether similar differences in receptor degradation exist at early stages of neuromuscular junction formation, when AcChoRs are present in both junctional and extrajunctional regions. Burden (9, 10) has reported that junctional and extrajunctional AcChoRs are both degraded rapidly in young chickens and that junctional receptors do not become metabolically stable until 2 or more weeks after hatching. Berg and Hall (8), on the other hand, have found that junctional AcChoRs of neonate rat diaphragm apparently are more stable than are extrajunctional receptors. We studied this question more thoroughly in diaphragms from rats, 1 day old to adult, and found that junctional AcChoRs are metabolically more stable than extrajunctional AcChoRs at all ages examined.

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MATERIALS AND METHODS

Preparation of ^{125}I-Labeled α-Bungarotoxin. α-Bungarotoxin was purified from the venom of the krait *Bungarus multicinctus* (Ross Allen Serpentarium, Miami, FL). It was iodinated with ^{125}I by the iodine monochloride method, and the labeled diiodo α-BuTx (^{125}I-αBuTx) was purified by chromatography (11).

AcChoR Degradation in Organ Culture. The degradation rate of AcChoR in rat diaphragms was determined by the method of Berg and Hall (8) with some modifications. Lewis rats of various ages were injected in the thoracic cavity with ^{125}I-αBuTx (about 0.2 nmol/100 g of body weight). This dose was sublethal and labeled 5–20% of the AcChoR in adult rats. At 15–18 hr after the injection the diaphragms were placed in organ culture (see ref. 12 for details). The rate of total AcChoR degradation was determined by measuring the amount of radioactivity released into the culture medium. At the end of the experiment the radioactivity remaining bound was determined. The release data were then expressed as the percentage of total radioactivity bound to the muscle at the various times.

Denervation. Denervated hemidiaphragms were obtained by sectioning the left phrenic nerve under ether anesthesia 8–12 days before the ^{125}I-αBuTx injection.

Estimation of Junctional and Extrajunctional AcChoR. In some experiments the percentage of AcChoR that was extrajunctional was estimated. Muscles were labeled with ^{125}I-αBuTx *in vivo* or *in vitro* (see below) and fixed with 4% paraformaldehyde in 50 mM sodium phosphate buffer at pH 7.2; then, junctional regions were stained by using a histochemical method to demonstrate cholinesterase activity (13). Bundles of fibers were removed and the number of fibers in the squashed bundles was counted at ×100–×200 magnification. The bundles were cut into measured lengths and the radioactivity was determined by gamma counting. The extrajunctional binding per fiber per unit length was calculated from the radioactivity bound to regions of the fibers that did not stain for cholinesterase. This value was then used to calculate the additional binding per muscle fiber that occurred in regions that stained for cholinesterase ("junctional" binding).

Some of the muscles were labeled *in vivo*, as described above. Other muscles were labeled *in vitro* by incubation for 2 hr at room temperature in 20 nM ^{125}I-αBuTx in Dulbecco's modified Eagle's medium buffered to pH 7.0 with 15 mM Hepes and containing 1 mg of bovine serum albumin per ml (hereafter

Abbreviations: AcChoR, acetylcholine receptor; ^{125}I-α-BuTx, ^{125}I-labeled α-bungarotoxin; dTC, *d*-tubocurarine.

referred to as "medium"). When muscles were labeled *in vitro*, control muscles were preincubated in 1 mM *d*-tubocurarine (dTC) in medium for 15 min and then incubated in 20 nM ^{125}I-αBuTx in the presence of dTC. After labeling, muscles were rinsed three times with medium, either containing 1 mM dTC or not as appropriate, and then washed for 2 hr at room temperature with medium either containing 1 mM dTC or not, with the medium changed every 20 min. The dTC-protectable binding was calculated and assumed to represent binding of ^{125}I-αBuTx to AcChoR. At all ages the junctional binding was greater than 80% protectable (85 ± 5%, mean ± SD) whereas extrajunctional binding was 80% protectable at 4 and 8 days, decreasing to 69 and 61% at 12 and 15 days, respectively (3).

There are several sources of error in these procedures. The overall error is probably ±10% in the estimated percentage of extrajunctional receptors, based on the scatter of results shown in Fig. 3. An error also arises from the definition of junctional binding. The method used to calculate junctional binding assumes that the density of extrajunctional AcChoRs is uniform along the fiber length; this is certainly not the case at young ages (3). In the sternomastoid muscle from 3-day-old rats, as much as 10–15% of the total extrajunctional AcChoRs may be located in a perijunctional gradient. These AcChoRs would be counted as junctional by the methods used. The data have not been corrected for this distortion because the appropriate correction is not known as a function of age, but its effects are considered in the *Results*.

Analysis of Release Data. Release data were analyzed on a PDP-11/34 minicomputer (Digital Equipment Corp., Waltham, MA) using standard curve-fitting procedures. In each analysis, equations containing two free variables were fitted to the data (see *Results*) and the values that minimized the sum of the squared deviations between predicted and experimental data were determined. Nonlinear regressions were performed by using an iterative procedure that stepped values until a ±0.6% change of variable values (together or separately) did not decrease the sum of squared deviations. The programs were tested by using theoretical "release" curves and also by starting the search at various initial values. In each case the search terminated at the calculated minimal values.

RESULTS

Apparent Metabolic Stability of the Total AcChoR Content. Our initial experiments examined the time course of the degradation of the total AcChoR content in the diaphragm as a function of the age of the rat at the time of labeling with ^{125}I-αBuTx. We labeled the diaphragms of young rats with ^{125}I-αBuTx and, after placing the muscles in organ culture, observed the rate at which radiolabeled low molecular weight material appeared in the culture medium. Several investigators have shown that the appearance of degradation products of bound ^{125}I-αBuTx accurately reflects the rate of degradation of AcChoR to which ^{125}I-αBuTx was bound (14–16).

Isotope was rapidly lost from diaphragm muscles of young rats. The rate of release decreased with age for the first 3–4 weeks after birth (Fig. 1*A*). When a single exponential equation was fitted to the data, the estimated time constant increased with age in the fashion shown in Fig. 2. Even at the earliest age, the mean (±SD) time constant for release (42.7 ± 1.5 hr; n = 4) was significantly longer than the time constant found for adult extrajunctional receptors (28.7 ± 4.1 hr; n = 15); the difference in mean τ is significant at $P < 0.01$ by the t test. This observation implies that at least some AcChoRs in neonate muscles are degraded at a slower rate than are adult extrajunctional AcChoRs.

The observations shown in Fig. 2 may be explained in two

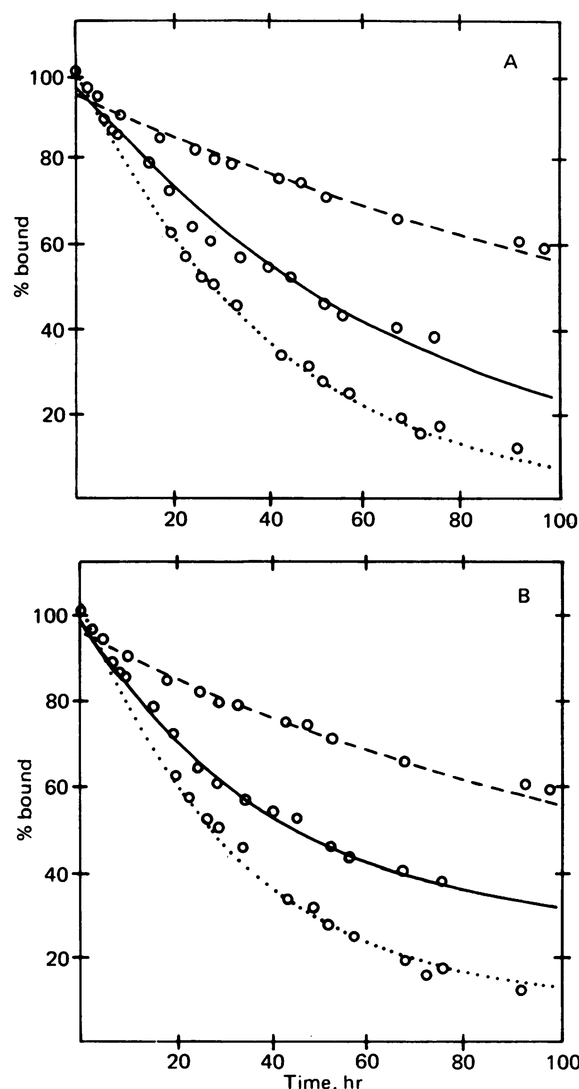

FIG. 1. Percentage of radioactivity remaining bound to diaphragm muscles from rats of different ages, as a function of time. The lines through the data in *A* are single exponential curves (Eq. 1); in *B* they are the sums of two exponentials (Eq. 2). The lines are least-squares fits generated by computer analysis. ·····, From a diaphragm labeled in 1-day-old rat; ——, from 7-day-old rat; - - -, from 19-day-old rat.

simple ways. The first is that there is a metabolically homogeneous population of AcChoRs at all ages but the first-order rate of AcChoR degradation changes as a function of age. Mathematically, this may be expressed as

$$Y = A \exp(-t/\tau) \quad [1]$$

in which Y is the percentage of the initial radioactivity remaining bound, A is the initial amount bound (ideally, 100%), and τ is an age-dependent time constant for receptor degradation. In fitting this equation, both A and τ were varied. Eq. 1 fits the data quite well (Fig. 1) and gives the estimates for τ shown in Fig. 2.

The second explanation is that there are two populations of AcChoRs which have age-independent rates of degradation but which are present in varying amounts depending on the age of the rat. In this case,

$$Y = C \exp(-t/\tau_x) + D \exp(-t/\tau_j) \quad [2]$$

in which C and D are the initial percentages of the two AcChoR populations and τ_x and τ_j are fixed age-independent time constants for receptor degradation in the two populations. In

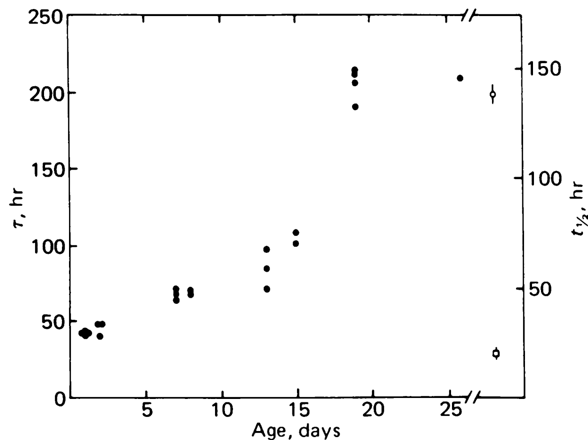

FIG. 2. Estimated τ for release of radioactivity from single diaphragms from rats labeled at different ages. O, Mean ± SEM for release from innervated diaphragms from rats >30 days old (τ_j = 199.7 ± 7.8 hr; range, 137–341 hr; n = 41). □, Mean ± SEM for degradation of extrajunctional AcChoRs on denervated adult muscles (τ_x = 28.7 ± 1.0 hr; range, 19.0–32.4 hr; n = 15). The age plotted is the age at which the rats were injected; the release determinations were begun about 15 hr later.

fitting this equation, C and D were varied while τ_x and τ_j were fixed at the values found for adult extrajunctional and junctional AcChoRs, respectively.‡

To determine the ability of Eq. 2 to describe the data, τ_j and τ_x were determined for AcChoRs on normal and denervated adult muscles. The mean τ_j for adult diaphragm AcChoRs (rats older than 30 days) is shown in Fig. 2 (τ_j = 199 hr; $t_{1/2}$ = 138 hr). To determine τ_x, it was assumed that denervation did not alter the rate of degradation of junctional AcChoRs on denervated muscles. Hence, the release of radioactivity from denervated adult muscle was analyzed in terms of

$$Y = E \exp(-t/\tau_x) + (100 - E) \exp(-t/\tau_j) \qquad [3]$$

in which E is the percentage of AcChoRs that are extrajunctional and are metabolized with a time constant τ_x; the remaining percentage $(100 - E)$ of AcChoR is assumed to be metabolized at the mean rate τ_j. The estimated τ_x is shown in Fig. 2 (τ_x = 29 hr; $t_{1/2}$ = 20 hr). The mean (±SD), value estimated for E was 90 ± 11%.

By using these values of τ_x and τ_j, the release data for diaphragms from young rats (<30 days old) were fitted with Eq. 2 (see Fig. 1*B*). Overall, Eqs. 1 and 2 were equally good at describing the data. As an estimate of their relative ability to describe the data, the sum of the squared deviations (S) between the actual data and the predicted curves were compared for the two equations. Of 22 release experiments on diaphragms labeled in rats less than 30 days old, Eq. 1 had the lower S in 10 cases and Eq. 2 had the lower S in 12. It should be noted that the same number of free variables (two) was used in fitting either equation. Thus, overall degradation of the total muscle AcChoR can be equally well described by either of the two hypotheses advanced. Further tests were necessary to determine which hypothesis is more reasonable.

Two tests were performed. Both tests used the observation that in adult muscle the metabolically distinct junctional and extrajunctional AcChoRs are located in different regions of the muscle. The first test was to determine the percentage of the total muscle AcChoRs that were located extrajunctionally, as a function of age of the rat. The second test was to determine the rate of loss of radioactivity from junctional and extrajunctional regions of diaphragms labeled in 2-day-old rats.

Proportion of Extrajunctional AcChoR. The proportions of extrajunctional and junctional AcChoRs were estimated directly. These determinations gave independent values for the two free variables used in fitting Eq. 2 to the release data. If the two-population hypothesis is to be accepted, the directly measured proportion of extrajunctional AcChoRs must be in reasonable agreement with the proportion predicted by the kinetic analysis.

Fig. 3 shows the predicted percentage of extrajunctional receptors obtained from fitting Eq. 2 to the release data and the directly measured percentages. Although there is some scatter in the data, the agreement is quite good. The agreement supports the idea that there are two populations of AcChoRs on neonatal rat diaphragm and that they have different metabolic rates.

Turnover of Extrajunctional and Junctional AcChoRs. The second and more direct test was to examine the rate of loss of

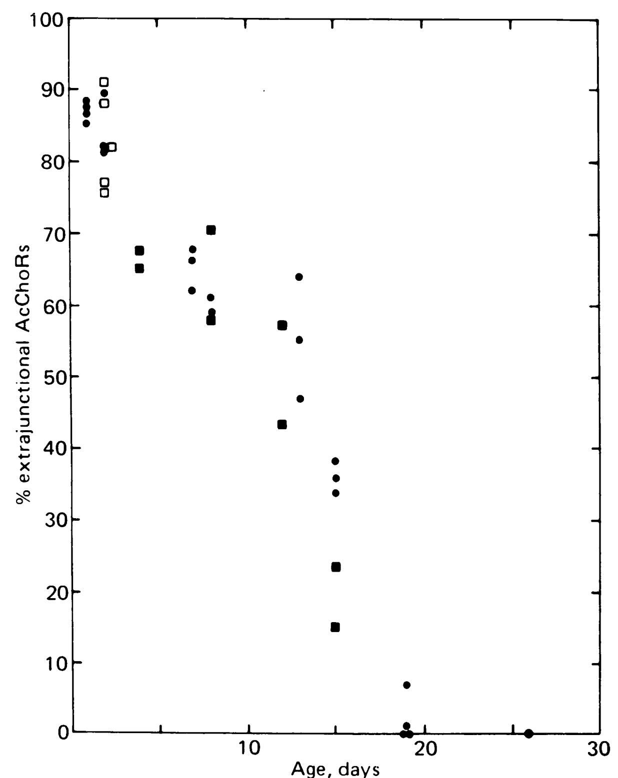

FIG. 3. Estimates of the percentage of the total AcChoRs that are extrajunctional, as a function of the age of the rat. ●, Individual predictions calculated from the degradation of the total AcChoR content by using Eq. 2; these predictions have been corrected for the lag between the time of injection with ^{125}I-αBuTx and the start of the release experiment. ■, Measurements made by determining the amount of binding to junctional and extrajunctional regions. □, Data obtained by using rats labeled *in vivo* and used in the turnover experiment described in Fig. 4; these data have been corrected for the 18-hr lag between the labeling of the muscle and its dissection. The filled squares show the proportion of the dTC-protectable ^{125}I-αBuTx binding that is located extrajunctionally after *in vitro* labeling. The estimates shown as squares have not been corrected for extrajunctional receptors located in the perijunctional gradient; thus, they are probably somewhat lower than the true values.

‡ The amplitude factors A, C, and D were allowed to vary because the percentage bound was an experimentally determined quantity. The mean (±SD) value for A was 98 ± 2%; for $C + D$ it was 103 ± 5%. The fact that the amplitude was a free variable explains why some theoretical curves in Fig. 1 do not pass through the point 0 hr, 100%.

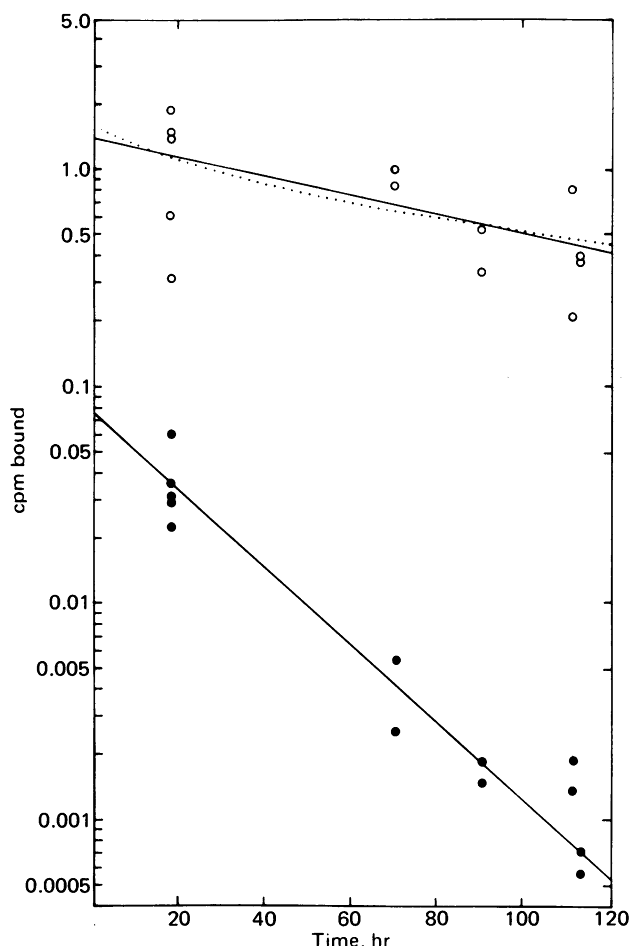

FIG. 4. Decrease in ^{125}I-αBuTx bound to junctional and extrajunctional regions of diaphragms from rats injected with ^{125}I-αBuTx at 2 days after birth (two litters were used). The rats were killed at various intervals after the injection (0 time in the figure) and the extrajunctional binding per fiber per unit length (●) and the junctional binding (○) were estimated. The solid lines show the best-fitting single exponential curves through the data. For junctional counts τ = 98 hr ($t_{1/2}$ = 68 hr); for extrajunctional, τ = 24 hr ($t_{1/2}$ = 17 hr). The slope of the line through the junctional data is significantly different from that through the extrajunctional data ($P < 0.01$, t test) but only marginally different from that expected from the rate of degradation of adult junctional AcChoR (τ = 200 hr; $0.05 < P < 0.10$, t test). Bevan and Steinbach (3) have shown that the density of extrajunctional AcChoRs is higher close to the junction on neonatal muscles than it is in distant extrajunctional regions. Thus, it is possible that the junctional AcChoR population measured by the techniques used here actually included some extrajunctional AcChoRs. For this reason, the junctional data were also fitted with Eq. 2 (dotted line through the junctional data). This fit predicted that 54% of the original junctional AcChoRs were metabolized at the adult junctional rate and 46% at the adult extrajunctional rate. Due to the scatter in the data it cannot be said that either the solid or the dotted line is a more adequate description of the data. The diaphragm muscle fibers grew in length by about 25% during the course of the experiment; the data shown have not been corrected for this dilution. If the extrajunctional data were corrected by assuming that growth was linear over the period of the experiment, the best-fitting single exponential had τ = 26 hr ($t_{1/2}$ = 17 hr). When the extrapolated 0-time binding values were used to calculate the percentage of extrajunctional AcChoRs, the solid lines gave a value of 83%. When the value for junctional AcChoRs predicted by the dotted line was used, 90% of the AcChoRs were predicted to be extrajunctional.

radioactivity from junctional and extrajunctional regions of diaphragms labeled in young rats. The first explanation, a single metabolic population of receptors, predicts that radioactivity would be lost equally rapidly from either region. The second explanation predicts that radioactivity should be lost rapidly from extrajunctional and slowly from junctional regions.

This experiment was conducted *in vivo*, by injecting 2-day-old rats intrathoracically with ^{125}I-αBuTx and allowing them to survive for various lengths of time (18 hr to 6 days). The extrajunctional binding per fiber per unit length and the junctional binding per fiber were calculated. The data in Fig. 4 show that the junctional AcChoRs were metabolically more stable than the extrajunctional AcChoRs. The extrajunctional binding decreased with the half-life expected for adult extrajunctional receptors. The junctional binding decreased a little more rapidly than expected on the basis of the rate for adult junctional AcChoRs. This could be due to the fact that some extrajunctional AcChoRs located in the perijunctional gradient (3) were counted as junctional AcChoRs (see Fig. 4). These data show that there are two populations of AcChoRs on neonatal rat diaphragm muscles and that they have different degradation rates.

It might be argued that the degradation of AcChoRs differs *in vivo* and *in vitro*. However, when Eq. 1 was fitted to the *in vivo* whole muscle binding data, the best-fitting τ was 38 hr, compared to the mean (±SD) τ of 45 ± 5 hr for release of radioactivity in organ culture from 2-day-old diaphragms. Eq. 2 fit the whole muscle data as well as Eq. 1 and predicted that 94% of the total radioactivity was bound to extrajunctional AcChoRs whereas a mean (±SD) percentage of 84 ± 4% was predicted by analysis of the organ culture release data. Thus, the degradation of the total AcChoR content of whole muscles was similar *in vivo* and in organ culture.

So far as could be determined, the injected rats developed normally: no rats in these litters died after the injection, they gained weight normally, and the weight of the sternomastoid muscle increased normally.

The injection *in vivo* clearly cannot provide a perfect pulse label. However, the period during which binding of ^{125}I-αBuTx occurred is certainly short, based on the following observations. First, the amount of ^{125}I-αBuTx injected did not saturate the available AcChoRs in these rats. In adult rats, similar injections label 5–20% of the AcChoRs in diaphragm (12). Also, at most, 20% of the AcChoRs were occupied on neonatal sternomastoid muscles removed 18 hr after the injection. Second, in four neonatal rats the blood level of ^{125}I-αBuTx was estimated 18 hr after the injection by taking a blood sample and determining the amount of radioactivity that was excluded on P-2 columns. The maximal blood concentration of ^{125}I-αBuTx calculated was 26 ± 2 pM (mean ± SD) 18 hr after the injection. The agreement between the loss of radioactivity from muscles *in vivo* and in organ culture also suggests that binding of ^{125}I-αBuTx did not occur at long times after the injection.

DISCUSSION

The following conclusions can be drawn from these results: First, the degradation of the total AcChoR content of neonatal rat diaphragm muscles can be accurately described by assuming that there are two populations of AcChoRs (junctional and extrajunctional) that are degraded at the rates found in adult innervated and denervated muscle. Second, the rate of degradation of the total AcChoR content changes during development because the proportion of extrajunctional AcChoRs changes. Third, even at young ages, junctional AcChoRs are metabolically more stable than extrajunctional AcChoRs. This conclusion is in agreement with the observation made by Berg and Hall (8).

Our data are not sufficiently accurate to demonstrate that there is no change in the degradation rate of junctional receptors

with development. However, the data are consistent with the idea that junctional AcChoRs on neonatal rat diaphragm muscles are as stable metabolically as they are in adult muscle. Furthermore, Brockes and Hall (6) have reported that some of the AcChoRs found in junctional regions of 1-day-old rat diaphragm have the isoelectric point characteristic of adult junctional receptors.

These conclusions contrast with observations on chicken muscles, in which junctional AcChoRs have a fast turnover ($\tau_{1/2} = 32$ hr) even 1 week after hatching (9, 10). Whether or not this difference between chicken and rat reflects some fundamental difference or just different rates of development remains to be seen.

Junctional and extrajunctional AcChoRs on adult muscle also differ in their mean open-time when activated by acetylcholine (7). It has recently been reported that junctional AcChoRs on fibers on neonatal rat diaphragm have mean open-times characteristic of adult extrajunctional AcChoRs (17). Only at about 6 days after birth are AcChoRs with the short open-time characteristic of adult junctional AcChoRs detected; between 6 and 11 days, many fibers show a mixed population of junctional AcChoRs apparently composed of two subpopulations that have junctional or extrajunctional mean open-times (17). Sakmann and Brenner (18) have shown that the shift in mean open-times occurs earlier in the omohyoid muscle and later in the soleus muscle. Our data on diaphragm muscle indicate that some junctional AcChoRs are metabolically stable even when biophysical data indicate that their mean open-time is characteristic of adult extrajunctional AcChoRs. These observations suggest that different properties of AcChoRs (location, degradation rate, and mean open-time) change at different times during development of the muscle. It is not known how the different properties are controlled. However, the data on mean open-times indicate that simple aggregation of extrajunctional AcChoRs is not sufficient to change this property (17–19). Furthermore, at least in chicken muscle, AcChoR aggregation does not alter the degradation rate (9, 19). It may be that some properties of the AcChoR are altered by the properties of the membrane patch in which they are located (e.g., the membrane lipid composition or the presence of submembranous structures). Alternatively, receptor proteins could be altered after synthesis, or the synthesis of the polypeptide chains composing the receptor could be independently regulated.

The rat diaphragm changes greatly during the first 2 weeks after birth. For instance, the multiple innervation of embryonic muscle fibers in the diaphragm is lost (20, 21). Similarly, the extrajunctional AcChoRs decrease in number in the first 2 weeks, presumably due to a decrease in synthesis induced by muscle contractile activity (22, 23). The myosin light chain complement also shifts to the adult pattern during the second week after birth (24). Our results show that junctional AcChoRs are stabilized metabolically shortly after birth, before these other changes occur. These observations suggest that the appearance of metabolically stable AcChoRs is controlled by a different process than are the later changes, although it is possible that the AcChoRs simply respond more rapidly.

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