Journal of Physiology (1990), **426**, *pp.* 281–296
With 8 *figures*
Printed in Great Britain

DEVELOPMENTAL CHANGES IN THE HALF-LIFE OF ACETYLCHOLINE RECEPTORS IN THE MYOTOMAL MUSCLE OF *XENOPUS LAEVIS*

By M. W. COHEN, P. F. FRAIR, C. CANTIN AND G. HÉBERT

From the Department of Physiology, McGill University, Montreal, Quebec, Canada

(*Received 13 November 1989*)

SUMMARY

1. Tail preparations, containing myotomal muscle and associated spinal cord, were isolated from embryos and tadpoles of *Xenopus laevis* between stages 25 and 49 (1·15–12 days) and were pulse-labelled with ^{125}I-α-bungarotoxin (^{125}IαBT) so that the half-life ($T_{\frac{1}{2}}$) of their acetylcholine receptors (AChRs) could be estimated in organ culture.

2. For the entire population of AChRs, estimates of $T_{\frac{1}{2}}$ based on a single exponential decline in radioactivity (but see item 4 below) increased from 53–55 h at stages 25–31 (1·15–1·56 days) to $\sim$ 135 h at stage 47 (5·5 days). Beyond stage 47 $T_{\frac{1}{2}}$ increased only slightly.

3. Radioautographic estimates of the $T_{\frac{1}{2}}$ of extrajunctional AChRs at stages 47–48 (5·5–7·5 days) were 41–50 h. It follows that the developmental change in the $T_{\frac{1}{2}}$ of the entire population of AChRs was due to the junctional AChRs.

4. At stages 47–49 (5·5–12 days) the decline in radioactivity for the entire population of AChRs was fitted well by a double exponential. Assuming a $T_{\frac{1}{2}}$ of 50 h for the extrajunctional AChRs and 210 h for the junctional AChRs, the correlation coefficient (r) was 0.9947 ± 0.0014 (mean $\pm$ S.E.M.; $n = 14$) and junctional AChRs were estimated to comprise 80 ± 3 % of the entire population. Similar analysis, as well as experiments in which the degradation of junctional AChRs was assessed by pulse-labelling with fluorescent α-bungarotoxin, suggested that at earlier stages of development the junctional AChRs have a shorter $T_{\frac{1}{2}}$ and comprise a smaller fraction of the entire population.

5. The developmental increase in $T_{\frac{1}{2}}$ occurred even when animals were raised in the anaesthetic tricaine or in tetrodotoxin, conditions which abolished all motor activity.

6. Developmental increases in $T_{\frac{1}{2}}$ also occurred in culture but were smaller than those *in vivo*. The increases in culture did not occur amongst those AChRs which were pre-labelled with ^{125}IαBT.

7. It is concluded that in *Xenopus* myotomal muscle the $T_{\frac{1}{2}}$ of junctional AChRs begins to increase within a day after the onset of innervation and that the increase does not require nerve or muscle impulse activity. We suggest, among other possibilities, that it may depend upon incorporation of a different molecular species of AChR into the postsynaptic membrane.

MS 8069

INTRODUCTION

Marked changes occur in the number, distribution and properties of AChRs in developing skeletal muscle (for reviews see Salpeter & Loring, 1985; Schuetze & Role, 1987). Early on there is a nerve-induced clustering of acetylcholine receptors (AChRs) so that the receptor density becomes much higher in the developing postsynaptic membrane than elsewhere along the muscle cell. This nerve-induced clustering of AChRs involves a mechanism whereby previously mobile AChRs are immobilized at the site of innervation. Subsequently there is a decline in receptor density in extrajunctional regions, a slowing of the degradation rate of junctional AChRs and, in some but not all muscles, a change in the channel properties of the AChRs.

These, and related, synaptogenic events have been studied most extensively in rodent muscle, chick muscle and *Xenopus* myotomal muscle. Whereas all of the muscles exhibit nerve-induced clustering of AChRs shortly after the onset of innervation there are some significant differences between muscles with respect to the other synaptogenic events. For example (a) the decline in the density of extrajunctional AChRs is much more pronounced in rodent muscle (Bevan & Steinbach, 1977; Steinbach, 1981) and in chick muscle (Burden, 1977*a*; Betz, Bourgeois & Changeux, 1980) than in *Xenopus* myotomal muscle (Chow & Cohen, 1983); (b) the channel properties of the AChRs undergo a developmental change in rodent muscle (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980; Michler & Sakmann, 1980) and in *Xenopus* myotomal muscle (Kullberg, Brehm & Steinbach, 1981) but not in chick muscle (Schuetze, 1980); and (c) the slowing of AChR degradation occurs much sooner after the onset of innervation in rodent muscle (Steinbach, Merlie, Heinemann & Bloch, 1979; Michler & Sakmann, 1980; Reiness & Weinberg, 1981; Steinbach, 1981) than in chick muscle (Burden, 1977*b*; Betz *et al.* 1980). What accounts for the similarities as well as the differences in the progression of synaptogenesis in different muscles is likely to be of importance in achieving an understanding of how neuromuscular synaptogenesis is regulated.

The present study focuses on the developmental slowing of AChR degradation. Recent findings have indicated that neuromuscular transmission plays an important role in regulating the degradation rate of junctional AChRs in *mature* rodent muscle (Salpeter, Cooper & Levitt-Gilmour, 1986; Cangiano, Fumagalli & Lømo, 1987; Avila, Drachman & Pestronk, 1989). However, there is no available information on the role that neuromuscular transmission plays in the conversion from a fast to a slow degradation rate in developing muscle. The present study describes the time course of changes in the degradation rate of AChRs in developing *Xenopus* myotomal muscle and provides evidence that these changes occur independently of neuromuscular transmission. The results also indicate that the change in degradation rate does not occur amongst AChRs which have ^{125}I-α-bungarotoxin (^{125}IαBT) bound to them. A preliminary account of some of this work has been published as an abstract (Frair & Cohen, 1981).

METHODS

Organ culture

Embryos of *Xenopus laevis* were obtained by induced breeding and raised in dechlorinated tap water at room temperature. They developed at about the same rate as described by Nieuwkoop & Faber (1967) and hatched after 2 days. Beginning on the fourth day the tadpoles were fed finely ground frog brittle.

Prior to dissection selected animals were kept overnight in dechlorinated tap water containing 100 μg/ml gentamicin and 100 i.u./ml nystatin to decontaminate the animals. The pH of the solution, assessed by phenol red, was adjusted to $\sim$ 7·2 by addition of NaOH. All subsequent procedures were carried out under sterile conditions.

For each experiment animals of the same developmental stage were selected according to the external criteria of Nieuwkoop & Faber (1967). Their range of lengths never exceeded 10 % of the mean length of the group. The animals were anaesthetized in 100–200 μg/ml tricaine methanesulphonate at pH $\sim$ 7·2 (Cohen, Greschner & Tucci, 1984) and dissected in a solution consisting of culture medium to which was added gentamicin (50 μg/ml) and nystatin (50 i.u./ml). Culture medium contained 67 % L15 and 0·5 % dialysed horse serum. The anaesthetic was obtained from Sigma Chemical Co. and the other agents and solutions from Grand Island Biological Co.

'Tail preparations', consisting of myotomal muscle and associated spinal cord and notochord, were isolated from the anaesthetized animals as described previously (Chow & Cohen, 1983). Smaller caudal myotomes and the first 2–4 rostral myotomes were excluded so that the tail preparations contained myotomes which were approximately similar in size.

After pulse-labelling with ^{125}IαBT (see below) and rinsing, the number of myotomes and the amount of radioactivity in each tail preparation were counted. The tail preparations were then cultured separately in capped tubes or covered wells which were placed on a tilted rotator to ensure continuous mixing. At daily intervals 10 μl samples of medium from each culture were examined for bacteria, contaminated cultures were discarded, and the remaining ones were rinsed and counted for radioactivity. The incidence of contamination was rare (< 0·5 %). Most experiments were terminated after 5 days and a few were continued for 6 days. All procedures were carried out at room temperature (23–25 °C).

Throughout the text the ages for each developmental stage are those of Nieuwkoop & Faber (1967).

^{125}I-α-bungarotoxin binding

For each experiment eight or more tail preparations were separated into two groups. One group was exposed for 30–60 min to 2–4 μg/ml αBT (0·25–0·5μM) and then both were exposed to ^{125}IαBT (0·3–1·1 μg/ml for 60 min) and rinsed for at least 2 h. Accordingly, total binding, non-specific binding and specific binding of ^{125}IαBT to surface AChRs could be assessed. During the rinse period the number of myotomes in each tail preparation was counted. The tail preparations were then placed in separate plastic vials and their radioactivity was measured in a γ-counter (Nuclear Chicago, Model 1185). Background radioactivity was also measured, and subtracted, so that the amount of bound ^{125}IαBT per myotome could be calculated. Non-specific binding per myotome was usually less than 6 % of total binding per myotome and in only one experiment, on stage 25 (1·15 day) tail preparations, was it as high as 12 %.

In fifteen experiments both groups of tail preparations were cultured so that $T_{\frac{1}{2}}$ could be estimated on the basis of total binding as well as specific binding. The estimates of $T_{\frac{1}{2}}$ based on specific binding were 98 ± 1 % of those based on total binding. Since the two estimates were so close, all values reported in the Results are based on total binding.

In some experiments the radioactivity in the culture medium was analysed on a Biorad P-2 chromatographic column in order to assess how much resulted from degradation and how much from unbinding of ^{125}IαBT (Bevan & Steinbach, 1983). By knowing how much ^{125}IαBT appeared in the culture medium during a given period of time, and the initial amount of bound ^{125}IαBT, the rate constant of unbinding could be readily calculated.

The potency of each new batch of αBT (kindly provided by M. Quik) and of ^{125}IαBT (New England Nuclear) was tested as described previously (Goldfarb, Cantin & Cohen, 1990). Based on these tests appropriate concentrations and exposure times were chosen to ensure saturation of AChRs.

Radioautography

Radioautography was carried out as described previously (Chow & Cohen, 1983; Goldfarb *et al.* 1990). All sections from a single experiment were dipped in emulsion at the same time and processed identically. Grains were counted at a magnification of 1250 × by viewing them through a Zeiss microscope fitted with an appropriate ocular grid. For each tail preparation grains were counted in longitudinal sections at twenty different extrajunctional sites, each covering an area of 50 × 50 μm. Only well-preserved regions were chosen. The extrajunctional regions were selected so that they were at least 20 μm away from the intermyotomal junctions, the main site of innervation. Typical values for tail preparations which were pulse-labelled with $^{125}I\alpha$BT and fixed on the same day were 200–500 grains/2500 μm^2. Background values, measured in neighbouring regions without tissue, were typically 15–25 grains/2500 μm^2 and were subtracted.

Measurements of grain density and relative tissue volume in radioautographs of transverse sections at stage 47 (5·5 days) indicated that less than 1% of the total binding of ^{125}IαBT was associated with the spinal cord and notochord. This is in agreement with previous findings that AChRs in *Xenopus* tail preparations are confined to the myotomal muscle (Baldwin, Yoshihara, Blackmer, Kinter & Burden, 1988; Goldfarb *et al.* 1990).

Fluorescent staining

Based on potency tests (Goldfarb *et al.* 1990), tetramethylrhodamine-conjugated α-bungarotoxin (RαBT) was used at appropriate dilutions and exposure times to pulse-label the AChRs in tail preparations to saturation. After rinsing, the tail preparations were cultured for different periods and fixed overnight in the refrigerator with 4% formaldehyde in 90 mM-phosphate buffer, pH 7·3. Fixed tail preparations were split along their mid-line into two sets of myotomal muscle, cleared and mounted in a solution consisting of 1 mg/ml *p*-phenylenediamine, 10 mM-sodium carbonate and 90% glycerol and stored at −16 °C until examined in the fluorescence microscope.

In some cases the same tail preparations were also stained for a synaptic vesicle antigen. Monoclonal antibody 48 (Mab 48, kindly provided by L. Reichardt and J. Bixby) is directed against a 65 kD protein in the membrane of synaptic vesicles of most, if not all, neurons including motoneurons (Matthew, Tsaveler & Reichardt, 1981; Cohen, Rodriguez-Marin & Wilson, 1987). The tail preparations were fixed for 5–10 min with 4% formaldehyde in phosphate buffer, rinsed with a solution consisting of 67% phosphate-buffered saline (PBS) and 1% goat serum (GS), and then exposed in the refrigerator for at least 5 h to Mab 48 diluted 300–1000 times with a solution consisting of 67% PBS, 1% GS and 1 mg/ml saponin. After rinsing, they were then exposed in the refrigerator for at least 5 h to affinity-purified fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories) diluted 100 times with 67% PBS and 1% GS. After rinsing again, the tail preparations were post-fixed overnight in the refrigerator with 4% formaldehyde in phosphate buffer and then split, mounted and examined as described above.

For each experiment, photographs of the fluorescent staining were taken at the same exposure time and printed under identical conditions.

Abolition of motor activity

During normal development *Xenopus* embryos begin to exhibit spontaneous motor activity by stage 26 (1·23 days). To determine whether this activity influences developmental changes in AChR degradation, in some experiments stage 20–26 embryos of the same clutch were hatched and raised in solutions with and without the anaesthetic tricaine (Cohen *et al.* 1984) or tetrodotoxin (Kullberg, Owens & Vickers, 1985). The concentration of tricaine was 200 μg/ml and its pH was adjusted to ∼ 7·2. The concentration of tetrodotoxin was 100 μg/ml for the first day and 20 μg/ml thereafter. Daily observations on the anaesthetic-reared animals revealed no motor behaviour, even in response to prodding with a pin, whereas control animals always exhibited vigorous motor activity. After 3–4 days, when the animals were at stages 43–46 (3·6–4·2 days), tail preparations were isolated from both groups and AChR degradation was measured as usual.

RESULTS

Entire population of acetylcholine receptors

Figure 1 shows the results of two experiments in which the degradation rate of AChRs in *Xenopus* tail preparations was estimated from the decline in radioactivity after pulse-labelling with $^{125}I\alpha BT$. In both experiments the radioactivity declined exponentially with time but the rate of decline was considerably faster for the stage

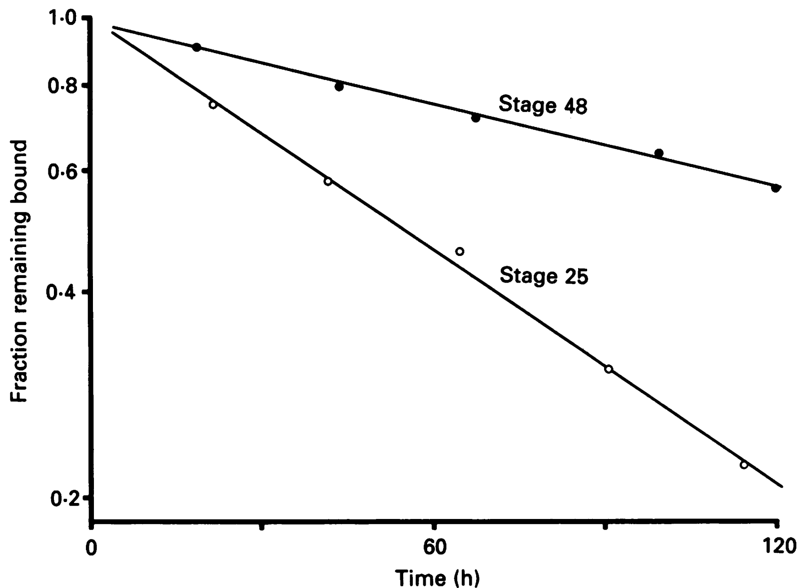

Fig. 1. The fraction of $^{125}I\alpha BT$ remaining bound to tail preparations is shown as a function of time in culture for two experiments. Each value is the mean for eight tail preparations in the stage 48 (7·5 day) experiment and seven tail preparations in the stage 25 (1·15 day) experiment. The standard errors of the means were all less than 1·5%. Based on regression analysis, the estimated degradation rate constants were 0·0046/h for the stage 48 experiment ($r = 0·9977$) and 0·0130/h for the stage 25 experiment ($r = 0·9985$).

25 (1·15 day) tail preparations than for the stage 48 (7·5 day) ones. Regression analysis based on the equation $y = e^{-\alpha t}$ (where y is radioactivity, t is time and α is the rate constant) yielded degradation rate constants of 0·0130/h and 0·0046/h for the stage 25 and stage 48 results, and correlation coefficients (r) of 0·9985 and 0·9977. The degradation rates correspond to half-life values of 53 h for stage 25 and 151 h for stage 48 ($T_{\frac{1}{2}} = \ln 2/\alpha$).

Most of the radioactive material which appeared in the culture medium during such experiments had a lower molecular weight than $^{125}I\alpha BT$ (Fig. 2). This is in agreement with previous studies which have indicated that during the degradation of the $^{125}I\alpha BT$-labelled AChR the entire complex is degraded thereby resulting in the appearance of ^{125}I-tyrosine in the culture medium (Berg & Hall, 1975; Devreotes & Fambrough, 1975). Based on the amount of radioactivity that did appear in the culture medium as $^{125}I\alpha BT$, it was possible to estimate that $^{125}I\alpha BT$ remained bound to the AChR with a half-life of 56 ± 10 days ($n = 5$). This value is about 50% greater

than that obtained for unbinding of $^{125}I\alpha BT$ from AChRs in rat muscle at 37 °C (Bevan & Steinbach, 1983). It follows that most of the decline in radioactivity in the tail preparations was due to degradation of the $^{125}I\alpha BT$-labelled AChRs and that unbinding of $^{125}I\alpha BT$ only marginally affected our $T_{\frac{1}{2}}$ estimates.

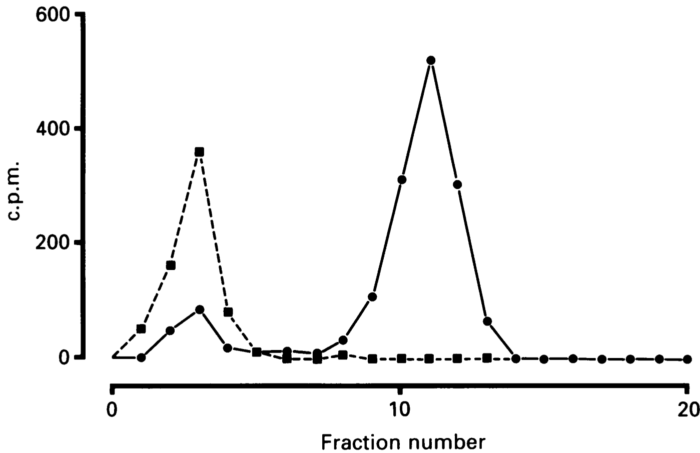

Fig. 2. Elution profiles for $^{125}I\alpha BT$ (■---■) and for culture medium in which stage 40 (2·75 day) tail preparations were kept for 18 h after being pulse-labelled with $^{125}I\alpha BT$ (●——●). The elution profiles were obtained using a Biorad P-2 chromatographic column (see Methods). Only 11 % of the radioactivity in the culture medium had the same elution profile as $^{125}I\alpha BT$. The remainder eluted after a longer delay, indicating that it was of lower molecular weight.

Estimates of $T_{\frac{1}{2}}$ were obtained in thirty-nine experiments similar to those illustrated in Fig. 1. The correlation coefficient for each set of data in these experiments was $0{\cdot}9960 \pm 0{\cdot}0007$. The resulting $T_{\frac{1}{2}}$ values are shown in Fig. 3. It can be seen that for tail preparations from embryos at stages 25–31 (1·15–1·56 days) the $T_{\frac{1}{2}}$ values were 53–55 h. They increased progressively during the next few days of development, attaining values of ~ 135 h (range: 120–158 h) by stage 47 (5·5 days). Thereafter $T_{\frac{1}{2}}$ increased only slightly (~ 4 h/day) whereas during the first few days of development the rate of increase was ~ 20 h/day. In fact in cases where two experiments were done on the same batch of animals, the first at early stages when $T_{\frac{1}{2}}$ was < 71 h and the second 1–3 days later, the actual rate of increase in $T_{\frac{1}{2}}$ was 19 ± 5 h/day ($n = 5$).

Junctional and extrajunctional acetylcholine receptors

Since *Xenopus* myotomal muscle cells have extrajunctional as well as junctional AChRs even at the latest developmental stages examined in this study (Chow & Cohen, 1983; Owens & Kullberg, 1989) it was of interest to determine whether the changes in $T_{\frac{1}{2}}$ involved both categories of AChRs. This was done by estimating the $T_{\frac{1}{2}}$ of extrajunctional AChRs by radioautography in stage 47–48 (5·5–7·5 day) tail preparations and comparing these values with those obtained for the entire population of AChRs. Figure 4 illustrates the decline in extrajunctional radioactivity

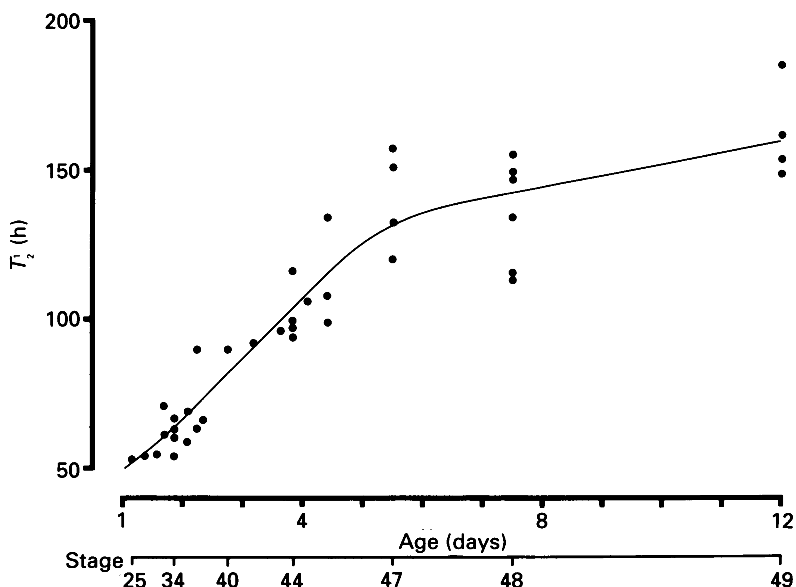

Fig. 3. Developmental changes in $T_{\frac{1}{2}}$. Each value represents a single experiment in which $T_{\frac{1}{2}}$ was estimated as shown in Fig. 1. The abscissa indicates the ages of the animals from which tail preparations were obtained and also shows some of the corresponding stages of Nieuwkoop & Faber (1967). Note that at early stages $T_{\frac{1}{2}}$ increased $\sim$ 20 h for each additional day of normal development whereas after stage 47 (5·5 days) there was relatively little increase. The smooth curve was drawn on the basis of linear regression analysis between stages 32–47 (1·67–5·5 days) and stages 47–49 (5·5–12 days).

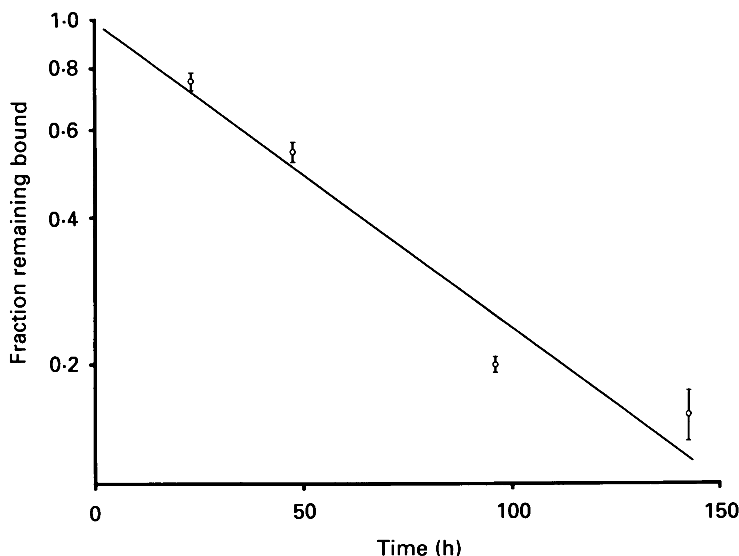

Fig. 4. Degradation of extrajunctional AChRs in stage 47 (5·5 day) tail preparations. After pulse-labelling fifteen tail preparations with ^{125}IαBT and counting their radioactivity, one group of three was fixed immediately while other groups of three were cultured for the times indicated and then counted and fixed. Subsequently the fixed tail preparations were processed for radioautography and grain counts were made in extrajunctional regions. Regression analysis of the data indicated that the degradation rate of the extrajunctional AChRs was 0·0139/h ($T_{\frac{1}{2}}$ = 50 h, r = 0·9822).

in one of three such experiments. Based on regression analysis the $T_{\frac{1}{2}}$ for the extrajunctional AChRs was 50 h whereas for the entire population of AChRs it was 152 h. In two other experiments the $T_{\frac{1}{2}}$ values for the entire population were 135 and 113 h whereas the values for the extrajunctional AChRs were 41 and 46 h respectively. These $T_{\frac{1}{2}}$ estimates for the extrajunctional AChRs at stages 47–48 are close to the value of 52 h obtained for the $T_{\frac{1}{2}}$ of AChRs in aneural cell cultures of embryonic *Xenopus* myotomal muscle (Brehm, Yeh, Patrick & Kidokoro, 1983) and

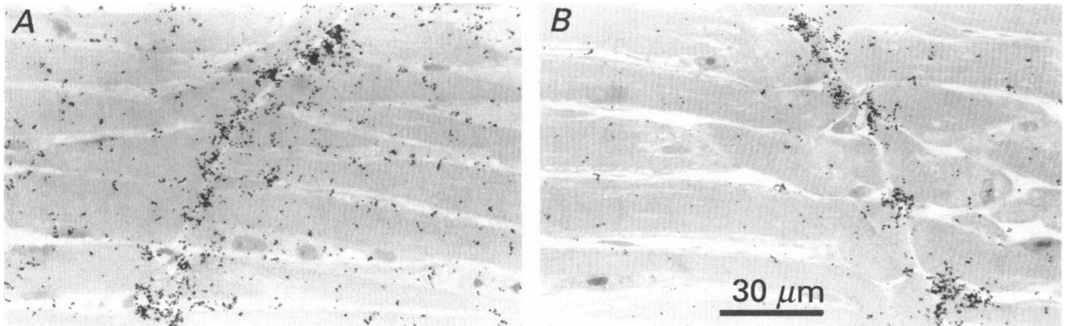

Fig. 5. Radioautographs of stage 48 (7·5 day) tail preparations. Both tail preparations were pulse-labelled with $^{125}I\alpha BT$; one was fixed on the same day (*A*) and the other after 5 days of AChR degradation (*B*). Junctional sites of high grain density can be seen at the ends of the muscle cells of two adjacent myotomes, and extrajunctional grains can be seen elsewhere along the length of the cells. Note that after 5 days of AChR degradation (*B*) there was a large decline in extrajunctional grain density whereas dense clusters of grains were still apparent in the junctional region.

are also close to the lowest $T_{\frac{1}{2}}$ values obtained for the entire population of AChRs at the earliest stages (Fig. 3). It follows that in *Xenopus* myotomal muscle, as in rat and chick muscle (Burden, 1977*b*; Steinbach *et al.* 1979; Michler & Sakmann, 1980; Reiness & Weinberg, 1981; Steinbach, 1981), it is only the junctional AChRs which undergo a developmental increase in $T_{\frac{1}{2}}$. That junctional AChRs at stages 47–48 have a long $T_{\frac{1}{2}}$ is apparent from the fact that sites of very high grain density were still observed at the ends of the myotomal muscle cells (the main site of innervation) even after 5 days of degradation (Fig. 5).

According to the above findings, the loss of radioactivity due to degradation of the entire population of AChRs should follow a double exponential given by the relationship

$$y = J\mathrm{e}^{-\alpha_J t} + (1-J)\mathrm{e}^{-\alpha_E t},$$

where y is radioactivity in the tail preparation, t is time, α_J and α_E are the degradation rate constants for the junctional and extrajunctional AChRs, and J is that fraction of the AChR population which is junctional. Using this relationship one can solve for J by regression analysis after assigning values to α_J and α_E. For this purpose $T_{\frac{1}{2}}$ was assumed to be 50 h ($\alpha_E = 0{\cdot}0139$/h) for the extrajunctional AChRs and 210 h ($\alpha_J = 0{\cdot}0033$/h) for the junctional AChRs, similar to the $T_{\frac{1}{2}}$ value for junctional AChRs in rat muscle (Salpeter & Loring, 1985). Such analysis for stages 47–49 (5·5–12 days) yielded values of 80 ± 3 % ($n = 14$) for the proportion of the

junctional AChRs. Furthermore the correlation coefficients based on this regression analysis were $0{\cdot}9947 \pm 0{\cdot}0014$, thereby indicating that the decline of radioactivity for the entire population of AChRs was consistent with the two different degradation rates for junctional and extrajunctional AChRs at stages 47–49.

When the results for earlier stages were analysed in the same way the estimated proportion of junctional AChRs decreased with decreasing stage. However, the correlation coefficients (a) were not as high, (b) became progressively poorer as the stage decreased, and (c) improved when the $T_{\frac{1}{2}}$ of the junctional AChRs was assumed to be less than 210 h. Presumably in *Xenopus* myotomal muscle, as in rat muscle (Reiness & Weinberg, 1981), it takes a few days before the junctional AChRs reach their ultimate $T_{\frac{1}{2}}$ value.

Supporting evidence in this regard was obtained in experiments in which the AChRs were pulse-labelled with RαBT. When this was done on stage 47 (5·5 day) tail preparations, brightly fluorescent sites were still apparent at the ends of almost all myotomes even 5 days later (Fig. 6*A* and *B*). By contrast, when stage 32–34 (1·67–1·85 day) tail preparations were pulse-labelled with RαBT, the fluorescence at the ends of all myotomes was barely detectable 3 days later (Fig. 6*D*) even though it was initially bright (Fig. 6*C*). This more rapid disappearance of the fluorescence at stages 32–34 than at stage 47 was not associated with a corresponding widespread loss of junctional sites during culture of the early stage tail preparations. In fact, when the stage 32–34 tail preparations were freshly stained with RαBT even after 5 days in culture brightly fluorescent sites of high AChR density were still apparent at the ends of most myotomes (Fig. 6*E*) and more than 80% of the myotomes ($n = 514$) still exhibited at least partial innervation as judged by immunofluorescent staining of a synaptic vesicle antigen (Fig. 6*F*). Taken together these results support the conclusion that the $T_{\frac{1}{2}}$ of the junctional AChRs was shorter at the earlier developmental stages.

Changes in $T_{\frac{1}{2}}$ during organ culture

As noted above, the $T_{\frac{1}{2}}$ for the entire population of AChRs increased at a rate of ~ 20 h/day up to stage 47 (5·5 days; see Fig. 3). Since in individual experiments $T_{\frac{1}{2}}$ was estimated from measurements over a period of 5 days (see Fig. 1), the early stage tail preparations might have been expected to exhibit a progressively decreasing degradation rate with time in culture. But this was not the case. For example the stage 25 data in Fig. 1 reveal no apparent slowing in the rate of degradation. In fact based on the measurements between 0 and 65 h (day 0–day 3), 22–91 h (day 1–day 4), and 42–114 h (day 2–day 5) the $T_{\frac{1}{2}}$ values were 58 h, 55 h and 52 h respectively. Other experiments also failed to show any consistent increase in the $T_{\frac{1}{2}}$ of the ^{125}IαBT-labelled AChRs with time in culture (Fig. 7, filled symbols). On the other hand when early stage tail preparations were first cultured for 1–2 days and then pulse-labelled with ^{125}IαBT, the $T_{\frac{1}{2}}$ values (Fig. 7, open symbols) were significantly larger than those of the control preparations which were pulse-labelled immediately after dissection. These findings indicate that even in culture the AChRs underwent a developmental increase in $T_{\frac{1}{2}}$ but not when they had ^{125}IαBT bound to them.

Whereas the increase in $T_{\frac{1}{2}}$ at early stages was ~ 20 h/day for *in vivo* development

(see Fig. 3), the increase in $T_{\frac{1}{2}}$ after 1 day in culture was only 8 h and after 2 days in culture it was only 13 h (Fig. 7). That overall development was reduced in culture is also apparent from the results in Fig. 8 which shows developmental increases in the number of ^{125}IαBT binding sites/myotome in culture (open symbols) and *in vivo* (filled symbols). In contrast to the case for *in vivo* development, the increases in

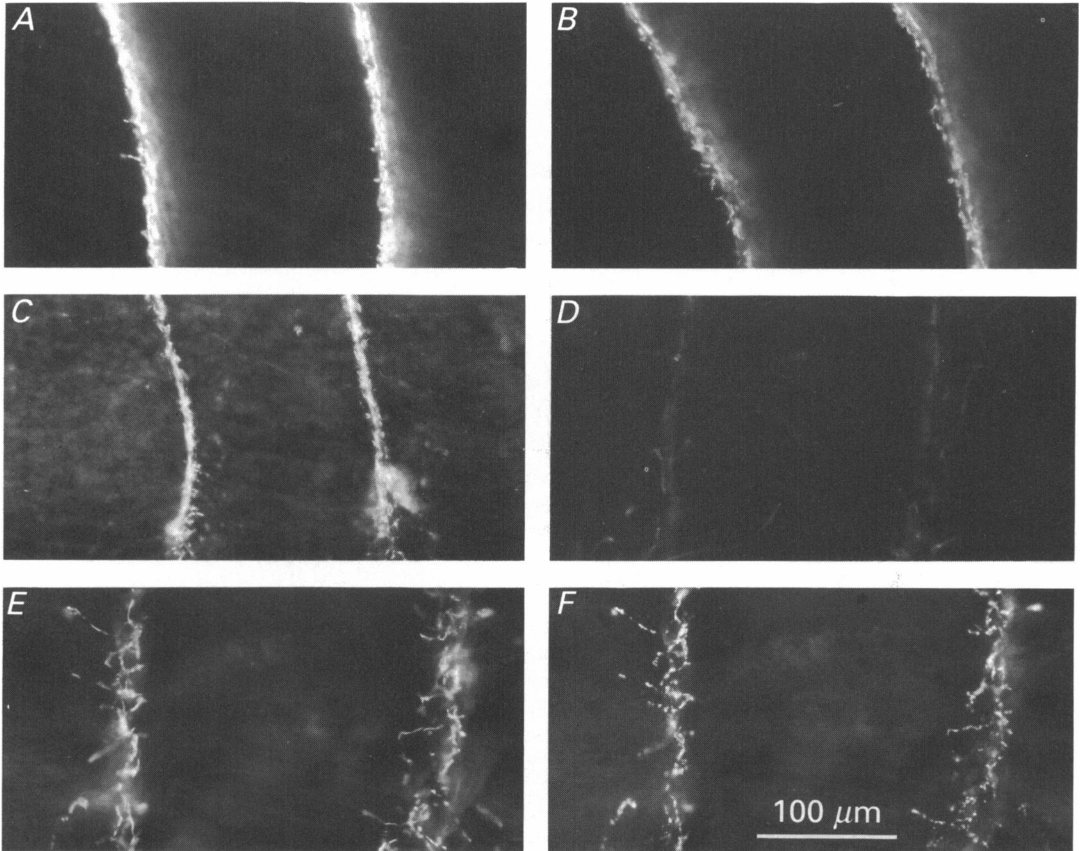

Fig. 6. Degradation of junctional AChRs revealed by pulse-labelling with RαBT. At stage 47 (*A* and *B*) brightly fluorescent sites of high AChR density were seen at the ends of the myotomes immediately after pulse-labelling (*A*) and were still apparent after 5 days of degradation (*B*). At stages 32–34 (*C–F*) the fluorescence was bright immediately after pulse-labelling (*C*) but was barely detectable after 3 days of degradation (*D*). Yet even after 6 days in culture fresh labelling with RαBT revealed that sites of high AChR density were still present at the ends of the myotomes (*E*) and that many were still innervated as judged by co-localized sites exhibiting bright immunofluorescent stain for a synaptic vesicle antigen (*F*).

culture were smaller and did not continue beyond the first day. The extensiveness of innervation also exhibited variable reductions in culture although some myotomes still appeared densely innervated even after 5 days (Fig. 6*F*). In view of these findings it is perhaps not unexpected that the developmental increases in $T_{\frac{1}{2}}$ were smaller in culture than *in vivo*.

Influence of motor activity

Tail preparations in culture did not exhibit any spontaneous motor activity. It was therefore possible that motor inactivity was an additional contributing factor in limiting the developmental increase in $T_{\frac{1}{2}}$ in culture. To test this possibility stage 20–26 (0·91–1·23 day) animals were maintained in the anaesthetic tricaine or in

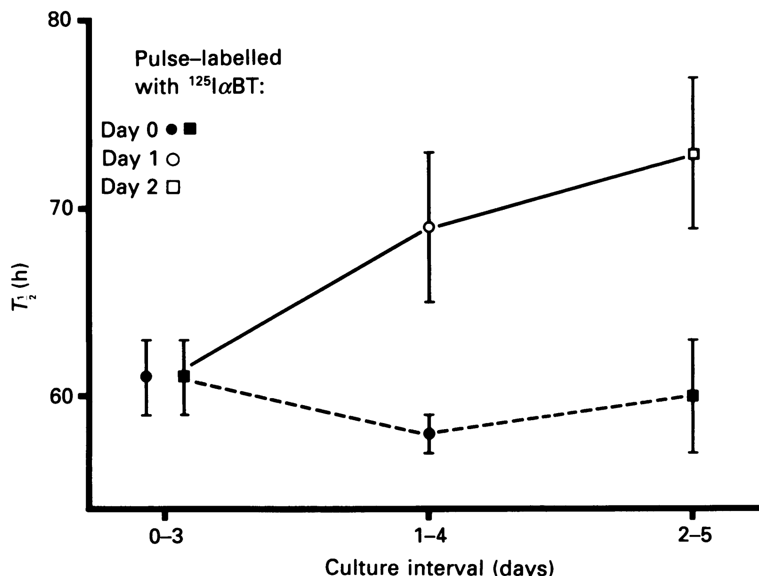

Fig. 7. Changes in $T_{\frac{1}{2}}$ during culture of early stage (stage 28–36) tail preparations. For one set of four experiments (● and ○) tail preparations were divided into two groups. One group was pulse-labelled with ^{125}IαBT at the time of dissection, as usual, and two estimates of $T_{\frac{1}{2}}$ were obtained (●): one was based on measurements between day 0 and day 3 and the other on measurements between day 1 and day 4. The other group of tail preparations was pulse-labelled with ^{125}IαBT after 1 day in culture and $T_{\frac{1}{2}}$ was estimated from measurements between day 1 and day 4 (○). In a second set of six experiments (■ and □) the tail preparations were again divided into two groups. Two estimates of $T_{\frac{1}{2}}$ were obtained from one group which was pulse-labelled at the time of dissection (■): one estimate was based on measurements between day 0 and day 3 and one on measurements between day 2 and day 5. The other group was pulse-labelled after 2 days in culture (□). Note that $T_{\frac{1}{2}}$ increased with time in culture only for the case where pulse-labelling with ^{125}IαBT was delayed for 1 or 2 days. The correlation coefficients (see Fig. 1) for each of the six estimates of $T_{\frac{1}{2}}$ shown in the figure were not less than $0{\cdot}9942 \pm 0{\cdot}0028$.

tetrodotoxin for 3–4 days during which time even prodding with a pin failed to evoke any motor responses (see also Cohen *et al.* 1984; Kullberg *et al.* 1985). Yet tail preparations from these animals exhibited the same developmental increase in $T_{\frac{1}{2}}$ as those from control animals (Table 1). It follows that developmental increases in $T_{\frac{1}{2}}$ can occur in the absence of motor activity and that motor inactivity was not responsible for the smaller increases in culture than *in vivo*.

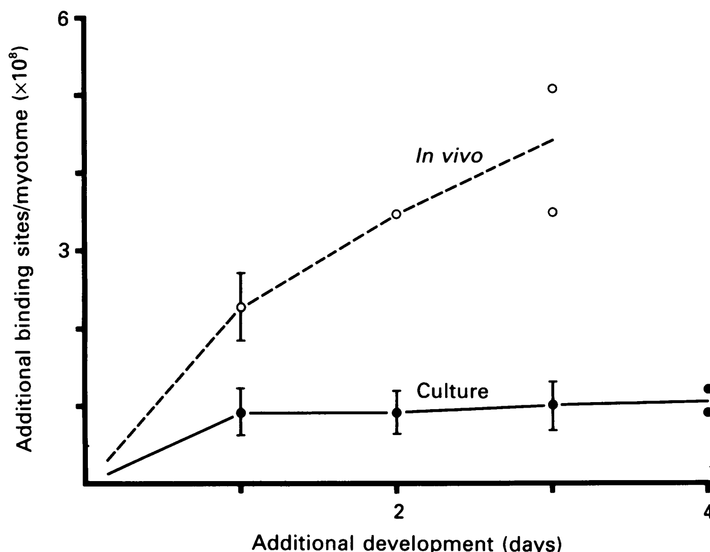

Fig. 8. Developmental increases in the number of AChRs *in vivo* (○) and in culture (●). For development *in vivo* specific binding of ^{125}IαBT/myotome was measured first in a set of tail preparations at an early stage (stages 25–38) and then again in a second set of tail preparations obtained from the same batch of animals after 1–3 days of further development. The latter measurement was subtracted from the former one in order to obtain the developmental increase in binding sites/myotome. Note that the daily increase *in vivo* was 1·5–2 × 10^8 sites/myotome per day, which is in agreement with values obtained in previous studies(Chow & Cohen, 1983; Goldfarb *et al.* 1990). For development in culture a set of tail preparations was obtained at an early stage (stages 32–38) and specific binding/myotome was measured immediately after dissection as well as after 1–4 days in culture. Note that the developmental increase in culture was smaller than *in vivo* and did not continue beyond the first day. Where standard error bars are included, the values are based on three to six experiments.

TABLE 1. $T_{\frac{1}{2}}$ in tail preparations from animals raised in anaesthetic

		$T_{\frac{1}{2}}$ (h)	
Anaesthetic	Number of experiments	Control animals	Anaesthetic-reared animals
Tricaine	4	104 ± 2	102 ± 4
Tetrodotoxin	1	104	106

As described in the Methods, stage 20–26 animals were kept in tricaine or tetrodotoxin for 3–4 days until they reached stage 43–46. During the entire period they exhibited no motor activity even in response to prodding with a pin. Control animals from the same clutches were not exposed to the anaesthetics.

DISCUSSION

Degradation of extrajunctional and junctional acetylcholine receptors

At stage 25 (1·15 days), when the $T_{\frac{1}{2}}$ of the entire population of AChRs was found to be 53 h, the innervation of the myotomes is less than 8 h old (Blackshaw & Warner, 1976; Kullberg, Lentz & Cohen, 1977; Chow & Cohen, 1983). In addition the junctional sites of high AChR density are relatively small and sparse so that in

radioautographs the grains appear to be essentially uniformly distributed along the entire length of the myotomes (Chow & Cohen, 1983). It follows that at this early stage the great majority of AChRs are extrajunctional. Indeed the $T_{\frac{1}{2}}$ of 53–55 h at stages 25–31 (1·15–1·56 days) matches well with the $T_{\frac{1}{2}}$ of 52 h obtained for AChRs on embryonic *Xenopus* myotomal muscle cells grown in culture without nerve cells (Brehm *et al.* 1983).

Even at stages 47–48 (5·5–7·5 days), when the apparent $T_{\frac{1}{2}}$ of the entire population of AChRs had increased markedly, the $T_{\frac{1}{2}}$ of the extrajunctional AChRs was still $\sim$ 2 days. It is apparent that the increase in $T_{\frac{1}{2}}$ was restricted to the junctional AChRs and that in *Xenopus* myotomal muscle, as in rodent muscle (Steinbach *et al.* 1979; Michler & Sakmann, 1980; Reiness & Weinberg, 1981; Steinbach, 1981), there is no accompanying developmental increase in the $T_{\frac{1}{2}}$ of the extrajunctional AChRs.

The $T_{\frac{1}{2}}$ of extrajunctional AChRs in mammalian muscle and in chick muscle is $\sim$ 1 day at 37 °C (Fambrough, 1979), and for the case of chick muscle it is more than 10 days at 24 °C (Devreotes & Fambrough, 1975). That the corresponding value for *Xenopus* myotomal muscle at 23–25 °C is $\sim$ 2 days presumably reflects a poikilotherm–homeotherm difference.

Comparison with other synaptogenic events

The results of Fig. 3 suggest that $T_{\frac{1}{2}}$ has begun to increase by about stage 32 (1·67 days), less than 1 day after the onset of innervation. In rat muscle too the $T_{\frac{1}{2}}$ of the junctional AChRs begins to increase shortly after innervation. For example, the $T_{\frac{1}{2}}$ of the junctional AChRs in rat diaphragm at fetal day 17, 3 days after the onset of innervation, was found to be $\sim$ 34 h as compared with a value of $\sim$ 24 h for the extrajunctional AChRs (see Fig. 4 in Reiness & Weinberg, 1981). These findings for *Xenopus* and rat muscle contrast with those for chick muscle where the $T_{\frac{1}{2}}$ of the junctional AChRs does not begin to increase until $\sim$ 1 month after the onset of innervation (Burden, 1977*b*; Betz *et al.* 1980).

In addition to the increase in $T_{\frac{1}{2}}$, AChRs having short channel open times also begin to appear at about stage 32 (Kullberg & Kasprzak, 1985; Owens & Kullberg, 1989). This conversion from slow to fast channels is essentially complete by stage 47 (5·5 days), the same stage that we find the conversion to a long $T_{\frac{1}{2}}$ to be complete. That both changes follow a similar time course in *Xenopus* myotomal muscle may be fortuitous. In rat muscle the change in AChR degradation precedes the change in channel open time (Michler & Sakmann, 1980; Schuetze & Role, 1987) and in chick muscle there is no conversion at all from slow to fast channels (Schuetze, 1980). Clearly, in the latter muscles the two developmental changes are regulated differently. This is probably also the case for *Xenopus* myotomal muscle since the conversion from slow to fast channels involves all AChRs (Kullberg *et al.* 1981; Kullberg & Kasprzak, 1985; Owens & Kullberg, 1989) whereas we find that, as in rodent and chick muscle, the conversion from a short to a long $T_{\frac{1}{2}}$ is restricted to the junctional AChRs.

Regulation of acetylcholine receptor degradation

The developmental change in AChR degradation was not affected when the animals were raised in tricaine or tetrodotoxin (Table 1) which abolished all motor

activity. Previous studies have indicated that nerve and muscle action potentials are also eliminated under these rearing conditions (Cohen *et al.* 1984; Kullberg *et al.* 1985). It follows that in *Xenopus* myotomal muscle the developmental change in AChR degradation does not require nerve or muscle impulse activity or muscle contraction.

A developmental increase in $T_{\frac{1}{2}}$ also occurred during organ culture, but only amongst those AChRs which were not pre-labelled with ^{125}IαBT. To explain this finding we suggest three possibilities. (1) It may be that the increase in $T_{\frac{1}{2}}$ depends upon insertion of new, molecularly different AChRs into the surface membrane. If this is the case then it is also necessary to assume that these new AChRs are inserted directly into the postsynaptic membrane (since extrajunctional AChRs do not exhibit a developmental increase in $T_{\frac{1}{2}}$) or become metabolically stable only in the postsynaptic membrane as a result of an interaction with other molecular components of the synapse. (2) Alternatively, it is unnecessary to invoke a new molecular species of AChR. Instead it could be that the conversion mechanism involves activation of the AChR by acetylcholine. With ^{125}IαBT bound to the AChR there would be no activation by acetylcholine and therefore no conversion. Since conversion occurred in tricaine- and tetrodotoxin-reared animals activation by spontaneously released acetylcholine would have to be sufficient to trigger the conversion. (3) Another possibility is that conversion to a long $T_{\frac{1}{2}}$ involves an interaction between the external portion of the AChR and other molecules in the synaptic region, and that this interaction is sterically hindered when ^{125}IαBT is bound to the unconverted AChR.

At present it is not known for other muscles whether the developmental conversion from a short to a long $T_{\frac{1}{2}}$ can occur under conditions which abolish nerve and muscle impulse activity and whether it is prevented when the AChRs have ^{125}IαBT bound to them. Studies on *mature* rodent muscle have indicated that denervation (Loring & Salpeter, 1980; Bevan & Steinbach, 1983), treatment with tetrodotoxin (Cangiano *et al.* 1987) and treatment with botulinum toxin or αBT (Avila *et al.* 1989) all cause the $T_{\frac{1}{2}}$ of mature junctional AChRs to decline from ~ 10 to ~ 3 days. Additional findings indicate that even when they have ^{125}IαBT bound to them, mature junctional AChRs in denervated rodent muscle reacquire a long $T_{\frac{1}{2}}$ upon reinnervation (Salpeter *et al.* 1986). In the latter study only $\sim 20\%$ of the AChRs were labelled with ^{125}IαBT so that reinnervation was presumably associated with the return of neuromuscular transmission. Considered altogether the findings suggest that some event associated with neuromuscular transmission, such as muscle contraction but not activation of AChRs by acetylcholine, regulates the $T_{\frac{1}{2}}$ of mature junctional AChRs in rodent muscle. However, muscle activity may not be the only factor. Whereas the $T_{\frac{1}{2}}$ of the mature junctional AChRs falls to ~ 3 days following denervation, newly inserted AChRs at the same denervated neuromuscular junctions have a $T_{\frac{1}{2}}$ of ~ 1 day (Shyng & Salpeter, 1989).

Perhaps the simplest way to reconcile the findings on mature rodent muscle with our findings on developing *Xenopus* myotomal muscle is to assume that in rodent muscle conversion of the $T_{\frac{1}{2}}$ of junctional AChRs from ~ 1 to ~ 3 days involves a mechanism which is independent of muscle activity and further conversion to a $T_{\frac{1}{2}}$ of ~ 10 days is activity dependent. That the entire conversion is independent of muscle

activity in *Xenopus* myotomal muscle is consistent with studies which have revealed that muscle activity plays a much less important role in regulating other properties, such as synaptic cholinesterase, in *Xenopus* myotomal muscle (Cohen *et al.* 1984: Kullberg *et al.* 1985) than in rodent muscle (Weinberg & Hall, 1979; Lømo & Slater, 1980).

This work was supported by the Medical Research Council of Canada.

REFERENCES

AVILA, O. L., DRACHMAN, D. B. & PESTRONK, A. (1989). Neuromuscular transmission regulates stability of acetylcholine receptors at the neuromuscular junction. *Journal of Neuroscience* **9**, 2902–2906.

BALDWIN, T. J., YOSHIHARA, C. M., BLACKMER, K., KINTER, C. R. & BURDEN, S. J. (1988). Regulation of acetylcholine receptor transcript expression during development in *Xenopus laevis*. *Journal of Cell Biology* **106**, 469–478.

BERG, D. & HALL, Z. W. (1975). Loss of α-bungarotoxin from junctional and extrajunctional acetylcholine receptors in rat diaphragm muscle *in vivo* and in organ culture. *Journal of Physiology* **252**, 771–789.

BETZ, H., BOURGEOIS, J.-P. & CHANGEUX, J.-P. (1980). Evolution of cholinergic proteins in developing slow and fast skeletal muscle in chick embryo. *Journal of Physiology* **302**, 197–218.

BEVAN, S. & STEINBACH, J. H. (1977). The distribution of α-bungarotoxin binding sites on mammalian skeletal muscle developing *in vivo*. *Journal of Physiology* **267**, 195–213.

BEVAN, S. & STEINBACH, J. H. (1983). Denervation increases the degradation rate of acetylcholine receptors at end-plates *in vivo* and *in vitro*. *Journal of Physiology* **336**, 159–177.

BLACKSHAW, S. & WARNER, A. (1976). Onset of acetylcholine sensitivity and endplate activity in developing myotome muscles in *Xenopus*. *Nature* **262**, 217–218.

BREHM, P., YEH, E., PATRICK, J. & KIDOKORO, Y. (1983). Metabolism of acetylcholine receptors on embryonic amphibian muscle. *Journal of Neuroscience* **3**, 101–107.

BURDEN, S. (1977*a*). Development of the neuromuscular junction in the chick embryo: the number, distribution and stability of acetylcholine receptors. *Developmental Biology* **57**, 317–329.

BURDEN, S. (1977*b*). Acetylcholine receptors at the neuromuscular junction: developmental change in receptor turnover. *Developmental Biology* **61**, 79–85.

CANGIANO, A., FUMAGALLI, G. & LØMO, T. (1987). Acetylcholine receptors at rat neuromuscular junction turn over rapidly after nerve block by TTX. *Journal of Physiology* **390**, 174*P*.

CHOW, I. & COHEN, M. W. (1983). Developmental changes in the distribution of acetylcholine receptors in the myotomes of *Xenopus laevis*. *Journal of Physiology* **339**, 553–571.

COHEN, M. W., GRESCHNER, M. & TUCCI, M. (1984). *In vivo* development of cholinesterase at a neuromuscular junction in the absence of motor activity in *Xenopus laevis*. *Journal of Physiology* **348**, 57–66.

COHEN, M. W., RODRIGUEZ-MARIN, E. & WILSON, E. M. (1987). Distribution of synaptic specializations along isolated motor units formed in *Xenopus* nerve–muscle cultures. *Journal of Neuroscience* **7**, 2849–2861.

DEVREOTES, P. N. & FAMBROUGH, D. M. (1975). Acetylcholine receptor turnover in membranes of developing muscle fibers. *Journal of Cell Biology* **65**, 335–358.

FAMBROUGH, D. M. (1979). Control of acetylcholine receptors in skeletal muscle. *Physiological Reviews* **59**, 165–227.

FISCHBACH, G. D. & SCHUETZE, S. M. (1980). A post-natal decrease in acetylcholine channel open time at rat end-plates. *Journal of Physiology* **303**, 125–137.

FRAIR, P. M. & COHEN, M. W. (1981). Degradation of acetylcholine receptors in innervated myotomes of *Xenopus laevis* maintained in organ culture. *Society for Neuroscience Abstracts* **7**, 838.

GOLDFARB, J., CANTIN, C. & COHEN, M. W. (1990). Intracellular and surface acetylcholine receptors during the normal development of a frog skeletal muscle. *Journal of Neuroscience* **10**, 500–507.

KULLBERG, R. W., BREHM, P. & STEINBACH, J. H. (1981). Nonjunctional acetylcholine receptor channel open time decreases during development of *Xenopus* muscle. *Nature* **289**, 411–413.

KULLBERG, R. & KASPRZAK, H. (1985). Gating kinetics of nonjunctional acetylcholine receptor channels in developing *Xenopus* muscle. *Journal of Neuroscience* **5**, 970–976.

KULLBERG, R. W., LENTZ, T. L. & COHEN, M. W. (1977). Development of the myotomal neuromuscular junction in *Xenopus laevis*: an electrophysiological and fine-structural study. *Developmental Biology* **60**, 101–129.

KULLBERG, R., OWENS, J. L. & VICKERS, J. (1985). Development of synaptic currents in immobilized muscle of *Xenopus laevis*. *Journal of Physiology* **364**, 57–68.

LØMO, T. & SLATER, C. R. (1980). Control of junctional acetylcholinesterase by neural and muscular influences in the rat. *Journal of Physiology* **303**, 191–202.

LORING, R. H. & SALPETER, M. M. (1980). Denervation increases turnover rate of junctional acetylcholine receptors. *Proceedings of the National Academy of Sciences of the USA* **77**, 2293–2297.

MATTHEW, W. D., TSAVELER, L. & REICHARDT, L. F. (1981). Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *Journal of Cell Biology* **91**, 257–269.

MICHLER, A. & SAKMANN, B. (1980). Receptor stability and channel conversion in the subsynaptic membrane of the developing mammalian neuromuscular junction. *Developmental Biology* **80**, 1–17.

NIEUWKOOP, P. D. & FABER, J. (1967). *Normal Table of Xenopus laevis* (*Daudin*), 2nd edn. North-Holland, Amsterdam.

OWENS, J. L. & KULLBERG, R. (1989). *In vivo* development of nicotinic acetylcholine receptor channels in *Xenopus* myotomal muscle. *Journal of Neuroscience* **9**, 1018–1028.

REINESS, C. G. & WEINBERG, C. B. (1981). Metabolic stabilization of acetylcholine receptors at newly formed neuromuscular junctions in rat. *Developmental Biology* **84**, 247–254.

SAKMANN, B. & BRENNER, H. R. (1978). Change in synaptic channel gating during neuromuscular development. *Nature* **276**, 401–402.

SALPETER, M. M., COOPER, D. L. & LEVITT-GILMOUR, T. (1986). Degradation rates of acetylcholine receptors can be modified in the postjunctional plasma membrane of the vertebrate neuromuscular junction. *Journal of Cell Biology* **103**, 1399–1403.

SALPETER, M. M. & LORING, R. H. (1985). Nicotinic acetylcholine receptors in vertebrate muscle: properties, distribution and neural control. *Progress in Neurobiology* **25**, 297–325.

SCHUETZE, S. M. (1980). The acetylcholine channel open time in chick muscle is not decreased following innervation. *Journal of Physiology* **303**, 111–124.

SCHUETZE, S. M. & ROLE, L. W. (1987). Developmental regulation of nicotinic acetylcholine receptors. *Annual Review of Neuroscience* **10**, 403–457.

SHYNG, S.-L. & SALPETER, M. M. (1989). Degradation rate of acetylcholine receptors inserted into denervated vertebrate neuromuscular junctions. *Journal of Cell Biology* **108**, 647–651.

STEINBACH, J. H. (1981). Developmental changes in acetylcholine receptor aggregates at rat skeletal neuromuscular junctions. *Developmental Biology* **84**, 267–276.

STEINBACH, J. H., MERLIE, J., HEINEMANN, S. & BLOCH, R. (1979). Degradation of junctional and extrajunctional acetylcholine receptors by developing rat skeletal muscle. *Proceedings of the National Academy of Sciences of the USA* **76**, 3547–3551.

WEINBERG, C. B. & HALL, Z. W. (1979). Junctional form of acetylcholinesterase restored at nerve-free end plates. *Developmental Biology* **68**, 631–635.