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CHANGES IN KINETICS OF ACETYLCHOLINE RECEPTOR CHANNELS AFTER INITIAL EXPRESSION IN *XENOPUS* MYOCYTE CULTURE

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SUMMARY

1. Developmental changes in single acetylcholine (ACh) receptor channel properties were analysed in cultured *Xenopus* myocytes at low temperatures (11–16 °C) using the cell-attached patch clamp technique. Single-channel recordings were done at fourteen stages of development ranging from several hours (stage 21) to 5 days (stage 47) after ACh receptors first appear in the muscle membrane.

2. Two types of channels, low conductance and high conductance, which have been described previously, were observed at all stages. At low concentrations of ACh, channel events often occurred as bursts of openings separated by closures briefer than 1 ms. Such bursts were treated as one event. Many brief, isolated channel openings, which were described in the preceding paper, were also observed. Developmental changes in burst duration, brief openings and brief closures were assessed for the period studied in culture.

3. Throughout development, most of the burst duration histograms for the low-conductance channel were not well fitted by a single exponential, having an excess of brief openings. The brief component could be largely accounted for by singly liganded openings, as described in the preceding paper. The burst durations reported here represent the main component of the distribution. At 150 nM-ACh, high-conductance channel burst duration histograms were well fitted by single exponentials.

4. There was a developmental increase in the percentage of single-channel events belonging to the high-conductance class. The percentage of high-conductance events remained low (less than 10%) for the first day after ACh receptors appeared (stages 20–34), and increased to 60% at stage 47.

5. In addition to a shift in channel population, there was a decrease in both low- and high-conductance channel burst durations during early stages of development (mostly within the first day in culture). The effect was more dramatic for the low-conductance channel; overall, low-conductance channel burst duration at 50 mV hyperpolarization over the resting potential decreased from 24 ms at stage 24/25 to 6 ms at stage 47, a 4-fold decrease. During the same period, high-conductance burst duration decreased from 10·6 to 4 ms, a 2·5-fold decrease. In contrast to the burst duration, the mean open time of 60 μs for the brief isolated class of opening in the low-conductance channel did not change with development.

6. Neither the single-channel conductance nor the voltage dependence of burst

duration changed with development. Conductances were 29 and 43 pS for the low- and high-conductance channels, respectively; the voltage producing an e-fold change in burst duration was about 80 mV for the low-conductance channel and about 60 mV for the high-conductance channel.

7. The developmental decrease in burst duration was observed only at early stages. When receptors at later stages were blocked with α-bungarotoxin, the burst duration of newly inserted receptor channels was already shortened. The percentage of high-conductance events for the new receptor population was over three times greater than for the existing population in unblocked control cells of the same stage.

8. The frequency of channel events declined with recording time, probably due to desensitization. The rate of this decline at 150 nM-ACh was similar for both young and old myocytes, suggesting that the desensitization process does not change during this developmental period.

9. Closed time analysis was done at high temporal resolution for patches hyperpolarized 100 mV over the resting potential. For the low-conductance channel, the mean number of brief closures (gaps) within a burst decreased from 2·81 to 1·57 over development, while the mean closed time of brief closures (about 25 μs) remained stationary. For the same patches, the burst duration decreased by 5-fold. Therefore, the closing rate constant α was the major kinetic parameter that changed during the shortening of the burst duration. Development changes in these parameters were not measured for the high-conductance channel.

INTRODUCTION

Single-channel analysis of acetylcholine (ACh)-induced activity in skeletal muscle has shown that two types of nicotinic acetylcholine receptors appear during development in various preparations. The embryonic or extrajunctional ACh receptor has a longer channel open time and lower unitary conductance; the adult or junctional ACh receptor has a brief open time and a unitary conductance about 50% larger than the low-conductance channel (Hamill & Sakmann, 1981; Brehm, Steinbach & Kidokoro, 1982; Kidokoro, Brehm & Gruener, 1982; Brehm, Kidokoro & Moody-Corbett, 1984*a*; Brehm, Kullberg & Moody-Corbett, 1984*b*; Leonard, Nakajima, Nakajima & Takahashi, 1984; Siegelbaum, Trautman & Koenig, 1984; Greenberg, Nakajima & Nakajima, 1985; Vicini & Schuetze, 1985; Auerbach & Lingle, 1986; Kullberg, Owens & Brehm, 1986; Igusa & Kidokoro, 1987; Leonard, Nakajima, Nakajima & Carlson, 1988; Owens & Kullberg, 1989).

We have used the *Xenopus* muscle culture system to study developmental changes in ACh receptor function. In myotomal muscle which is dissociated prior to innervation, and maintained in aneural culture, both classes of ACh receptor channels appear (Clark & Adams, 1981; Brehm *et al.* 1984*a*; Greenberg *et al.* 1985; Leonard *et al.* 1984, 1988). In culture, there is a developmental transition from the low-conductance channel, which predominates in early stages, to the high-conductance channel (Brehm *et al.* 1984*a*; Leonard *et al.* 1984, 1988), similar to the transition also observed *in vivo* (Kullberg *et al.* 1986; Owens & Kullberg, 1989). This replacement process is believed to involve the molecular substitution of the adult-

type ϵ-subunit for the γ-subunit (Mishina, Takai, Imoto, Noda, Takahashi, Numa, Methfessel & Sakmann, 1986).

A second developmental change which has been demonstrated recently in *Xenopus* myocyte culture (Leonard *et al.* 1984, 1988) is that the burst duration of the low-conductance receptor channel shortens dramatically during the first several days after ACh receptors initially appear in the membrane. A nearly 4-fold decrease was observed, with a majority of the decrease occurring during the first 24 h in culture. Similarly, Owens & Kullberg (1989) found that the apparent mean open time of low-conductance channels *in vivo* decreased by 50% during development.

Although these two changes in the kinetics of ACh receptor channels were documented previously in *Xenopus* myocyte culture, the time courses of both processes were not examined in the same experimental condition. In the same culture conditions, we found that between stages 34 and 40, these two changes overlap. The relation to other developmental events, such as functional synapse formation and receptor accumulation, is of particular interest when we consider the functional significance of these kinetic changes.

Another question which remains from these previous reports is whether the shortening of the burst duration in early development reflects a change in the channel closing rate constant α, or in the number of reopenings in one burst. Using the modified activation scheme shown in Fig. 1 of the preceding paper (Kidokoro & Rohrbough, 1990), we have analysed developmental changes in ACh receptor channel kinetics to address this question. Here we have examined the time course of the shortening of ACh receptor channel burst duration, with particular attention to the first two days in culture where this change primarily takes place.

It was reported for *Xenopus* muscle culture that the apparent mean open time varied from one patch to another, and that the apparent mean open time of the high-conductance channel was positively correlated with that of the low-conductance channel in the same patch (Igusa & Kidokoro, 1987). This suggests that whatever the mechanism which affects the mean open time, it influences both types of channel in the same direction. If the developmental change in burst duration does not follow this rule, the underlying mechanism must be different from the mechanism causing variations in the mean open time. We were therefore particularly interested in the differential development of low- and high-conductance channels reported by Leonard *et al.* (1984, 1988), who found that while the mean burst duration of the low-conductance channel shortened, the mean burst duration of the high-conductance channel did not change. In this study, in addition to the decrease in low-conductance burst duration, we also found a parallel decrease in the high-conductance channel burst duration. The burst durations for the two channel types were positively correlated throughout development. These findings are consistent with the possibility that both receptor types are similarly modified during development. A preliminary report of this study has been published elsewhere (Rohrbough & Kidokoro, 1989).

METHODS

The methods and experimental conditions were as described in the preceding paper except as noted here.

Cell culture procedures

In this developmental study, we used embryos at stages 15–18. This developmental stage precedes the innervation of muscle *in vivo* (Kullberg, Lentz & Cohen, 1977); therefore, muscle cells in culture were removed from direct neural influence. In most cases, embryos were dissected at stage 15, but in several experiments which were done within 5–7 h after plating, stage 18 embryos were used so that cultured cells would have ACh receptors present in the surface membrane by this time. Embryos were carefully staged at the beginning of the dissection. For all cultures, at least three sister embryos from the same batch were grown intact for the time the cultures were maintained. The developmental stage of cells in culture was therefore expressed in terms of the stage of the intact embryos. At the time the electrophysiology was done, the control embryos did not vary by more than one stage, and experiments were in some cases classified as a range of two stages, such as stage 24/25. The cultures and control embryos were maintained at room temperature (21–23 °C) until the time of the experiment.

Electrophysiology

Single-channel current events activated by low concentrations of ACh were recorded from myocytes ranging from several hours (stage 21/22) to 5 days (stage 47) in culture. Cells were considered to be of the same developmental stage for the length of the experiment, which was of 4 h duration or less to minimize developmental changes during the experiment.

The concentrations of ACh used for burst duration analysis were 100–200 nM, with the majority of experiments done with 150 nM-ACh. The frequency of channel openings at this concentration was typically 2–3 s^{-1} or less, usually with modest desensitization.

Data analysis

Single-channel currents were recorded at 5 kHz (in a few cases, 10 kHz) filter settings onto FM tape at a tape speed of 15 in s^{-1}. At this speed, the frequency response of the tape is 8 kHz. Data was digitized from the FM tape for burst duration analysis. Recordings were done at 20, 50, 90 and 100 mV applied hyperpolarization over the resting potential. The burst duration data reported here, except in Fig. 7*A* and Table 1, was recorded at 50 mV applied hyperpolarization. In experiments using young muscle cells, many single-channel openings at this potential exceeded 100 and 200 ms in duration. In these cases, the data was digitized at 5 kHz, at which events of up to 200 ms could be displayed and measured. Records from older muscle were digitized at 10 kHz.

Digitized data was processed on a DEC 11/23 computer as previously described (Igusa & Kidokoro, 1987). The threshold for detection was usually set at 60–70% of the unitary current amplitude. In some cases, records were analysed separately for low-conductance and high-conductance events when the latter occurred in low numbers. Up to five events could be displayed on the screen at one time. Obvious noise and artifacts were eliminated by the operator. Overlapping events were not analysed for burst duration unless they were of separate amplitude classes and could be unambiguously distinguished.

At low concentrations of ACh (150 nM), single-channel events often occurred as a burst of several openings separated by closures briefer than 1 ms. Such closures within a burst were defined as 'gaps' and these bursts were treated as one event (Igusa & Kidokoro, 1987; Leonard *et al.* 1988).

Amplitude and burst duration histograms of each class of opening were made for each record. Burst duration histograms were fitted by eye with a semi-logarithmic scale to the best single exponential. For the low-conductance class, duration histograms were rarely fitted satisfactorily by a single exponential, usually having an excess of short-duration openings (less than 1–2 ms), as reported previously (Brehm *et al.* 1984*a*; Igusa & Kidokoro, 1987; Leonard *et al.* 1988; Owens & Kullberg, 1989). As demonstrated in the preceding paper (Kidokoro & Rohrbough, 1990), a great majority of brief openings were due to singly liganded receptor channels. For the purpose of analysing burst durations, the brief component was not studied and the fitting procedure was performed on the main (slow) component of the distribution. Duration histograms were also fitted to a single exponential by the method of maximum likelihood, in which the shortest duration bin (less than 1 or 2 ms) was disregarded. Values of the time constant from both of these methods were usually in close agreement.

In young cultures, where the number of high-conductance events was usually very low, the arithmetic mean burst durations of these events were also computed. For patches where a high-conductance class of openings was clearly present with at least twenty events, the mean burst

durations were used and are reported here. When 100–150 high-conductance events could be analysed, the burst duration normally fitted well to a single exponential, with a time constant usually within 10% of the arithmetic mean. Therefore, for this study, 'mean burst duration', where it is used, is defined as the arithmetic mean burst duration. When the number of high-conductance events was large (several hundred or more), the arithmetic mean and the time constant of the burst duration were the same.

In a few experiments, a third, lower amplitude class of opening was observed, similar to that previously reported (Brehm *et al.* 1984*b*; *Leonard et al.* 1988; Owens & Kullberg, 1989), that resembled the two major classes of events, but this class was not analysed. In addition, a fourth type of opening was occasionally seen which had a ragged, flickery appearance and rapid kinetics. This activity could be increased with negative pressure, and usually declined with time after seal formation. This channel resembled the pressure-activated channel which has been described for this preparation (Auerbach & Lingle, 1986; Leonard *et al.* 1988). Channel events of this type could usually be easily recognized and discarded. In a few cases, where such pressure-activated activity did not decline, the entire record was excluded from analysis.

Measurement of brief openings and gaps within a burst

A total of sixteen patches from myocytes of various stages were examined at high temporal resolution for assessment of developmental changes in kinetic parameters. The concentration of ACh ranged from 20 nM to 1 μM. These patches were hyperpolarized 100 mV over the resting potential and the current was filtered at 10 kHz and digitized at 40 kHz. These are the same patches used in the preceding paper for high-resolution kinetic analysis of brief openings and brief closures. Recording conditions and protocol for brief events analysis are the same as previously described (Kidokoro & Rohrbough, 1990).

RESULTS

Developmental changes in ACh receptor channel properties were studied in cultured *Xenopus* muscle ranging in age from several hours to 5 days in culture. The stage of the cells in culture was expressed as the stage of the intact control embryos at the beginning of the experiment. Because of slight variations in the rate of control embryo development, some experiments were classified as a range of two stages as stated in the Methods, such as stage 24/25. The youngest muscles recorded from were at stage 21; this is within 2 h after ACh receptors first appear in the muscle membrane around stage 19, as measured by macroscopic ACh sensitivity (Blackshaw & Warner, 1976; Kullberg *et al.* 1977), and by single-channel records in our laboratory. At this early stage, ACh receptor channel events often occurred at very low frequency or were occasionally not detected. Records were taken from six closely spaced developmental groups between stages 21/22 and 30, a 12 h period, to study the entire time course of the developmental changes in ACh receptor channel properties during this period. Most recordings were done with 150 nM-ACh in the pipette, which usually induced a sufficient number of events in the recording period (5–20 min) for kinetics analysis. The event frequency and relative proportions of the channel types varied among different experiments and with developmental age. A gradual decrease in event frequency, probably due to desensitization, was usually observed with time after seal formation even at 150 nM-ACh (see Kidokoro & Rohrbough, 1990).

In Fig. 1, representative single-channel current records are shown from three stages of cultured muscle. Records of ACh-activated single-channel events showed two discrete channel types, low conductance and high conductance, which differed in their single-channel current amplitude and conductance throughout the devel-

opmental period studied. These channel events closely resembled those previously described for this preparation (Brehm *et al.* 1982, 1984*a*, *b*; Igusa & Kidokoro, 1987; Leonard *et al.* 1984, 1988; Auerbach & Lingle, 1986; Owens & Kullberg, 1989). In records from younger muscle (stages 21–27), the high-conductance event occurred at low frequency or was not observed. The low-conductance event had a single-channel conductance of about 29 pS at the lowered temperatures (11–16 °C), while the high-conductance channel had a conductance of about 43 pS. The low-conductance events had longer burst durations on average than high-conductance events at all stages.

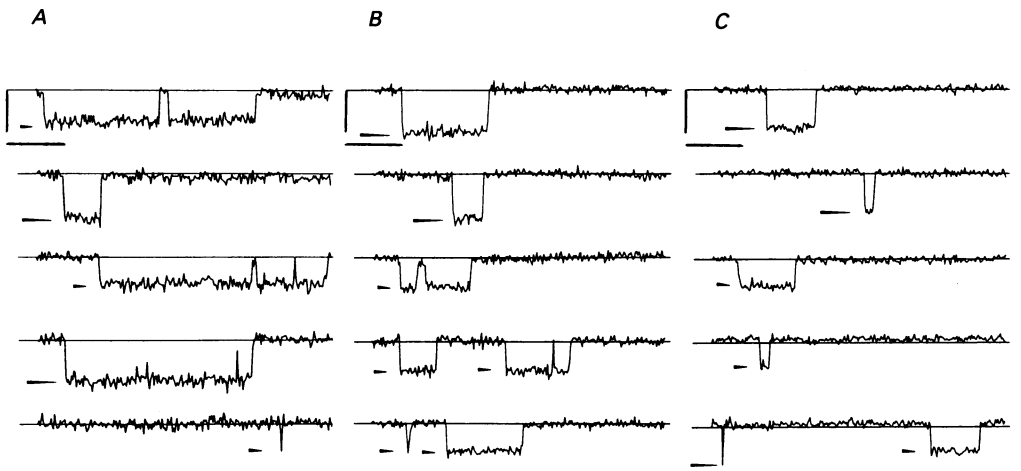

Fig. 1. Single ACh receptor channel current records from three stages of cultured muscle. Channel openings are shown as downwards deflections (inward current); scale bars are 5 pA (vertical bars at the left end of the uppermost traces) and 10 ms (horizontal bars). Both low- and high-conductance channels are present at each stage. Short arrow-heads mark low-conductance events and long arrow-heads mark high-conductance events. Note the presence of one or more brief closures in several of the bursts, and several very brief openings (less than 1 ms). *A* (stage 24/25, 14·2 °C) was recorded with 10 kHz filter; *B* (stage 37/38, 14·2 °C) and *C* (stage 44/45, 12·00) were recorded at 5 kHz. All traces were recorded with 50 mV applied hyperpolarizing potential over the resting potential and 150 nM-ACh.

Amplitude and burst duration histograms were constructed for each record. Figure 2 shows two types of ACh receptor channel events recorded at three developmental stages. The amplitude histogram was used to divide the low-conductance and high-conductance classes. For some young cultures (earlier than stage 30), a high-conductance amplitude peak was not clearly distinguishable. Such records were occasionally reanalysed separately for high-conductance events. The conductance and burst duration of the high-conductance event were included only if there were sufficient openings to make a clear measurement.

Developmental shift of channel population from the low- to high-conductance channel

Two developmental changes are evident in Fig. 2. The percentage of openings belonging to the high-conductance class increased with developmental age in culture (Fig. 2, first column), as has been previously established (Brehm *et al.* 1984*a*; Leonard *et al.* 1988). The percentage of high-conductance events for an entire

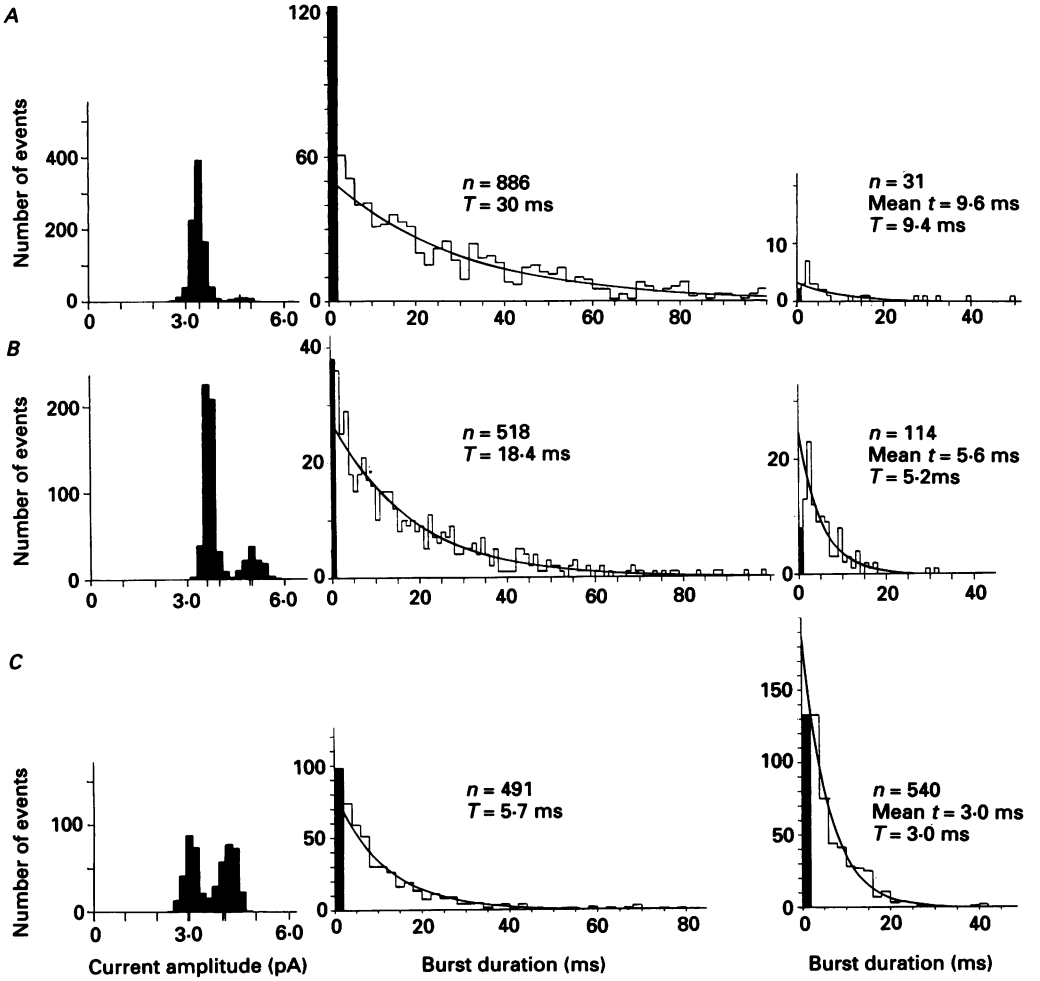

Fig. 2. Amplitude and burst duration histograms of single ACh receptor channel events. Records are from the same cells shown in Fig. 1. *A*, stage 24/25; *B*, stage 37/38; *C*, stage 44/45. First column, amplitude histograms for the three developmental stages. Two amplitude classes are present from the earliest stages studied, but in many experiments with young muscle, the high-conductance class was not present in sufficient numbers for analysis. The number of high-conductance events increases with the time in culture. Second and third columns, low- and high-conductance channel burst duration histograms. The best single exponential fit to the distribution, determined by the method of maximum likelihood, is given for each histogram (T). The shortest-duration bin (shown as the filled bin) was disregarded for curves fitted by the method of maximum likelihood. For the high-conductance channel burst duration records, the arithmetic mean burst duration (Mean t) is also given. For records with less than 100–150 high-conductance events, the mean burst duration was reported for high-conductance channel burst duration. The burst duration for both low- and high-conductance events decreased with development.

recording period was used. High-conductance openings did not begin to constitute a significant fraction until about stages 32–34, after which the percentage of high-conductance events began to rise. High-conductance openings accounted for 60% of all events recorded at stage 47. The time course of the increase in high-conductance

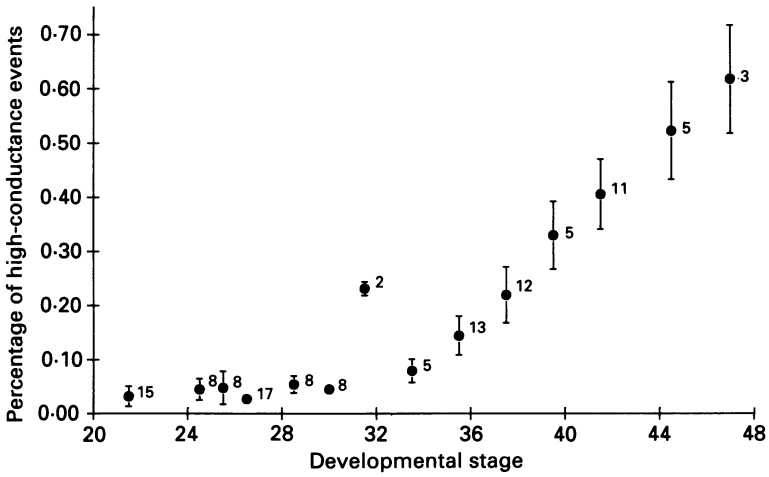

Fig. 3. The increase in the percentage of high-conductance events with successive stages of development. For each experiment, the percentage of high-conductance events during the recording period was determined. Each point is the mean ± S.E.M. for the patches from the indicated developmental stage(s). The experiments correspond to the stages also shown in Fig. 4. The number of patches examined is indicated for each point. The percentage of high-conductance events began to increase appreciably around stage 34.

events is shown in Fig. 3. This increase compared similarly with measurements made with development *in vivo* (Owens & Kullberg, 1989). It should be noted that the increase did not appear to occur more slowly in aneural culture than for innervated myocytes *in vivo*.

Developmental shortening of burst duration in both types of channel

The second developmental change evident in Fig. 2 is the decrease in burst duration of ACh receptor channel events with increasing developmental age (Fig. 2, second and third columns). This change was particularly pronounced for the low-conductance channel, which underwent a decrease in burst duration from 24·2 ± 5·2 ms (mean ± S.D.; $n = 7$; all data are expressed in this format) at stage 24/25 to 6·1 ± 2·0 ms ($n = 3$) at stage 47. The time course of this decrease is plotted in Fig. 4. The decrease occurred rapidly in the first 24–36 h in culture, and continued gradually over the range of stages examined.

The time courses of the changes in channel population and burst duration had a region of overlap in the range of stages 34–40. In Figs 3 and 4, it should be noted that the abscissa (stage) is not linear with time; the time between successive stages increases later in development (see lower axis in Fig. 4). There was a significant

decrease in burst duration before the percentage of high-conductance events had increased appreciably.

Nearly all the low-conductance burst duration histograms were best fitted by two or more exponentials. There was, in almost every case, an excess of short-duration openings (less than 1–2 ms). The nature of these brief openings was discussed in detail in the preceding paper (Kidokoro & Rohrbough, 1990).

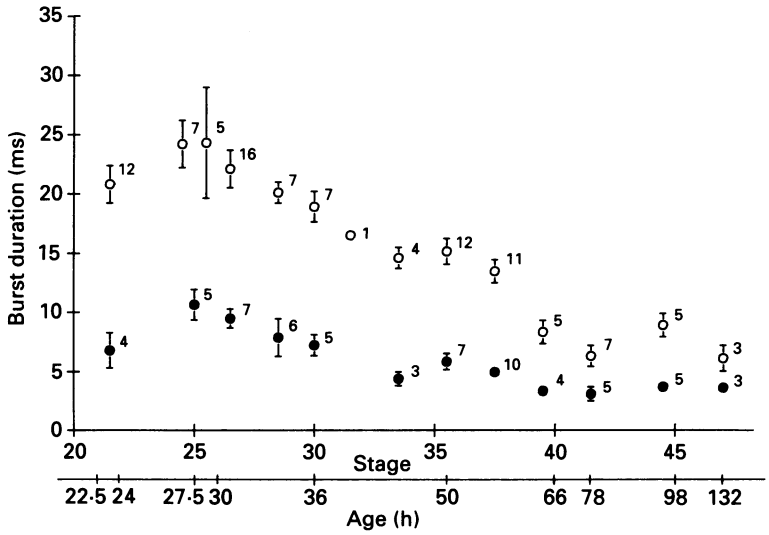

Fig. 4. The decrease in burst duration with development. Low-conductance channels are shown with open circles and high-conductance channels with filled circles, all burst durations were measured at 50 mV applied potential. Each point is the mean burst duration (±S.E.M.) of the patches from the indicated stage. For muscle past stage 35/36, most patches yielded both low- and high-conductance channel burst durations. Each point shown represents one stage (e.g. stage 30) or a two-stage group (e.g. stage 24/25) with one exception, stages 24–26 (plotted at stage 25) for the high-conductance class. The corresponding developmental age (in hours post-fertilization; Nieuwkoop & Faber, 1967) for the stages studied is shown on the lower axis. Note that the stages are not linearly related to time. ACh concentration was 150 nM in most cases (a few experiments were done at 100 or 200 nM).

In contrast to the previous report by Leonard *et al.* (1988), the mean burst durations of high-conductance channels were also found to shorten with development, though not as dramatically. The high-conductance burst duration was $10{\cdot}6 \pm 2{\cdot}9$ ms at stage 25 ($n = 5$), decreasing to $3{\cdot}6 \pm 0{\cdot}4$ ms ($n = 3$) by stage 47 (Fig. 4). This decrease is significant (at $P = 0{\cdot}01$, Student's t test, two tails). The decrease took place mainly during the first 24 h in culture, at stages when the occurrence of high-conductance events was rare. For culture stages 21–30, the number of high-conductance events used to compute mean burst duration varied from twenty-two to several hundred (average number of events: 84 ± 77, $n = 27$). A minimum of twenty events was arbitrarily established as a criteria for including the data.

Because of the low numbers of high-conductance events at early stages, there was

a possibility that in these records, the mean burst durations observed consistently overestimate the true burst duration. Although burst duration measurements made with fewer events at early stages are not as reliable and have greater variation than at later stages, we believe the longer high-conductance burst durations in young muscle are genuine for several reasons.

The duration histograms for the high-conductance channel have been demonstrated in previous studies to fit well to a single exponential (Brehm *et al.* 1984*a*, *b*; Leonard *et al.* 1984, 1988). Single-channel records were taken from later stage muscle, where the high-conductance burst duration histograms were well fitted by single exponentials. Four sets of twenty-five high-conductance channel events were analysed from different sections of the total record, and the mean burst durations for each set was computed. Although there was variation among the mean values, there was no tendency to consistently overestimate the overall burst duration for the entire record. More sets (eight of twelve) underestimated the overall value in three experiments. The mean burst duration for all 100 events agreed well with the value for the whole record (less than 15% error). For comparison, we used a program using a pseudo-random number generator which generated values to a single-exponential function, and compared the mean for records of twenty to fifty points with the known time constant. Even when as few as twenty-five values were averaged, the mean generally was within 20% of the true value. There was again no tendency for the means to consistently overestimate the actual time constant; in fact, most of the mean values underestimated the true time constant.

In addition, it was possible to record 100 or more high-conductance events in several experiments stages 21–29. When 100–150 events could be recorded, the distribution usually fitted well to a single exponential. Both the mean and fitted burst durations were compared for such cases, and for older cultures where many hundreds of events were recorded. Values obtained by these procedures rarely differed by more than a few per cent. Mean burst durations from these records were consistent with the values obtained from records with fewer events.

Correlation between low- and high-conductance channel burst durations

High- and low-conductance burst durations were also found to be positively correlated, as were high- and low-conductance channel mean open times (Igusa & Kidokoro, 1987). When the burst durations of high- and low-conductance channel events recorded from the same patch were plotted in several developmental groups, there was good correlation within each group as well as for all the combined data (Fig. 5). Patches with prolonged low-conductance burst duration tended to have high-conductance events with longer burst duration (correlation coefficient = 0·84); both types of channels had shorter burst durations with increasing developmental age.

Voltage dependence of the single-channel conductance

Recordings were done at 20, 50 and 90 or 100 mV hyperpolarization over the resting potential. The single-channel conductance of both the high- and low-conductance channels tended to increase at greater hyperpolarized potentials, as was

reported for *Xenopus* myocytes *in vivo* (Brehm *et al.* 1984*b*; Owens & Kullberg, 1989) and in culture (Brehm *et al.* 1984*a*); that is, the chord conductances between 50 and 90 mV or 50 and 100 mV were usually greater than the conductances between 20 and 50 mV for a given patch; over all the data, the voltage dependence of the

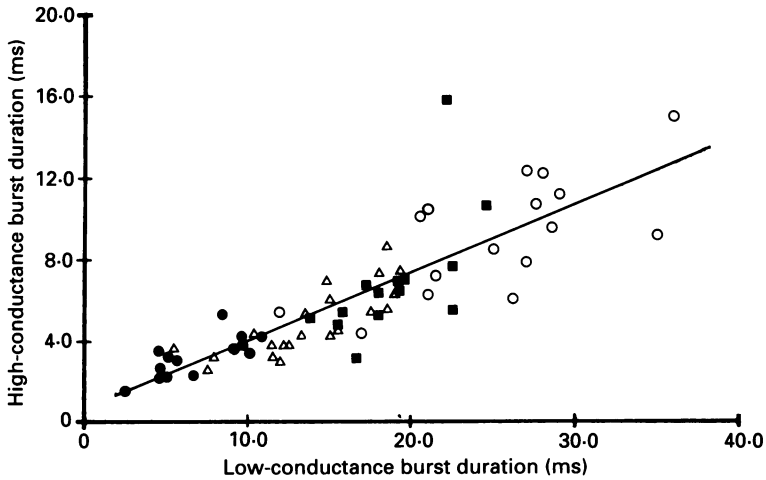

Fig. 5. The correlation of the high- and low-conductance channel burst duration. The burst durations are plotted for all patches from which both low- and high-conductance channel burst durations (at 50 mV applied hyperpolarizing potential) were measured. The symbols divide the data into four developmental groups for comparison. Open circles: stages 21–27; filled squares: stages 28–34; open triangles: stages 35–40; filled circles: stages 41–47. (Correlation coefficient = 0·84.)

conductance was more evident. The chord conductances between 20 and 50 mV applied hyperpolarization were 26·4 ± 3·9 pS (n = 15) and 38·3 ± 5·5 pS (n = 12) for low- and high-conductance channels, respectively; between 50 and 90 mV, conductances were 29·3 ± 3·9 pS (n = 31) and 43·2 ± 5·6 pS (n = 22), respectively. The conductance between 50 and 90 mV was significantly greater than between 20 and 50 mV for both channel types (at P = 0·05). Conductance and single-channel current amplitudes did not change during development.

Voltage dependence of burst duration

As shown in previous studies, the burst durations of both classes of ACh receptor channels increased with greater hyperpolarization (Fig. 6*A* and *B*). The high-conductance channel was more sensitive to voltage. About 60 mV hyperpolarization produced an e-fold change in high-conductance channel burst duration, while about 80 mV produced an e-fold change in low-conductance channels. The voltage dependence of the high-conductance channel was found to be somewhat greater than was previously measured in this culture system (Brehm *et al.* 1984*a*), but similar to that found *in vivo* (Owens & Kullberg, 1989). The voltage dependence of channel burst duration showed considerable variation among different experiments; this is evident in Fig. 6*A* and *B*, where it appears that the voltage dependence of the high-conductance channel burst duration changes. However, on average there was no

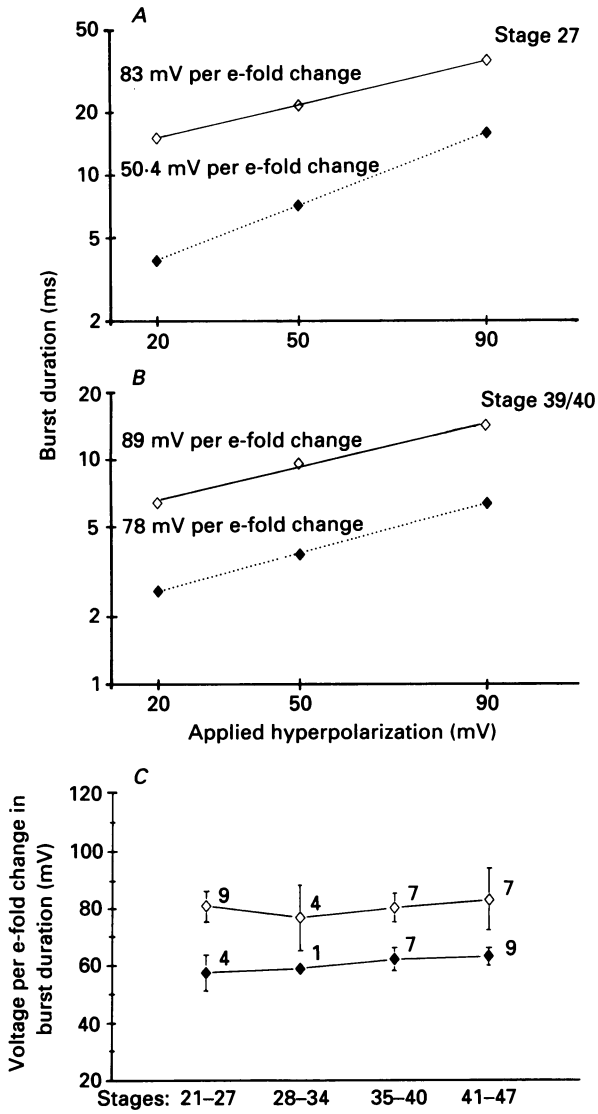

Fig. 6. The voltage dependency of burst duration. The burst durations at 20, 50 and 90 mV hyperpolarizing potentials are shown for cells at stages 27 and 39/40. Open symbols are low-conductance channels and filled symbols are high-conductance channels. The low-conductance channel burst duration was less sensitive to voltage for nearly every patch for which both low- and high-conductance channel burst durations were measured at two or more potentials. *A*, stage 27; *B*, stage 39/40. Voltage dependencies were obtained by linear regression. *C*, voltage dependence of burst duration (mean ± S.E.M.) is plotted for four developmental groups; stages 21–27, 28–34, 35–40 and 41–47. The ordinate is the potential change for an e-fold change in burst duration.

significant change during development in the voltage dependencies of either channel type, as shown in Fig. 6*C*.

Properties of newly incorporated receptors

The observed decrease in burst duration is largely complete after the second day in culture. By this time, muscle cells have a mixed population of older and younger ACh receptors present in the membrane due to the degradation of receptors, which has a half-time of about 50 h (Brehm, Yeh, Patrick & Kidokoro, 1983). Greenberg *et al.* (1985) found that newly synthesized receptors in cultured *Xenopus* muscle had similar channel properties to pre-existing ACh receptors. We also verified this finding in these experiments, and further observed that the percentage of high-conductance channel events was greater in the newly inserted receptor population.

Muscle cells in culture were treated with 2 μM-α-bungarotoxin, which binds to ACh receptors essentially irreversibly (Devreotes & Fambrough, 1975), for 60 min. The cells were then washed thoroughly to remove unbound toxin. Single-channel records were taken 6 h after removal of toxin, at stage 33/34 (which is about 45 h after ACh receptors first appear). In two cells a sufficient number of events was recorded for burst duration analysis.

In these cells, receptors were six or less hours old, but their channel gating properties were characteristic of ACh receptors which had been in the membrane for 45–50 h. In control stage 33/34 cultures, the low-conductance and high-conductance channel burst durations were $14{\cdot}6 \pm 1{\cdot}8$ ms ($n = 4$) and $4{\cdot}4 \pm 1{\cdot}1$ ms ($n = 3$), respectively, while for α-bungarotoxin-treated cells, burst durations were $11{\cdot}3 \pm 2{\cdot}4$ ms ($n = 2$) and $2{\cdot}7 \pm 0{\cdot}1$ ms ($n = 2$), respectively. The percentage of high-conductance events in the newly incorporated receptors was nearly three times greater than in stage 33–36 control cells ($37 \pm 16\%$, $n = 4$, *vs.* $13 \pm 13\%$, $n = 18$), reflecting the greater fraction of high-conductance receptors being newly synthesized and inserted at later stages of development.

Correlation of burst duration changes to kinetic parameters

For a number of muscle cells of various stages, recordings were done at low temperatures (11·2–15·8 °C) at 100 mV hyperpolarization over resting potential, and filtered at 10 kHz. Data records were then digitized at 40 kHz and analysed for developmental changes in brief openings, and brief closures (gaps) within bursts, as described in the preceding paper (Kidokoro & Rohrbough, 1990). Brief openings and brief closures were observed throughout development for both the low-conductance and high-conductance channels. Because of the difficulty in recording high-conductance events in sufficient numbers for the first day in culture, only the low-conductance channel was analysed for developmental changes in these kinetic parameters.

For the patches analysed for brief openings and closures, channel burst duration at 100 mV hyperpolarization was also measured (Fig. 7*A*). The burst durations shown include the pooled data from 20 nM to 1 μM concentrations of ACh. While not as extensive as Fig. 4, Fig. 7*A* demonstrates the dramatic period in which brief openings and closures were measured.

At 150 nM concentration of ACh, nearly all the low-conductance burst duration histograms had an excess of short openings, as noted above (Fig. 2). Most of the excess could be accounted for by the brief component which was analysed in the preceding paper. These brief openings, as described, almost always occurred in isolation. Under these high-resolution analysis procedures, the brief component could be easily separated from the main component of the burst duration. At 20 and 50 nM concentrations of ACh, an excess of brief events was also observed in high-conductance burst duration histograms (Fig. 2*B*, Kidokoro & Rohrbough, 1990). The low-conductance brief event had a mean open time of $63{\cdot}1 \pm 18{\cdot}1$ μs ($n = 15$), which did not vary with concentration. For data pooled from 20 nM to 1 μM concentrations of ACh (the brief opening was rarely observed with $10^{-}$$\mu$M-ACh), the mean open time did not change with developmental age (Fig. 7*B*).

The time constant of brief closures within a burst, as found in the preceding paper, was $25{\cdot}0 \pm 5{\cdot}1$ μs ($n = 16$) for the low-conductance channel and $32{\cdot}0 \pm 10{\cdot}2$ μs ($n = 10$) for the high-conductance channel. The brief closure time constant did not change with ACh concentration, and also remained stationary with development for the low-conductance channel (Fig. 8*A*). The measured mean closed time and total number of brief closures were corrected for missed events. The corrected number of gaps per burst was always greater for the low-conductance channel than for the high-conductance channel. As shown in Fig. 8*B*, the number of gaps per burst for the low-conductance channel decreased over the range of stages examined (stages 23–42). In early stages (23–28), the number of gaps per burst was $2{\cdot}81 \pm 0{\cdot}89$ ($n = 5$). At the latest stage analysed (stage 41/42), the number of gaps per burst was $1{\cdot}57 \pm 0{\cdot}15$ ($n = 3$). This difference between early and late stages was not statistically significant (at $P = 0{\cdot}05$). However, the decrease in the number of gaps per burst was significantly correlated with development over all the data (at $P = 0{\cdot}005$; correlation coefficient = 0·68), indicating that the observed decrease is real. For the high-conductance channel, the number of gaps per burst was $0{\cdot}85 \pm 0{\cdot}23$ ($n = 9$), for stage 35/36 and later.

The observed decrease in channel burst duration could be due to a decrease in the mean open time, $1/\alpha$, a decrease in the number of openings per burst ($\beta/k_{-2} + 1$), or a combination of both. The agonist dissociation rate k_{-2} increased significantly between early and late stages (at $P = 0{\cdot}05$), while β did not change. The channel closing rate α increased by over 3-fold from early to late stages, while burst duration decreased by 5-fold in the same patches. The apparent decrease in the number of openings per burst by a factor of about 1·5 accounts for only one-third of the change in burst duration. Therefore, α is the major kinetic parameter changing during the stages when the burst duration is decreasing.

The kinetic measurements and rate constants for channel opening, closing and agonist dissociation are summarized in Table 1 for early, intermediate- and late-stage muscle.

Desensitization

The frequency of channel openings recorded with 150 nM-ACh declined during the recording period after seal formation. The observed decline was almost completely

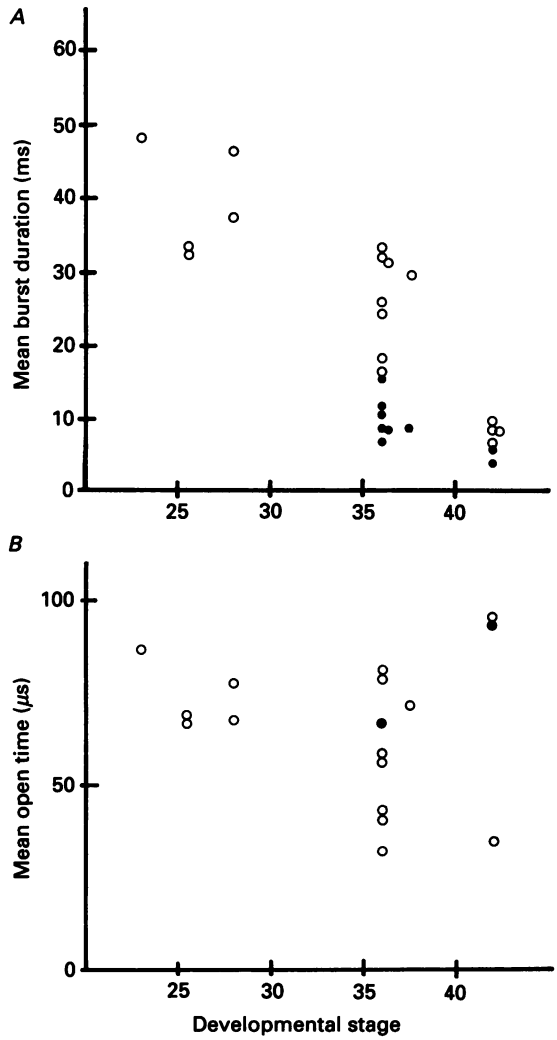

Fig. 7. Burst duration and mean open time of brief openings with development. Conditions for recording and analysis were as described in the text (100 mV hyperpolarization over resting potential, 10 kHz filter, low temperature). These records are from the same patches analysed for brief closures. Single-channel records were digitized at 5 or 10 kHz for burst duration measurement, and at 40 kHz for analysis of brief openings. The data for all concentrations of ACh (20 nM–1 μM) are pooled. Open symbols denote the low-conductance channel and filled symbols the high-conductance channel. The abscissa in each case is developmental stage. *A*, burst duration at 100 mV hyperpolarization *vs.* developmental stage. This figure is similar to Fig. 4. *B*, mean open times for brief events *vs.* developmental stage.

confined to low-conductance events, and is most likely due to desensitization, as described in the preceding paper (Kidokoro & Rohrbough, 1990). The decay time course roughly fits a single exponential. No difference in the decay time constant was observed between young and old myocytes (see also Fig. 10 in the preceding paper).

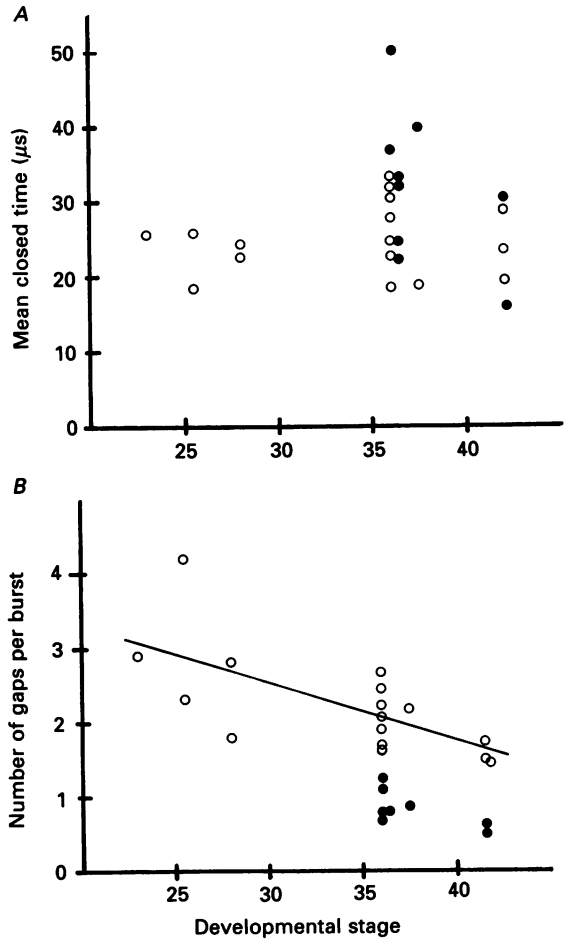

Fig. 8. Mean closed times of brief closures (gaps) and number of gaps per burst with development. Data were recorded at 100 mV hyperpolarization and digitized at 40 kHz. Analysis procedures were described in Kidokoro & Rohrbough (1990). Open symbols correspond to low-conductance channels and filled symbols to high-conductance channels. Data were pooled for 20 nM–1 μM concentrations of ACh as in Fig. 7. *A*, mean closed times of brief closures with development. Each point represents one patch at the indicated developmental stage. The pooled mean closed times were $25{\cdot}0 \pm 6{\cdot}1$ μs for the low-conductance channel and $32{\cdot}0 \pm 10{\cdot}2$ μs for the high-conductance channel. *B*, number of gaps per burst *vs.* development. The number of gaps per burst was corrected for missed closures. For the low-conductance channel, the number of gaps per burst was significantly correlated with development (at $P = 0{\cdot}005$); the continual line was fitted by linear regression (correlation coefficient = 0·68). For the high-conductance channels, there were $0{\cdot}85 \pm 0{\cdot}23$ gaps per burst for data from stage 35 and later.

TABLE 1. Summary of the developmental changes for the patches analysed at high resolution for fast kinetics

	n	Burst duration (ms)	τ_{closed} (μs)	No. gaps per burst	α $\times 10^2$ s^{-1}	β $\times 10^4$ s^{-1}	k_{-2} $\times 10^4$ s^{-1}	α' $\times 10^4$ s^{-1}
Low-conductance channel								
Early (stage 23–28)	5	39·08 ± 7·53	23·48 ± 3·09	2·81 ± 0·89	1·02 ± 0·37	3·17 ± 0·72	1·16 ± 0·17	1·39 ± 0·17
Intermediate (stage 35–38)	8	25·96 ± 6·32	26·30 ± 6·20	2·11 ± 0·35	1·30 ± 0·50	2·73 ± 0·80	1·28 ± 0·21	1·91 ± 0·67
Late (stage 41–42)	3	7·70 ± 1·10	23·80 ± 4·96	1·57 ± 0·15	3·41 ± 0·75	2·64 ± 0·55	1·69 ± 0·38	2·43 ± 1·07 ($n = 2$)
High-conductance channel								
Late (stage 35–42)	9	8·77 ± 3·16	31·98 ± 10·23	0·85 ± 0·23	2·52 ± 0·78	1·63 ± 0·66	1·89 ± 0·69	1·29 ± 0·30 ($n = 2$)

Values for the low-conductance channel during development are separated into early, intermediate and late stages. The decrease in burst duration during development was primarily due to the over 3-fold increase in the channel closing rate α. The rate constant for channel closing for the brief class of opening is represented by α' as in the activation scheme (Fig. 1 in the preceding paper). For the high-conductance channel, only stages 35 and later were analysed.

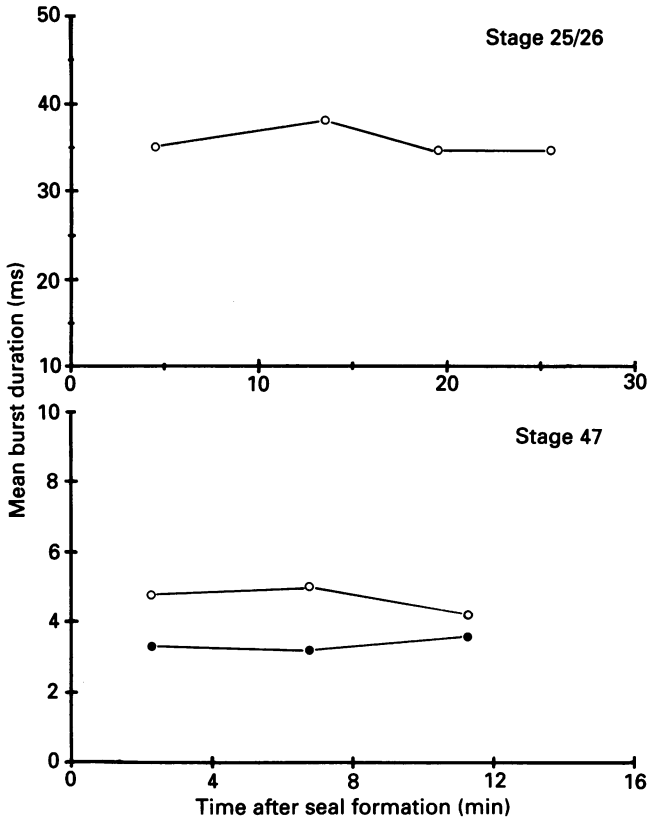

Fig. 9. Channel burst duration was stationary with recording time. Channel burst durations for different portions of the recording period are plotted. The upper part is for a cell at stage 25/26 and the lower part is for a cell at stage 47. Both recordings were done at 50 mV hyperpolarization over resting potential. ACh concentration was 150 nM. The data points are plotted midway through the segment of the record to which they correspond, and represent consecutive segments of the total record. Open symbols are low-conductance channel burst durations and filled symbols are high-conductance channel burst durations. The channel event frequency declined during the recording period due to desensitization.

The burst duration remained constant over the course of the recording period, which was sometimes longer than 30 min (Fig. 9). The desensitization of ACh receptors over time, therefore, did not cause the burst duration to be altered.

DISCUSSION

Early in the development of *Xenopus* muscle cells, after initial insertion of ACh receptor molecules, there are changes in the receptor channel kinetics which are not observed at later stages. Recent studies have shown that the burst duration of low-conductance channels shortens dramatically during the first day after they appear in the membrane, both in culture (Leonard *et al.* 1984, 1988) and *in vivo* (Kullberg *et al.* 1986; Owens & Kullberg, 1989). In this paper, we have followed the developmental

changes in channel kinetics with high time resolution, in an attempt to identify the kinetic parameters associated with this change.

In this study, we found that the burst durations of both low-conductance and high-conductance channels shortened during development in culture. During this developmental period, there was no change in the brief closed time (gap duration). The number of openings per burst decreased with development, but only by a factor that accounts for about one-third of the 4-fold decrease in low-conductance channel burst duration. We therefore conclude from our estimations of the rate constants for channel opening (β), closing (α) and agonist dissociation (k_{-2}) (Kidokoro & Rohrbough, 1990), that an increase in α is the major kinetic change over the period studied.

Burst duration changes

A discrepancy between our results and those of recent studies is our finding that high-conductance channel burst durations are longer during the initial stages after ACh receptors appear than at later stages. High-conductance channel mean burst duration decreased by a factor of about 2·5 over the course of development in culture, with most of the change occurring in the first 24 h in culture. Leonard *et al.* (1988), also using *Xenopus* myotomal muscle culture, reported no significant change in high-conductance channel mean burst duration throughout development (corresponding to stages 21–48); their developmental groupings were somewhat different from ours, pooling data into six broader ranges of stages (see Fig. 4, Leonard *et al.* 1988). Owens & Kullberg (1989), recording from the extrajunctional regions of myotomal muscle *in vivo*, also reported no developmental change in high-conductance channel apparent open time (at resting potential and room temperature). However, high-conductance channel apparent open time measurements were reported only for stage 33 and older. In our experiments, nearly all of the change had occurred by stage 33/34. We also found a dramatic decrease in low-conductance channel burst duration of about 4-fold. Most of this decrease occurs by the second day in culture, in agreement with both of these recent reports.

We have reported the mean burst duration for the high-conductance channel when the number of events recorded was twenty to 100–150; this was the case for most records from stages 21–30. For records with greater numbers of events, high-conductance burst duration histograms were nearly always well fitted by single exponentials, and the mean burst durations agreed well with the fitted time constants. The observed decrease in high-conductance channel burst duration occurs in early developmental stages (21–33) when the high-conductance event frequency in single-channel records is very low. Therefore, it can be argued that mean burst measurements made at these stages are not reliable. We have attempted to rule out possibilities of systematic bias due to the low numbers of events, as described in the Results. Although the numbers of high-conductance events at early stages were lower, there is no indication that this caused consistent overestimation of true channel burst duration. Neither was this the case at later stages; mean burst durations of twenty-five events did not overestimate the actual mean, but were in reasonable agreement.

It was previously found that high-conductance channel open time was positively

correlated with low-conductance channel open time in the same patch (Igusa & Kidokoro, 1987). Here, we also found burst durations to be correlated. Whatever the mechanism involved in the shortening of ACh receptor burst duration, it does not seem to be developmentally specific for the low-conductance channel.

Mechanism of kinetics changes

A question which remained from the previous studies cited is how to interpret the observed decrease in burst duration using the kinetic model for ACh receptor activation in the preceding paper (Fig. 1, Kidokoro & Rohrbough, 1990). The decrease in burst duration could be due to a change in α, the rate constant for the transition from the open to the doubly liganded closed state, or to changes in β and k_{-2}, which would be reflected in the time constant of the brief closures and the number of gaps per burst.

The analysis of brief openings and closures did not detect a developmental change for the low-conductance channel in the mean open time of brief openings or the brief closed time (gap duration). The decrease in the number of openings per burst ($\beta/k_{-2}+1$) is not the major mechanism. This therefore suggests that it is primarily α, the channel closing rate, and thus the true mean channel open time, which is changing.

The molecular basis of this change is not clear. It has been suggested that ACh receptor channels which are inserted into the membrane at early stages undergo post-translational or post-insertional modifications to produce the shortening of burst duration (Carlson, Leonard & Nakajima, 1985; Leonard *et al.* 1988). The developmental decrease of low-conductance burst duration, at least, occurs without protein synthesis or incorporation of new ACh receptors into the membrane (Carlson *et al.* 1985). Some form of modification of existing ACh receptors is also suggested since the decrease in burst duration occurs more rapidly than the rate of ACh receptor turnover, whereas the time course of the appearance of the high-conductance receptor agrees with ACh receptor turnover rate (Brehm, 1986).

Desensitization

The slow desensitization of single-channel events was noted here and in the preceding paper. The decline in event frequency, when observed, was almost completely due to a decline in frequency of low-conductance events, and was observed even at 20–150 nM concentrations of ACh. ACh receptor channel desensitization upon exposure to high (micromolar) concentrations of ACh is increased by phosphorylation of the receptor (Huganir, Delcour, Greengard & Hess, 1986), and by agents such as forskolin which are believed to stimulate cyclic AMP-dependent phosphorylation of ACh receptors in intact muscle (Albuquerque, Deshpande, Aracaua, Alkondon & Daly, 1986; Miles, Anthony, Rubin, Greengard & Huganir, 1987; Middleton, Rubin & Schuetze, 1988). We were interested in determining (1) whether the type of short-term modification that may occur when a receptor undergoes desensitization affected burst duration, and (2) whether the rate of channel desensitization changed noticeably during development. The rate of the decline in low-conductance burst and brief opening frequency with recording time was similar for early and later stage muscle. The burst durations of both low-conductance and high-conductance channels were shown to be stationary for the

course of the recording period. Therefore, the desensitization process, which is probably the mechanism of the decrease in event frequency, does not appear to alter burst duration.

This observation is supported by recent reports that agents which increased ACh receptor channel desensitization did not alter channel open time (Middleton, Jaramillo & Schuetze, 1986; Middleton *et al.* 1988). Therefore, phosphorylation of the receptor would not seem to be a candidate for the mechanism involved in the decrease in burst duration during development. The effects of altering receptor phosphorylation has not been examined in young muscle with prolonged burst duration, however.

Relation with other developmental changes

ACh receptor expression begins in *Xenopus* myocytes by stages 19–20 *in vivo* (Blackshaw & Warner, 1976; Kullberg *et al.* 1977), before spinal motoneurones reach the myotomal muscle. It has been demonstrated previously (Brehm *et al.* 1982, 1984*a*; Leonard *et al.* 1984, 1988; Igusa & Kidokoro, 1987) that muscle and ACh receptor channel development proceeds in the absence of functional innervation. Muscle cells dissociated and cultured at stages prior to innervation begin to express ACh receptors with a timing comparable to the intact embryo, and to exhibit developmental changes in ACh receptor channel properties.

The open time of ACh receptor channels, both at the endplate and extrajunctionally, decreases by 3- to 4-fold during skeletal muscle development in frog (Kullberg, Brehm & Steinbach, 1981; Kullberg & Kasprzak, 1985; Kullberg *et al.* 1986; Kullberg & Owens, 1986; Owens & Kullberg, 1989) and rat (Sakmann & Brenner, 1978; Fishbach & Schuetze, 1980; Vicini & Schuetze, 1985; also see reviews by Kidokoro (1988) and Brehm & Henderson (1988)). Single-channel studies have shown that two distinct channel types exist in frog and mammalian muscle, and also appear in cultured frog and rat muscle. The decrease in open time at the endplate is largely due to the developmental transition from the low-conductance channel with a long open time to the high-conductance channel with a brief open time (Brehm *et al.* 1984*b*; Kullberg *et al.* 1986). Inhibitors of transcription and protein synthesis block the developmental increase in the percentage of high-conductance channel events (Brehm, Kream & Moody-Corbett, 1987). There is now strong evidence that this transition involves the molecular substitution of a new subunit, ϵ, in the high-conductance channel for the γ-subunit found in the low-conductance channel (Mishina *et al.* 1986).

Similarly, both receptor channel types appear in cultured muscle, even without the influence of nerve. We found both classes of receptor channels to be present from the earliest stages examined, but the high-conductance class constituted only a few per cent of the total channel events for the first day in culture, in agreement with Leonard *et al.* (1988). The percentage of high-conductance channel openings increased gradually with muscle age, beginning about stage 34, to above 60% by stage 47. Apparently, a low level of the high-conductance ACh receptor transcription or translation product is present in very young muscle, but expression seems not to increase significantly for at least 24 h in both cultured *Xenopus* muscle and *in vivo* (Kullberg *et al.* 1986; Owens & Kullberg, 1989).

It was previously reported that when nerve was added to *Xenopus* myocytes which

had been cultured for several days, the low-conductance channel events again became predominant (Brehm *et al.* 1982). One proposed role of innervation in regulating receptor function is the suppression of low-conductance channel synthesis (Brehm, Lechleiter, Henderson, Owens & Kullberg, 1988). Denervation results in an increase in the number of low-conductance channels in the extrajunctional membrane. A possible explanation for the result observed after adding nerve to older muscle cultures is that some factor(s) released by dead and damaged nerve cells has a denervation-like effect on the expression of low-conductance channels in the muscle membrane. Denervation apparently does not result in the reappearance of the early embryonic-type low-conductance receptors with prolonged burst duration.

The shortening of ACh receptor channel burst durations observed in culture, like the appearance of the high-conductance channel, may occur under the developmental programme of the muscle alone. We have studied the decrease in burst duration within each class of receptor channel, a change which is separate and distinct from the transition to faster kinetics due to the population shift to the high-conductance channel. The prolonged burst durations in *Xenopus* have so far been shown to be exclusively an embryonic phenomenon characteristic of ontologically young, rather than metabolically young receptors (Greenberg *et al.* 1985; Leonard *et al.* 1988). Newly synthesized receptors following α-bungarotoxin block of existing ACh receptors have properties characteristic of the muscle age.

A difference between embryonic and adult ACh receptor channel types has been reported in mouse skeletal mouse ACh receptors (Steele & Steinbach, 1986). In isolated fibres from C3H mice, single-channel recordings revealed three apparent classes of ACh receptor channel events, according to kinetics, during development. Adult denervated fibres had both a high-conductance, brief burst duration class and a low-conductance, longer burst duration class of event. In neonatal muscle, a single low-conductance class event was observed, with a burst duration 2–3 times longer than the low-conductance event in adult muscle. This suggests that modulation of channel burst kinetics during development may take place in other types of skeletal muscle.

The receptor channel burst durations in myotomal muscle are decreasing during the period when functional innervation is becoming established in the embryo. It has been postulated that the prolonged low-conductance receptor channel burst durations at very early stages may be more effective in the detection of nerve activity during the initial phase of synaptic contact, when the receptor density in the postsynaptic membrane is still low (Leonard *et al.* 1988).

The high-conductance channel, with its greater current amplitude and brief kinetics, mediates transmission at the adult neuromuscular junction. The shortening of the low-conductance channel kinetics occurs largely before the high-conductance channel becomes dominant. In *Xenopus*, a significant percentage of receptors seems to remain as the low-conductance channel type well into stages where the tadpole is free-swimming (see Owens & Kullberg, 1989). A situation where the low-conductance channel open time is nearly as brief as for high-conductance channels may improve the temporal efficiency of junctional transmission in this period. The fact that the change in open time occurs in aneural culture presents an uncertainty as to the role of nerve or nerve-associated factors in the regulation of this change.

The presence of longer high-conductance channel burst durations at early stages seems to have little functional significance, since these openings occur with low frequency. It suggests, however, that both types of ACh receptor channels are modified by the same mechanism after insertion during early embryogenesis.

In this study, we have described developmental changes in the kinetic properties of ACh receptor channels in embryonic myocytes. Some of these changes have been previously reported. Here we have considered all the developmental changes as a whole for the first time, including both burst duration and brief closed time analyses. As a result, we have been able to identify the apparent parameters associated with the developmental kinetics change. We are interested in further investigating the molecular basis for the modifications which may be responsible for these kinetic changes.

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