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TWO TYPES OF ACETYLCHOLINE RECEPTOR CHANNELS IN DEVELOPING *XENOPUS* MUSCLE CELLS IN CULTURE: FURTHER KINETIC ANALYSES

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SUMMARY

1. Further developmental changes in acetylcholine (ACh) receptor channel function were examined in *Xenopus* muscle cultures using the cell-attached patch-clamp technique.

2. Two types of acetylcholine (ACh) receptor channel events, low- and high-conductance channel events, were distinguished as reported earlier. Apparent open-time histograms for high-conductance channel events were well fitted by a single exponential but those for low-conductance channel events were sometimes fitted better by two exponentials.

3. In low-conductance channel events when the open-time histogram was well fitted by two exponentials, successive open times were correlated: an event with a long open time tended to be followed after a brief interval (less than 1 ms) by another long-duration event. A short-duration event was less frequently followed by an event within a short interval (1 ms) with a long-duration event.

4. Closed-time histograms for the interval between successive low-conductance channel events and between successive high-conductance channel events were both fitted by two exponentials. The fast time constant was 0·36 ms for the high-conductance channel event and 0·31 ms for the low-conductance channel event. There was an indication that a third and faster component was hidden in the first bin (0–200 μs) in the closed-time histogram of both types of channel events.

5. Defining a burst as successive openings separated by closures briefer than 1 ms, the number of gaps per burst was different for the two types of channel events. They were 0·16 for high- and 0·37 for low-conductance channel events. In both types of channels, neither the fast component in the closed-time histogram nor the number of gaps per burst changed with time in culture.

6. The apparent open time of both types of channels increased progressively as ACh concentration was increased, suggesting an increasing number of unresolved closures at higher concentrations. At 100 μM-ACh the apparent open time became shorter, probably due to channel blockade by ACh molecules. Closed-time histograms were fitted by two exponentials. The time constant of the fast component remained similar

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to that at low concentration (0·2 μM) up to 20 μM, but the relative number of closures belonging to this component increased with ACh concentration. In contrast, the slow component shortened its time constant as ACh concentration increased and the relative frequency decreased. Again, there was an indication that another faster component existed in the closed-time histogram.

7. The probability of being open (P_o) was measured at high concentrations and found to increase from 0·33 at 1 μM to 0·88 at 100 μM for the high-conductance channel event and 0·62 at 1 μM to 0·94 at 100 μM for the low-conductance channel event. P_o was smaller for the high-conductance channel event than for the low-conductance channel event at all ACh concentrations.

8. Generally, current fluctuations were slightly greater when a channel was open than when it was closed. These fluctuations were well fitted by a Gaussian distribution. But at 100 μM-ACh, current fluctuations during the open period became more prominent, especially in high-conductance channel events. They were asymmetrical and skewed toward the zero current level. These fluctuations were analysed according to a channel-block model. The blocking and unblocking rate constants were estimated as $1{\cdot}16 \pm 0{\cdot}24 \times 10^4$ s^{-1} ($1{\cdot}16 \pm 0{\cdot}24 \times 10^8$ s^{-1} M^{-1}) and $5{\cdot}04 \pm 1{\cdot}34 \times 10^4$ s^{-1}, respectively.

9. An attempt was made to estimate rate constants for channel closing, channel opening and agonist dissociation. Problems involved in this estimation are discussed.

INTRODUCTION

There are two types of acetylcholine (ACh) receptor channels in developing *Xenopus* muscle cells (Clark & Adams, 1981; Kidokoro, Brehm & Gruener, 1982; Brehm, Kidokoro & Moody-Corbett, 1984*a*; Brehm, Kullberg & Moody-Corbett, 1984*b*; Greenberg, Nakajima & Nakajima, 1985; Auerbach & Lingle, 1986*a*,*b*). They are different in their unitary conductance: one is about 46 pS (low-conductance channels) and the other is about 64 pS (high-conductance channels) at room temperature. They are also different in the apparent open time. Low-conductance channels have a mean apparent open time of approximately 3 ms at the resting membrane potential which, on average, is 2–3 times longer than that for high-conductance channels (Brehm *et al.* 1984*a*).

Two types of ACh receptor channels are ubiquitous among various species of animals. These two types of receptor channels are distributed at the extrajunctional region (Sakmann, Patlak & Neher, 1980; Kullberg, Brehm & Steinbach, 1981) as well as at the junction (Kullberg, Owens & Vickers, 1985). In the adult frog after denervation ACh receptors appear in the extrajunctional region. These receptor channels have an apparent open time of about four times greater value and somewhat smaller unitary channel conductance than the junctional receptor channels (Neher & Sakmann, 1976*a*,*b*). In the snake two types of channels were found at the junction of slow muscle fibres even before denervation (Dionne, 1986). In the embryonic rat two types of channels were also observed (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980; Siegelbaum, Trautmann & Koenig, 1984). The majority of channels in developing rat myoblasts and myotubes in culture had a unitary conductance of 35 pS and mean open time of 15 ms at room temperature. The minor type of channel

event had a larger conductance (55 pS) and briefer mean open time (2–3 ms) (Siegelbaum *et al.* 1984).

At very early stages of development (at stage 24, at most 4–7 h after first emergence of functional receptors in the muscle membrane: Blackshaw & Warner (1976); Kullberg, Lentz & Cohen (1977)), the apparent open time of the low-conductance channel is much longer (15·5 ms at 11–14 °C) which decreased to 5·3 ms after the embryos were kept at room temperature for 3 days and measured again at 11–14 °C. In contrast the apparent mean open time of the high-conductance channel events (4–5 ms) did not change during this period of development (Leonard, Nakajima, Nakajima & Takahashi, 1983).

The apparent open time of the low-conductance channel events was already shortened (3·3 ms at room temperature) in muscle cells cultured for 1 day after dissecting out from embryos at stage 15–19 (Brehm *et al.* 1984*a*). At this early stage the population of the low-conductance channel event was predominant (84%). As muscle cells developed in culture the relative proportion of the low-conductance channel event decreased and after 5–6 days in culture the percentage of the low-conductance channel event became 44%. During this period no change in the apparent open time within the two types of channels was observed. A similar change in the relative population of two types of channel events was also found *in vivo* in *Xenopus* embryos (Kullberg *et al.* 1981) and in the rat (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980; Siegelbaum *et al.* 1984).

A nagging question remained whether changes in the population of channel events reflected the changes of ACh receptor channel population. We found previously that the density of α-bungarotoxin binding sites did not change during the period when a shift in channel population took place (Kidokoro & Gruener, 1981). If the opening rate of one type of channel changes during development, that is, if one type of channel starts to open more frequently during development and the other remains at the earlier opening rate, the apparent shift of event population from one type to the other could happen without changes in the total number of ACh receptor channels in the membrane.

In order to know the opening rate of channels we need to characterize further the kinetic properties of these two types of ACh receptor channels and their developmental changes. We thus examined further the open time and the closed time of channel events. We measured the closed-time intervals and number of gaps per burst. The closed-time histograms were similar for the two types of channel events but the number of gaps per burst was about twice as great in the low-conductance channel event as in the high-conductance event. Channel behaviour at higher concentrations of ACh was also studied. These kinetic properties within each of the two channel types did not change with time in culture. Therefore, the developmental changes we described previously, namely a shift of population from low- to high-conductance channel events, is not likely to result from change in the kinetics of existing channels. Probably the number of low-conductance channel molecules increases during development while the other decreases.

We have briefly reported a part of our results elsewhere (Igusa & Kidokoro, 1984).

METHODS

Cell culture

The procedures for dissection and plating of myotomal muscle cells of *Xenopus laevis* embryos were the same as described previously (Anderson, Cohen & Zorychta, 1977; Kidokoro, Anderson & Gruener, 1980). Briefly, dorsal portions of embryos at stages between 19 and 23 (Nieuwkoop & Faber, 1956) were dissected using a pair of fine needles. Myotomal tissue was then separated from the rest in saline including 0·5 mM-$CaCl_2$ and 0·1 % w/v collagenase (Worthington). Finally, individual muscle cells were dissociated in Ca^{2+}–Mg^{2+}-free saline with 2 mM-EDTA. Cells were plated on collagen-coated cover-slips in high-serum media (5 % v/v horse serum). After 1 day cells were usually well spread and attached to the substrate. These cells were then transferred to low-serum media (0·5 % horse serum). Cultures were kept at room temperature (21–23 °C). The age of the culture was counted in days since dissociation.

Recording of single-channel events

Single-channel currents were recorded from ACh receptor channels in myotomal muscle cells in culture using the cell-attached patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Shortly before the recording the culture medium was slowly replaced with recording solution having the following ionic composition (in mM): NaCl, 120; KCl, 1·6; $CaCl_2$, 1·0; and HEPES–NaOH, 8; pH 7·4. All recordings were made at room temperature (21–23 °C) under a Nikon inverted microscope (Diaphot) with a 40 × water-immersion phase-contrast objective. The recording electrode was pulled from glass tubing with an internal glass fibre. The pipette was coated with Sylgard (Dow Corning) to reduce noise and fire-polished immediately before use. Electrical resistances of the recording electrode ranged between 5 and 12 MΩ after filling the normal saline described above, containing various concentrations of ACh. Seal resistances were between 30 and 100 GΩ and no difference in the seal resistance was noticed among cultures of different ages. We used a patch-clamp amplifier (Meyer and Renz, F.R.G.) with a feed-back resistance of 10 GΩ and a frequency compensation circuit. The data were stored without filtering on an FM tape-recorder (Racal) at a tape speed of 15 in s^{-1}. At this tape speed the recorder has a frequency response of 8 kHz (−3 dB). In the majority of experiments the inside of the electrode was maintained at +80 mV relative to the bath potential level unless otherwise described. The membrane patch under the electrode was thus experiencing the potential which was equal to 80 mV plus the resting membrane potential. The resting potential was previously monitored under identical condition as around −85 mV (Brehm *et al.* 1984*a*).

Data analysis

Data analysis was performed in three steps on a DEC 11/23 computer.

Data-fetching process. First, data on the tape-recorder were filtered with an 8-pole Bessel filter at 3 or 5 kHz (corner frequency) and digitized at 10 kHz. Digitized data were stored on floppy disks. Channel events were caught by setting a threshold at around 3 pA above the base-line level which was periodically updated during the fetch process. When an event was detected 256 points were digitized including 16 points before the trigger. 100 events were stored on the memory and then transferred to the floppy disk. Thus with this method open times of longer than 24 ms cannot be measured. When two or more events occurred in succession the last event could become unmeasurable even if it had a shorter duration. This problem, however, was not serious since in the great majority of cases the mean apparent open time was less than 4 ms and repetitive openings were rare. Furthermore, for estimation of the mean open time with the maximum-likelihood method only events which had an open time of less than 5 ms were used. For closed-time analysis, the measurement of the fast component (less than 1 ms) is reliable but that for the longer component is less reliable as a result of this technical restriction. We did not interpret the longer component in this study.

At high ACh concentrations (1–100 μM), channel events occurred in clusters of bursts; therefore, 15600 points (1·56 s) were stored at a time which was usually enough to cover a cluster. Since the cluster of bursts in high ACh concentrations occurred infrequently this method of catching events worked effectively.

A burst was defined as successive openings separated by closures briefer than 1 ms. A cluster of bursts was identified as a group of bursts separated by closed intervals shorter than 10 ms.

Monitoring process. Data thus stored were displayed on a cathode ray tube screen together with the base line and measured events. Obvious artifacts and superimposed events were eliminated from measurement during this process. The open and closed times were measured by threshold crossing at the half-height of the unitary event. Thus the level was different between high- and low-conductance events. The threshold level was different from the previous work (Brehm *et al.* 1984*a*) where the level was set at 2·5 times the standard deviation of base-line noise, which was closer to the base line than the half-height level (see Fig. 16). This difference in the threshold level probably accounted for the difference in the values for the apparent open time between this and previous work. The base line was calculated from the preceding eight points of each trace and was adjusted by eye when it obviously deviated.

Two types of channel events were clearly distinguished when the apparent open time of an event was long enough to reach a full amplitude. However, when the event was brief and did not reach 83 % of the full amplitude, a high-conductance channel event was misclassified as a low-conductance channel event and when an event did not reach a half-amplitude level of the low-conductance event, the event was totally missed.

Analysis. We used the maximum-likelihood technique to estimate the time constants and the relative amplitude of components (Colquhoun & Sigworth, 1983; Horn & Lange, 1983). Usually two exponentials fitted the data but in the case of the open time of the high-conductance channel event one exponential always fitted well. We also measured time constants in the conventional graphical method using semilogarithmic paper. When there was only one exponential, or when the two time constants in the two-exponential-fit were well separated, the maximum-likelihood and the graphical method gave virtually identical values.

Probability of being open (P_o) within a cluster of bursts

We used P_o as defined by Auerbach & Lingle (1986*b*), which was equal to the sum of the open durations divided by the time between the first and last open transition in a cluster of bursts. The last open interval in the cluster was excluded from the calculation.

Time resolution. The frequency response of the recording system was measured by analysing the rising phase of the recorded ACh receptor channel unitary events. The events were digitized at 80 kHz and eight to sixteen events were averaged by aligning at the rising phase. The rising phase was then fitted to one exponential to estimate the time constant of the over-all recording system. It was found to be 47·6 μs when filtered at 3 kHz and 39·6 μs when filtered at 5 kHz. Since we usually sampled at 10 kHz and the over-all time constant of the recording system was 47·6 μs (filtered at 3 kHz) the briefest event which crossed the half-amplitude level was 33 μs which was measured as 100 μs (3-fold over-estimation). Some events as long as 201 μs were also measured as 100 μs (100 % underestimation). This kind of ambiguity was less in the second bin (200 μs). The shortest and the longest period which were classified in this bin were 101 and 300 μs.

Analysis of open-channel noise. The blockade of channels by ACh molecules was prominent in the high-conductance channel at 100 μM-ACh. We therefore estimated the blocking and unblocking rate by the method described by Yellen (1985). After producing a histogram of digitized individual points, a Gaussian function was fitted to the base-line noise as well as to open-channel noise. When the distribution was obviously skewed, as in the second peaks in Fig. 16*C* and *D*, the values greater than the peak level were used to calculate the parameters for the Gaussian function. For fitting the beta function we needed to know the open-channel level. Fortunately, in our experimental conditions the low-conductance channel did not have too much excessive open-channel noise and the open level was accurately determined. To determine the open level of the high-conductance channel event we used the ratio of the open-channel current between two types of channels which was fairly constant, $1{\cdot}44 \pm 0{\cdot}05$ ($n = 20$) in lower concentrations of ACh. The beta function had to be convoluted with the Gaussian function of the open-channel noise when there was no channel block by ACh. We noticed that the open-channel noise was always slightly larger than that of the base line even when the open-channel noise was symmetrical. Therefore we empirically estimated the open-channel noise of the high-conductance channel from the base-line noise by multiplying by 1·16. Convolution was done numerically by multiplying each point of the beta function with the Gaussian noise function of the peak amplitude of one. The convoluted beta function was then rescaled by equalizing the area under the curve to that under the original beta function. Then the

parameters A and B of the beta function were changed with a trial-and-error method until the fit was satisfactory. Final judgement was done by eye.

Estimation of unresolved closures

Since the closed time which we were dealing with was substantially shorter than the apparent open time, the bias introduced by unresolved closures was greater than by unresolved opening; therefore, we will discuss only unresolved closures and not unresolved openings. For simplicity of discussion, we ignore the recording noise which was usually symmetrical (see Fig. 16). When the closure was brief, the current trace would not cross the half-height threshold level due to the filtering characteristics of the recording system. When the recording system had the over-all time constant of 47·6 μs (filtered at 3 kHz) the minimum duration which could reach the half-height level was 33·0 μs. Closures briefer than this value could not be detected by our recording system. When the estimated time constant of the closure was 360 μs, as in the high-conductance channel event, 8·9 % of events fell into this category. When a closure was longer than 100 μs in duration the event was always detected as the record was digitized at 10 kHz. The closures between 33 and 100 μs were detected in a stochastic manner as the sampling point fell into this period. For longer events the probability of being detected was greater. If we assumed a linear relation between the probability of detection and the duration of events then the number of events undetected could be easily estimated. When the time constant of the closure was 360 μs, 8·0 % of events fell into this category. Thus, altogether 16·9 % of events were estimated to be unresolved. This correction was used to estimate the number of gaps per burst and the open time in data obtained at 0·2 μM-ACh as follows. The average number of gaps per burst in high-conductance channel events in a patch was 0·15 which was corrected as 0·18 ($= 0{\cdot}15 \times 100/(100-16{\cdot}9)$). Thus 0·03 gaps per burst were estimated to be missed. The measured apparent open time for the high-conductance channel event was 1·60 ms which was corrected as 1·33 ms.

The term 'apparent mean open time' used in this article is different from the 'mean burst duration' because traditionally we measure the open time whenever we recognize the opening and closing by the threshold crossing. Therefore, the time spent in a closed state is not considered. If the time resolution of the recording system is sufficiently good then this apparent time corresponds to the real mean open time which is $1/\alpha$ (α is the channel closing rate). However, in reality, during the apparent open state there could have been brief closures which were not detected. Since in our preparation the re-opening rate is relatively small at 0·2 μM-ACh, at most 0·4 in the low-conductance channel event, our apparent open time is slightly shorter than the burst duration and slightly longer than the real open time.

RESULTS

We have demonstrated that there is a shift of population of two types of channel events with time in culture (Kidokoro *et al.* 1982; Brehm *et al.* 1984*a*). In young cultures, channel events with a low amplitude (low-conductance channel events) were predominant and in older cultures channel events with a high amplitude (high-conductance channel events) increased. Neither the amplitude nor the apparent open time of two channel types changed during development. Since experimental procedures and measurement criteria were slightly different from our previous study, we first repeated previous experiments in the present study. In the majority of experiments the recording electrode contained 0·2 μM-ACh and the cell-attached patch was hyperpolarized by 80 mV. The unitary current was $10{\cdot}3 \pm 0{\cdot}6$ pA (mean $\pm$standard deviation, $n = 37$) for the high-conductance channel event and $7{\cdot}2 \pm 0{\cdot}4$ pA ($n = 37$) for the low-conductance channel event. These values are somewhat greater than in the previous study (Brehm *et al.* 1984*a*).

Apparent open time within each type of ACh receptor channel did not change during development

As illustrated in Fig. 1 we confirmed the previous finding that the mean apparent open time of the two types of channel events did not change with time in culture. The mean apparent open time of the combined data was $1{\cdot}60 \pm 0{\cdot}35$ ms ($n = 36$) for

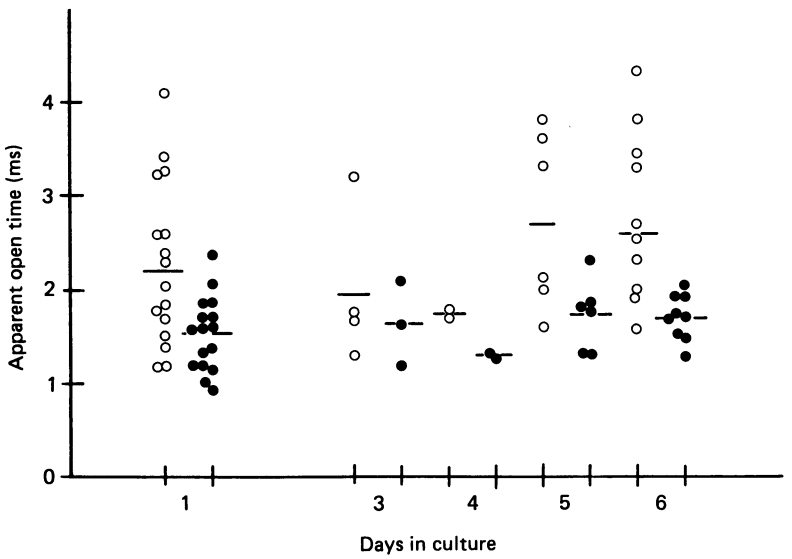

Fig. 1. Channel apparent open times *versus* days in culture. Muscle cells were dissociated at day 0 and were kept in culture at room temperature. Open and filled circles represent low- and high-conductance channel events, respectively. Bars indicate the mean of individual points. ACh concentration in the electrode was 0·2 μM and the membrane potential was hyperpolarized by 80 mV over the resting membrane potential. The resting membrane potential was previously measured as about −85 mV in the same experimental conditions (Brehm *et al.* 1984*a*).

the high-conductance channel event and $2{\cdot}23 \pm 0{\cdot}68$ ms ($n = 36$) for the low-conductance channel event. These two means are significantly different ($P = 0{\cdot}001$, Student's t test) and the variance is also different in these two populations ($P = 0{\cdot}0001$, F test). The variability of the channel open time will be discussed later. These apparent open times were shorter than the values previously published (in the previous study the apparent mean open time was about 5 ms for the low-conductance channel and 2 ms for the high-conductance channel at 80 mV hyperpolarization over the resting membrane potential level). This is at least partly due to the difference in the detection level as described in the Methods section. In the present study the level was set at the half-height of the unitary current amplitude. This procedure favoured the detection of brief closures which would have been missed if closures were detected at the level 2·5 times the standard deviation of base-line fluctuations, which was closer to the base line (see Fig. 16), as in the previous study.

The open-time histograms for high-conductance channel events were always well

fitted by a single exponential (Fig. 2*B*). We did not detect a component of brief openings (mean open time 0·13 ms at −130 mV and 10·5–11·5 °C) as described in Colquhoun & Sakmann (1981, 1985) but since the amplitude of brief events tended to be reduced due to the limited frequency response of the recording system those

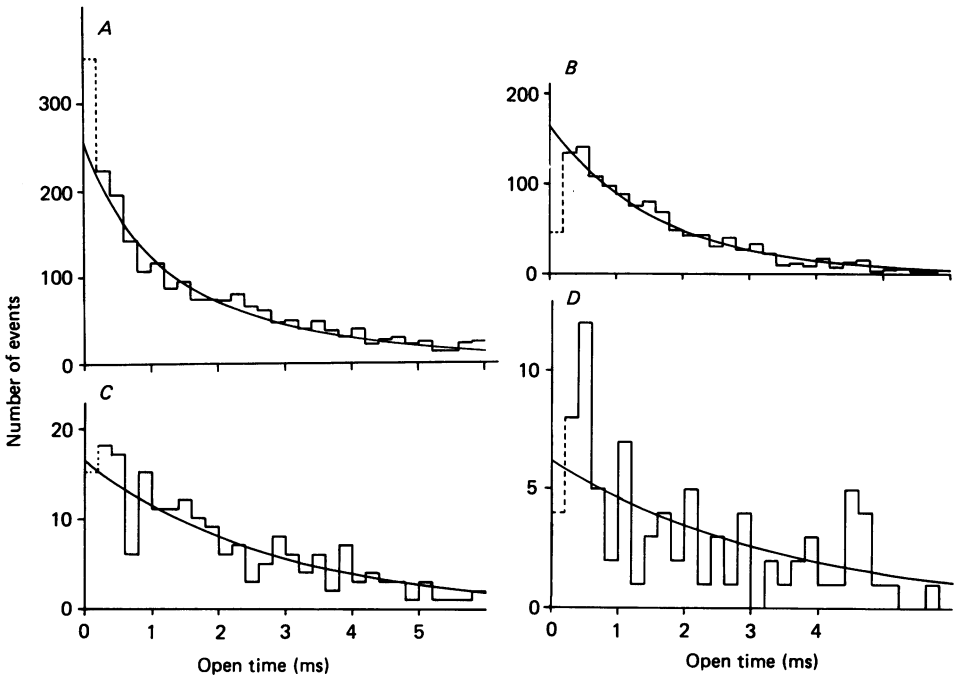

Fig. 2. Channel apparent open-time histograms. The ordinate is normalized number of events. The abscissa is intervals in milliseconds. The bin size is 0·2 ms. The curve was fitted with an exponential between 0·2 and 5·0 ms. Therefore, the first bin, depicted by dashed lines, was not used to fit the curve. *A*, histogram for low-conductance channel events. The histogram was fitted with two exponentials with time constants of 0·75 and 2·6 ms. 20 % of events belonged to the fast component. Total number of events used to fit the curve was 1810. *B*, histogram for high-conductance channel events which were recorded from the same patch as *A*. The continuous line has a time constant of 1·63 ms. Total number of events used to fit the curve was 1047. The culture was 1 day old. *C*, histogram of low-conductance channel events which followed within 1 ms an event with an open time of 1·5 ms or longer. The continuous line has a time constant of 2·77 ms. Total number of events used to fit the curve was 177. *D*, histogram of low-conductance channel events which followed within 1 ms an event with an open time of less than 0·7 ms. The continuous lines has a time constant of 3·45 ms. Total number of events used to fit the curve was 78.

events are likely to be misclassified as low-conductance channel events. This point will not be addressed further.

In general the low-conductance channel event had a longer apparent open time and had a larger variation among different patches. The difference in the apparent open time between two types of channel events was less than those reported previously in *Xenopus* muscle *in vitro* (Brehm *et al.* 1984*a*) or *in vivo* (Brehm *et al.* 1984*b*). Leonard *et al.* (1984) reported a similar mean open time for both types of channels after 3 days in culture.

Open-time histograms with two exponentials

In ten patches out of thirty-seven the histogram for the low-conductance channel event had a fast component which contained more than 5 % of total events (correction for unresolved events was done assuming only two exponentials were involved). One example of this kind of histogram is shown in Fig. 2*A*. The mean time constant of the fast component was $0{\cdot}73 \pm 0{\cdot}23$ ms ($n = 10$) and this population was 15 ± 11 % of the total events. The slower component in these patches had an average time constant of $3{\cdot}06 \pm 0{\cdot}97$ ms ($n = 10$). When values shown in Fig. 1 were tabulated only those for this slow component were included.

There was no tendency for the population of these low-conductance channel events with two time constants to change with time in culture.

The measured amplitude of events with shorter durations tends to be decreased due to the limited frequency response of the recording system (see Fig. 5*B* in Brehm *et al.* 1984*a*). It is, therefore, conceivable that excess events with short durations come from misclassified high-conductance channel events. Undoubtedly some attenuated high-conductance channel events contaminated the bins of short durations in the histogram. However, even patches which did not have many high-conductance channel events had a histogram with two exponentials for low-conductance channel events. Moreover, as shown in Fig. 2*A*, even bins with a sufficient duration for the amplitude to be measured accurately had excess entries (3rd to 4th bins from the left). Therefore, we believe that these histograms with two exponentials were not solely the result of contamination of high-conductance channel events. In spite of a higher temperature in this study (21–23 °C), this fast component is slower than that (130 μs at 10·5–11·5 °C) reported by Colquhoun & Sakmann (1981, 1985) in the frog neuromuscular junction and that (160 μs at 9–11 °C) reported by Sine & Steinbach (1984*a*, 1986*b*) in a mouse muscle cell line. Since this fast component was also observed at high concentrations of ACh a possibility that this arises from singly liganded receptor channels was eliminated (Colquhoun & Sakmann, 1985; Sine & Steinbach, 1986*b*).

Two similar components were noticed and analysed in rat myotubes in culture (360 μs for the fast component and 12·5 ms for the slow one with suberyldicholine at -100 mV and room temperature: Jackson, Wong, Morris, Lecar & Christian (1983)). They found that an event with a long duration tended to be followed after a brief interval by an event with a long duration and an event with a short duration tended to be followed by an event with a short duration. Among five models they excluded two which could not explain this correlation between the open duration of two successive events. One model in the remaining three was the one excluded as described above by Sine & Steinbach (1986*b*) and Colquhoun & Sakmann (1985). Therefore, only two models out of five postulated by Jackson *et al.* (1983) are left. One postulates two independent populations of channels and the other assumes two closed states, fully liganded, preceding two open states (Jackson *et al.* 1983).

We applied a similar analysis to our data from five patches which had histograms with two exponentials for low-conductance channel events. We selected events which followed an event with a long duration (longer than twice the time constant of the first component) in a brief succession (less than 1·0 ms) and generated a histogram (Fig. 2*C*). These histograms did not have a fast component and were fitted reasonably

well by one exponential. The time constant of these histograms ($3{\cdot}4 \pm 1{\cdot}5$ ms, $n = 5$) was close to that of the longer component of the original histogram ($3{\cdot}2 \pm 0{\cdot}8$ ms, $n = 5$). Conversely, we accumulated events which followed in a brief succession (less than 1·0 ms) an event with a brief open time (less than one time constant of the first component) and generated a histogram. The histogram thus generated (Fig. 2*D*) seems to have both components. That is, there are excess entries at lower bins. However, these events were relatively rare and no satisfactory conclusion could be reached. This observation prompted us to count the re-opening rate of events with different durations. In one example, among 1155 openings with a duration longer than twice the time constant of the fast component, 276 had an event following in a brief succession (less than 1·0 ms), giving a probability of rapid re-opening of 0·24 (= 276/1155). The average of five cases was $0{\cdot}26 \pm 0{\cdot}09$. In contrast, a similar parameter for openings less than one time constant of the fast component was $0{\cdot}11 \pm 0{\cdot}04$ ($n = 5$). These two values are statistically different (Student's t test, $P = 0{\cdot}01$). A similar comparison was made with the histogram of high-conductance channel events, giving corresponding numbers of $0{\cdot}12 \pm 0{\cdot}04$ ($n = 5$) and $0{\cdot}09 \pm 0{\cdot}03$ ($n = 5$), which are not significantly different. Therefore it is clear that low-conductance channel events with a short duration are less likely to be followed by an opening than those with a longer duration. These characteristics can be incorporated in either of the two models proposed by Jackson *et al.* (1983). Similarly, Sine & Steinbach (1984*a*, 1986*b*) and Colquhoun & Sakmann (1985) concluded that short openings tended to occur in isolation while closely spaced pairs appear to be long openings only.

In high concentrations of ACh, Auerbach & Lingle (1986*b*) reported three modes of channel activation in terms of open probability. The first two had relatively higher open probabilities whereas the third one had a low open probability and in this mode events were isolated from each other. They also suggested that one channel can assume different modes. If a similar switch of activation modes occurred at low ACh concentration an open-time histogram described above could result.

Closed-time analyses

Since we have two types of channel events we can measure four combinations of intervals between openings: (1) intervals can be between two low-conductance channel events (L–L) (Fig. 3, intervals marked with *a*); (2) intervals can be between two high-conductance channel events (H–H) (Fig. 3*B*, intervals marked with *b*); (3) intervals can be between high- and low-conductance channel event (H–L) (Fig. 3*B*, interval marked with *c*); (4) intervals can be between low- and high-conductance channel event (L–H) (not illustrated). When these intervals were measured and frequency histograms were generated there was a clear distinction between the former two combinations and the latter two. As shown in Fig. 4*A* and *B* these histograms have large numbers of closures with short intervals together with variable long intervals. The histogram in Fig. 4*A* was fitted with two exponentials of the time constant of 0·33 and 19·0 ms; 46% of closures belonged to the fast component (unresolved closures were corrected). Those for Fig. 4*B* were 0·43 and 8·0 ms; 33% of closures belonged to the fast component. The intervals between two different types of events did not have the fast component (Fig. 4*C*). It is also noteworthy that the

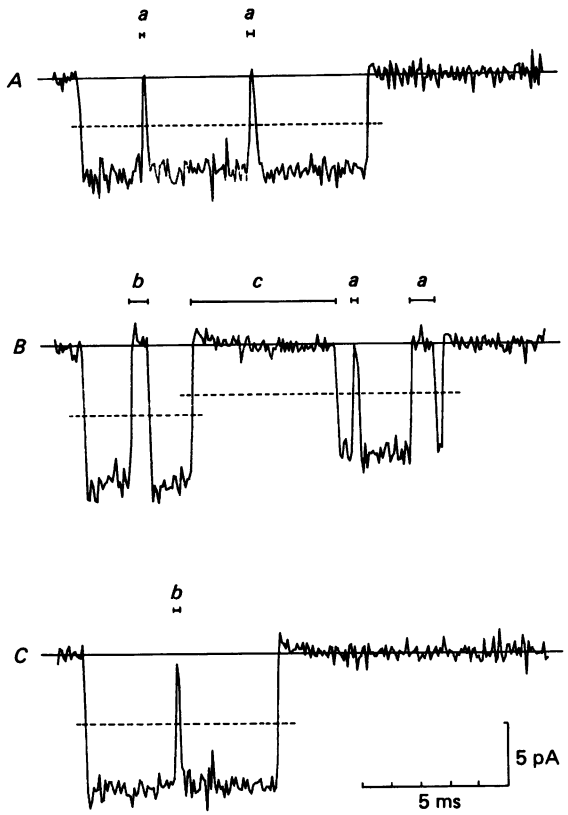

Fig. 3. Examples of current records depicting the method of how the closed time was measured. *a* indicates the closed time between two successive low-conductance events, *b* indicates one between two successive high-conductance events and *c* indicates one between high- and low-conductance event. Dashed lines indicate the level at which openings and closures were detected. The level was set at the half-height of the unitary current amplitude. The scale in *C* applies to all records.

first bin in Fig. 4*A* and *B* which accommodated closures less than or equal to 200 μs had excessive entries in spite of limited time resolution of the recording system. Therefore, it is probable that there is another component in this region similar to the 'nachschlags' reported by Colquhoun & Sakmann (1981, 1985). We expect that this component has a shorter time constant than 50 μs at room temperature since it was reported as 20 μs at 9–11 °C (Colquhoun & Sakmann, 1985) and 50 μs at 9–11 °C (Sine & Steinbach, 1986*b*). Therefore, we did not examine it any further. Instead we proceeded to study our fast component which was reliably resolved. Our fast component probably corresponds to the intermediate component of Colquhoun & Sakmann (1981, 1985) and Sine & Steinbach (1986*b*).

We could not interpret the longer component of the closed-time histogram since the number of channels in one patch was not determined. Thus we only analysed the combination between the same type of event (namely H–H and L–L). The average of the time constant of the first component was $0{\cdot}36 \pm 0{\cdot}11$ ms ($n = 25$) for H–H and $0{\cdot}31 \pm 0{\cdot}04$ ms ($n = 29$) for L–L.

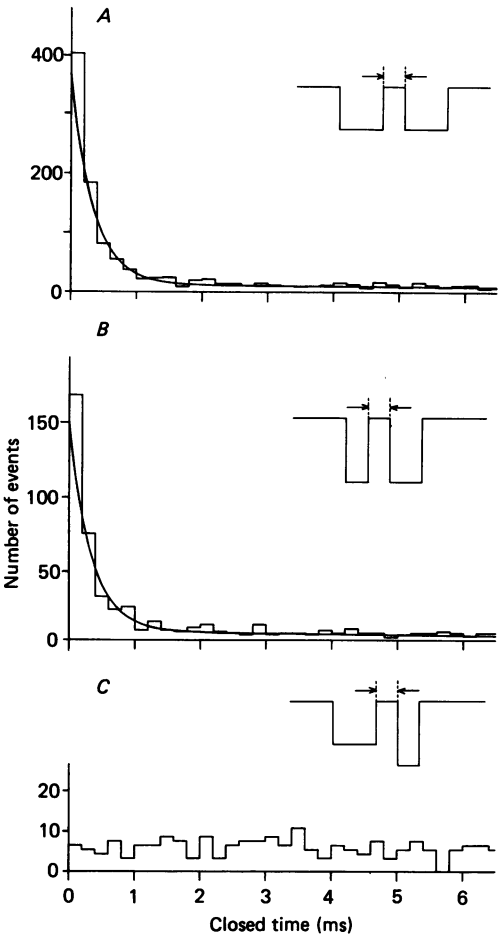

Fig. 4. Closed-time histogram between two successive low-conductance channel events (*A*), one between two high-conductance events (*B*) and one between low- and high-conductance events (*C*) as illustrated in schematic events in the upper right corner of each histogram. Two exponentials were fitted for *A* and *B*. Two time constants were 0·35 and 19·0 ms for *A* and 0·33 and 8·0 ms for *B*. The fast component was 33 % of total events in *A* and 46 % in *B*. Total number of events used to fit the curve was 686 for *A*, 277 for *B* and 151 for *C*. All data were obtained from one patch from a cell cultured for 1 day.

There was no change of this parameter in either of the channel types with time in culture as shown in Fig. 5.

Number of gaps per burst

We then measured the number of gaps per burst. Gaps were arbitrarily defined as closures lasting 1 ms or less. The great majority of gaps thus defined belong to the fast component of the closed-time histogram in Fig. 4 *A* and *B*. We found a significant difference in this parameter between H–H and L–L. They were $0{\cdot}16 \pm 0{\cdot}08$ ($n = 27$) for H–H and $0{\cdot}37 \pm 0{\cdot}16$ ($n = 29$) for L–L. These two means are statistically different ($P = 0{\cdot}001$). If these values are corrected for unresolved closures as described in Methods they are $0{\cdot}21 \pm 0{\cdot}09$ ($n = 24$) for H–H and $0{\cdot}46 \pm 0{\cdot}18$ ($n = 27$) for L–L.

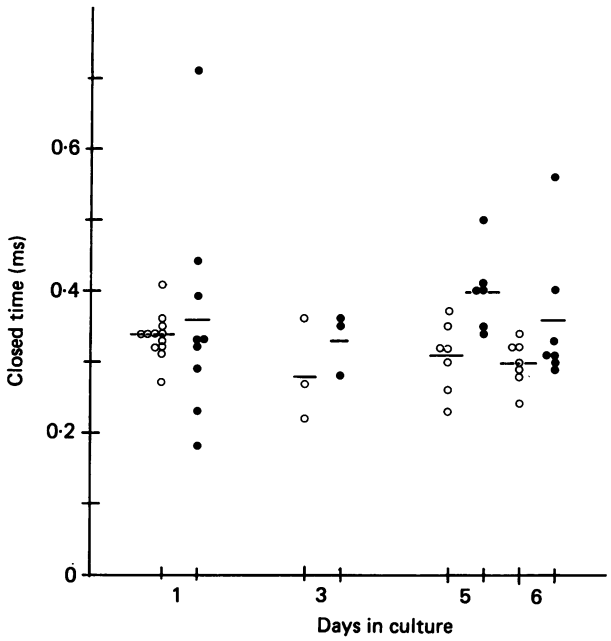

Fig. 5. Time constants of the fast component in the closed-time histogram *versus* days of muscle cells in culture. Open and filled circles represent low- and high-conductance channel events, respectively. Bars indicate the mean of individual points.

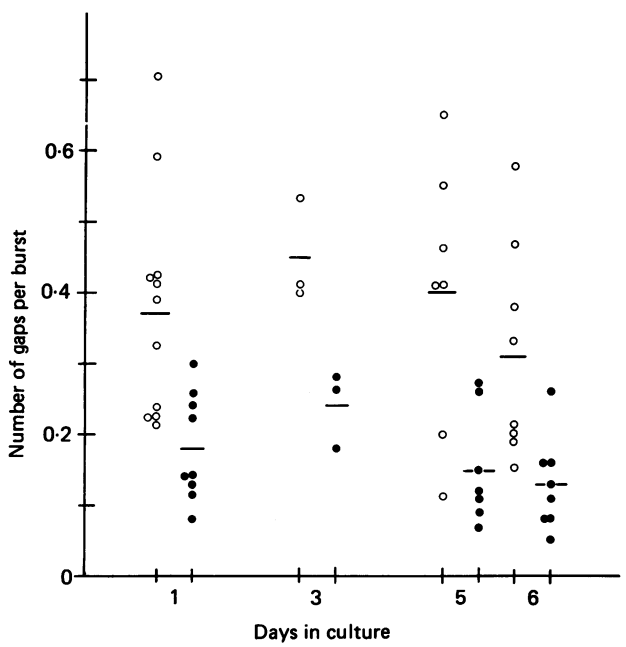

Fig. 6. Number of gaps per burst *versus* days in culture. Open and filled circles represent low- and high-conductance channel events, respectively. Bars indicate the mean of individual points.

For both types of channels this parameter did not change during development in culture (Fig. 6).

Voltage dependence of closed-time parameters

The voltage dependence of the apparent open time of ACh receptor channels (channel closing rate) has been demonstrated in many preparations (Anderson & Stevens,

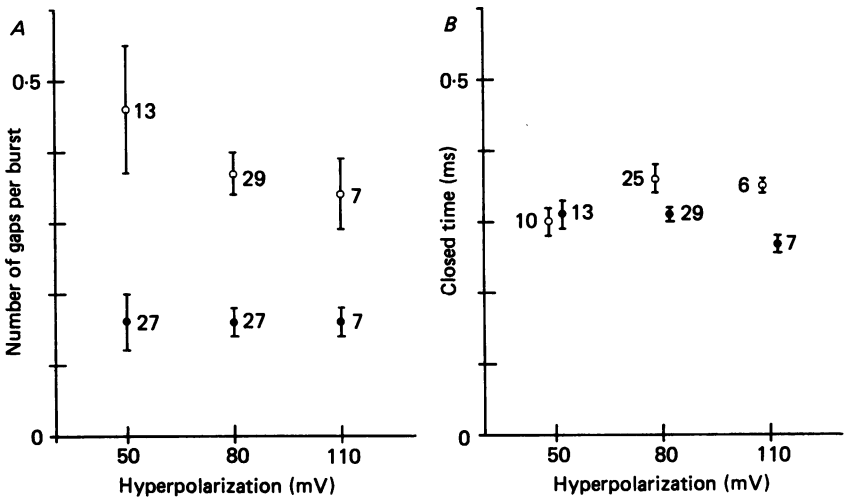

Fig. 7. Voltage dependence of number of gaps per burst and of the time constant of the fast component in the closed-time histogram. Open and filled circles represent low- and high-conductance channel events, respectively. Bars attached at each point indicate standard error of mean. Figures are number of samples. In the abscissa the applied potential at the electrode was plotted. The patch membrane was experiencing the potential value indicated on the abscissa plus the resting membrane potential of around −85 mV.

1973; Adams & Sakmann, 1978; Horn & Brodwick, 1980) including *Xenopus* myocytes in culture (Brehm *et al.* 1984*a*). Less-prominent voltage dependence of the channel opening rate has been implicated in rat myoballs in culture (Horn & Brodwick, 1980). Colquhoun & Sakmann (1985) reported in the frog neuromuscular junction that neither the number of gaps per burst nor the time constant of the fastest component depended on the membrane potential. However, the frequency of bursts increased slightly upon hyperpolarization. In contrast, Leibowitz & Dionne (1984) reported clear voltage dependence of the channel opening rate and agonist dissociation rate in the snake neuromuscular junction. Recently Sine & Steinbach (1986*b*) demonstrated in a mouse cell line (BC3H–1) with ACh as an agonist that the channel opening rate was potential dependent and increased with hyperpolarization, but the agonist dissociation rate was not potential dependent.

We measured the voltage dependence of the closed-time constant and the number of gaps per burst as defined above by changing the potential inside the recording electrode. As shown in Fig. 7, neither of these parameters in either type of channel changed significantly with voltage.

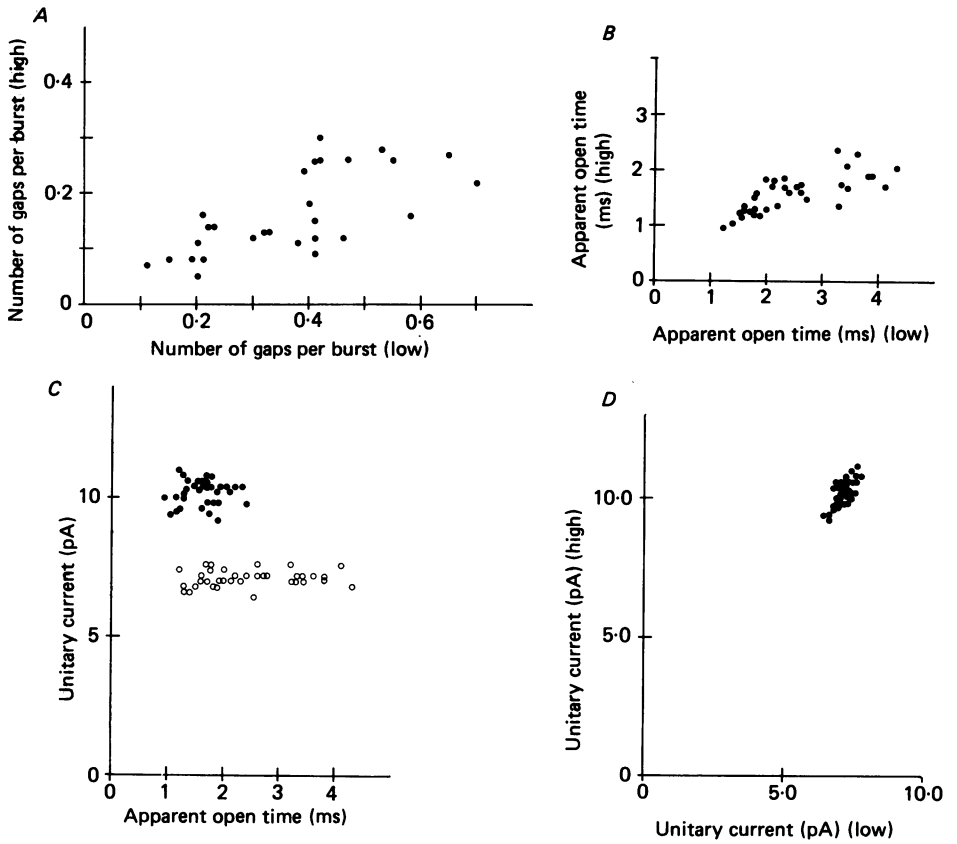

Fig. 8. Correlation between various kinetic parameters. *A*, number of gaps per burst of high-conductance channel events *versus* that of low-conductance channel events. These two parameters are significantly correlated ($P = 0{\cdot}0001$). Correlation coefficient = 0·69 ($n = 28$). Each point represents data for low- and high-conductance events from the same patch of membrane. *B*, apparent open time of high-conductance channel events *versus* that of low-conductance channel events from the same patch of membrane. Correlation coefficient = 0·73 ($n = 35$). This correlation is significant at $P = 0{\cdot}0001$. *C*, unitary current *versus* apparent open time. Open circles represent low-conductance channel events. These parameters are not correlated. *D*, unitary current of high-conductance channel events *versus* that of low-conductance channel events. Correlation coefficient = 0·77 ($n = 35$). These parameters are correlated at $P = 0{\cdot}0001$.

Variability of the apparent channel open time

The apparent channel open time was variable among different patches, especially in the low-conductance channel event. In order to assess the cause of the variability we examined correlations between various parameters. When the apparent open times of high- and low-conductance channel events were plotted there was a good correlation (Fig. 8*B*), the correlation coefficient being 0·73 ($n = 35$, significant at $P = 0{\cdot}0001$). This indicates that a patch which contained low-conductance channels with a long apparent open time tended to have high-conductance channels with a long apparent open time. Similarly there is correlation between unitary current of high- and low-conductance channel events (Fig. 8*D*, correlation coefficient = 0·77,

$n = 35$). Since we did not know the absolute membrane potential of the patch it was conceivable that the longer apparent open time was due to the larger membrane potential as it is known in this preparation that the open time is longer when the membrane is more hyperpolarized (Brehm *et al.* 1984*a*). This is, however, unlikely to be the case because the variability of the apparent open time is greater in the low-conductance channel events compared with that of the high-conductance channel events although the voltage dependence was found to be similar in these two types of channels (Brehm *et al.* 1984*a*). Furthermore, if that is the case there should be correlation between the unitary channel current and the apparent channel open time. Fig. 8*C* shows the plot between the unitary current and apparent open time. No correlation was found in these parameters (correlation coefficient is 0·11 for the low-conductance channel event ($n = 35$) and 0·006 for the high-conductance channel event ($n = 33$)). Therefore, it is unlikely that the variability of the apparent open time is due to the variability of the resting membrane potential.

The variability of the closed time is not correlated with any other parameters but as shown in Fig. 8*A*, the number of gaps per burst in low-conductance channel events was strongly correlated with that of high-conductance channel events (correlation coefficient = 0·69 ($n = 28$)).

Thus, apparently a factor or factors of a cell, such as the level of channel protein phosphorylation, can affect the channel kinetics in the same direction between high- and low-conductance channels.

ACh receptor channel kinetics at high ACh concentrations

So far we have measured and analysed receptor channel kinetics at a low ACh concentration (0·2 μM). In order to detect further kinetic differences between two types of channels and their developmental changes we studied channel kinetics at higher ACh concentrations.

The patterns of ACh receptor channel events at high concentrations have been described previously (Sakmann *et al.* 1980; Siegelbaum *et al.* 1984; Sine & Steinbach, 1984*b*; Auerbach & Lingle, 1986*a*,*b*). Channel events occurred in clusters of bursts as shown in Fig. 9 (10 μM-ACh) and Fig. 10 (100 μM-ACh) interspersed with seconds of silence. In general, within each cluster the unitary current amplitude was the same but among different clusters there were two types of events, corresponding to the low- and high-conductance channel described earlier. Usually channel events were not observed between clusters but occasionally events with a short duration were sporadically seen as described by Auerbach & Lingle (1986*b*). We did not analyse these channel events occurring outside of the major open activation mode. In 100 μM-ACh (Fig. 10) current noise in the open state was significantly greater than at the closed state. This was particularly prominent in the high-conductance channel event (Fig. 10*B*).

Recently ACh receptor channels in cultured *Xenopus* muscle cells were studied at high ACh concentrations (Auerbach & Lingle, 1986*b*). They found that these channels, both low- and high-conductance channels, have different modes in terms of the probability that a channel existed in an ion-conducting conformation during the burst (P_o). In both types of channels one mode is dominant. 80% or more of the clusters of bursts belonged to this mode. Occasional switching of modes was observed,

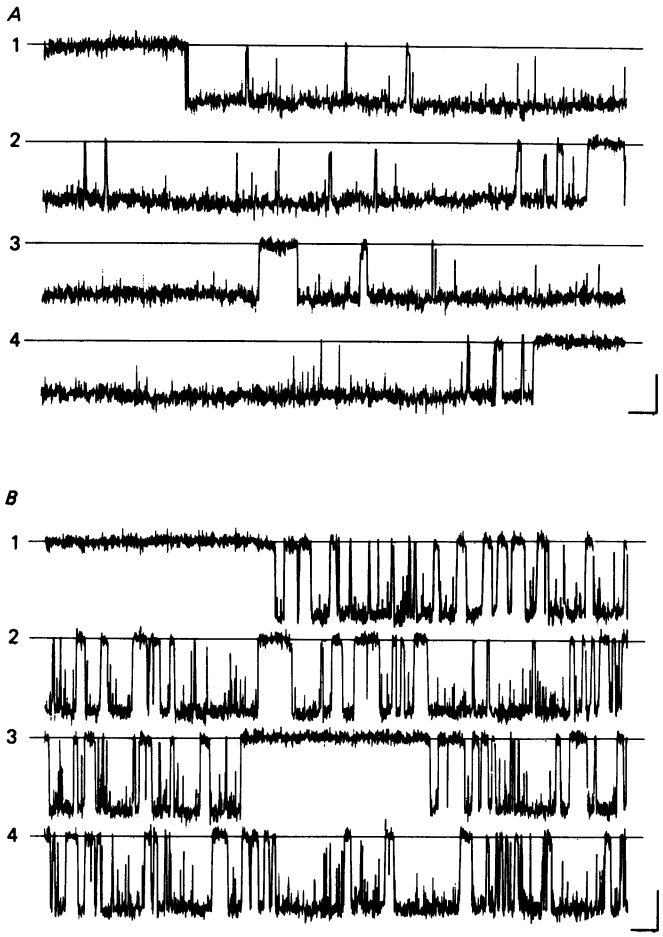

Fig. 9. Sample traces of single-channel current records with 10 μM-ACh in the patch electrode. *A*, low-conductance channel events. *B*, high-conductance channel events. Four traces in each panel are continuous. Continuous lines indicate the closed level. Scales at the lower right corner are 5 pA and 10 ms. Data in *A* and *B* were recorded from the same patch of a muscle cell cultured for 1 day after dissociation.

which suggests that these modes may represent different activity patterns of a homogeneous channel population. We did occasionally notice in high concentrations sporadic brief openings between clusters of bursts but did not study them because they were relatively rare. Therefore our analyses were carried out on channel events in the major mode of Auerbach & Lingle (1986*b*).

Concentration dependence of the apparent open time

If we were measuring true open times and only one open state existed then the histogram should have one exponential and the time constant should be independent of ACh concentration. The histograms for the apparent open time in high concentrations were similar to those in low concentration. The histograms for high-conductance channel events were fitted well with one exponential as shown in

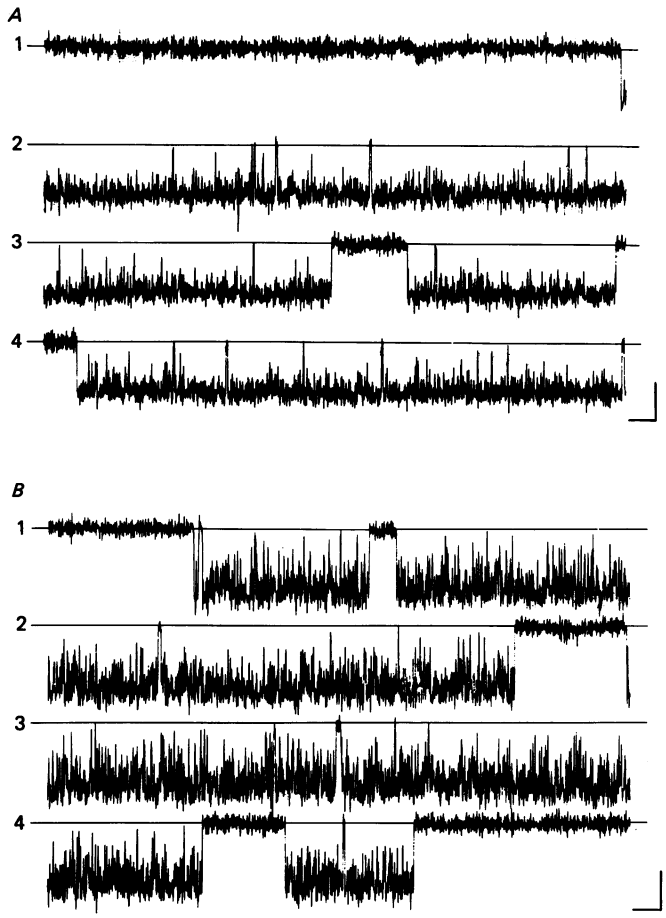

Fig. 10. Sample traces of single-channel current records with 100 μM-ACh illustrated in the same manner as in Fig. 9. *A* and *B* were recorded from the same patch from a cell after 1 day in culture.

Fig. 11 *B* whereas those for the low-conductance channel events tended to have extra entries in shorter-duration bins (Fig. 11 *A*). As shown in Fig. 12 the apparent open times for both types of channel events were slightly concentration dependent. The correlation coefficient for the high-conductance channel events was 0·70 which is highly significant ($P = 0{\cdot}0001$) and that for the low-conductance channel was 0·44 which is also significant ($P = 0{\cdot}001$). (Data at 100 μM-ACh were excluded from these calculations for reasons described below.) Correlation between apparent open time and ACh concentration was also reported by Auerbach & Lingle (1986*a*). This was considered to be due to undetected brief closures.

At 100 μM-ACh the apparent open time for high-conductance channel events decreased abruptly. As described below this is most likely due to blockade of ACh receptor channels with ACh molecules (Sine & Steinbach, 1984*b*; Ogden & Colquhoun, 1985). This blockade was more prominent in the high-conductance channel.

At 20 μM-ACh, Auerbach & Lingle (1986*b*) reported the mean open time for

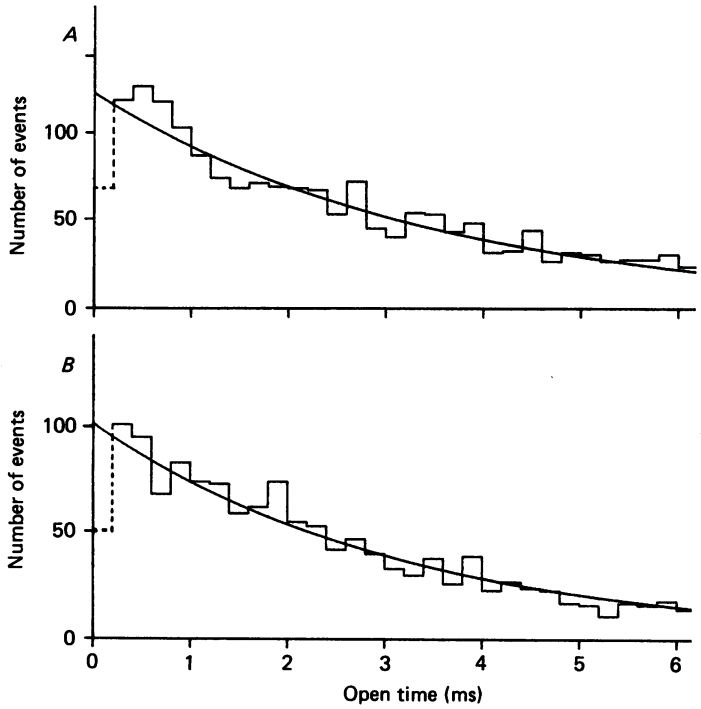

Fig. 11. Apparent open-time histograms of low- (*A*) and high- (*B*) conductance channel events at 10 μM-ACh. These data were obtained from the same patch of membrane of a muscle cell 5 days in culture. The continuous line has a time constant of 3·5 ms for *A* and 3·17 ms for *B*. Total number of events was 1567 for *A* and 1206 for *B*.

low-conductance channel events as 5·6 ms and that for high-conductance channel events as 1·7 ms at the estimated membrane potential of -120 mV and at room temperature. These values are similar to ours depicted in Fig. 12.

Concentration dependence of closed time

Closed-time histograms at higher ACh concentrations could be complicated due to multiple closed states. Qualitatively we wished to examine the difference in the behaviour between two types of channels at high concentrations. Examples of closed-time histograms at 20 μM-ACh are shown in Fig. 13. The histogram was fitted reasonably well by two exponentials. A fast component for high-conductance channel events had a time constant of $0{\cdot}35 \pm 0{\cdot}21$ ms and relative area of 50 ± 24% ($n = 9$, unresolved closures corrected, assuming only two exponentials). The slow component had a time constant of $2{\cdot}11 \pm 1{\cdot}0$ ms ($n = 8$). For low-conductance channel events these values were $0{\cdot}32 \pm 0{\cdot}07$ ms and 69 ± 17% ($n = 9$) for the fast component and $1{\cdot}33 \pm 0{\cdot}48$ ms ($n = 7$) for the slow component. The time constant for the fast component was similar to that for channel events at 0·2 μM-ACh described earlier. However, the relative area of closures belonging to the fast component was greater and increased with ACh concentration. Here again as we noted in low-concentration experiments the content of the first bin (less than or equal to 200 μs) is greater than

expected considering time resolution of the recording system. Thus, it is likely that there is another unresolved brief component in this region. Auerbach & Lingle (1986*b*) reported the mean closed time of 0·6 ms for low-conductance channel events and 1·1 ms for high ones at 20 μM-ACh. These values fell between our fast and slow

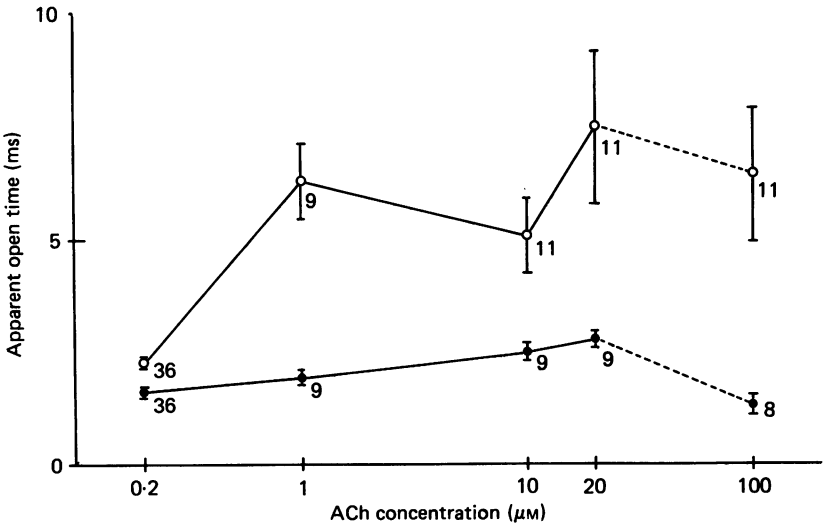

Fig. 12. Apparent open time *versus* ACh concentration. ACh concentration (μM) was plotted logarithmically on the abscissa. Open and filled circles represent low- and high-conductance channel events. Bars attached to each point indicate the standard error of the mean. Figures beside each point are the number of samples.

component. The concentration dependence of the time constant and the relative area of the component are depicted in Fig. 14. The fast time constant was not concentration dependent (filled circles) but the relative area of this component (squares) was. In contrast, the slow time constant (open circles) was concentration dependent. At 100 μM-ACh channel blockade by ACh molecules became prominent. There were many brief closures, particularly in the high-conductance channel event. This phenomenon will be analysed separately later. The time constant of the slow component progressively became shorter as the ACh concentration was increased.

The slow component for the high-conductance channel event had a longer time constant than that for the low-conductance channel event at all ACh concentrations. Other than this there was no discernible difference in these parameters between the high- and low-conductance channel event, nor was there a difference between young and old cultures in the channel behaviour at high ACh concentrations.

Probability of being open within a cluster of bursts (P_o)

In high concentrations of ACh (1–100 μM) ACh receptor channels undergo desensitization and occasionally open in a succession of bursts (cluster of bursts). The probability of a channel being open within a cluster (P_o) was defined by Auerbach & Lingle (1986*b*) as the sum of the open durations divided by the time between first

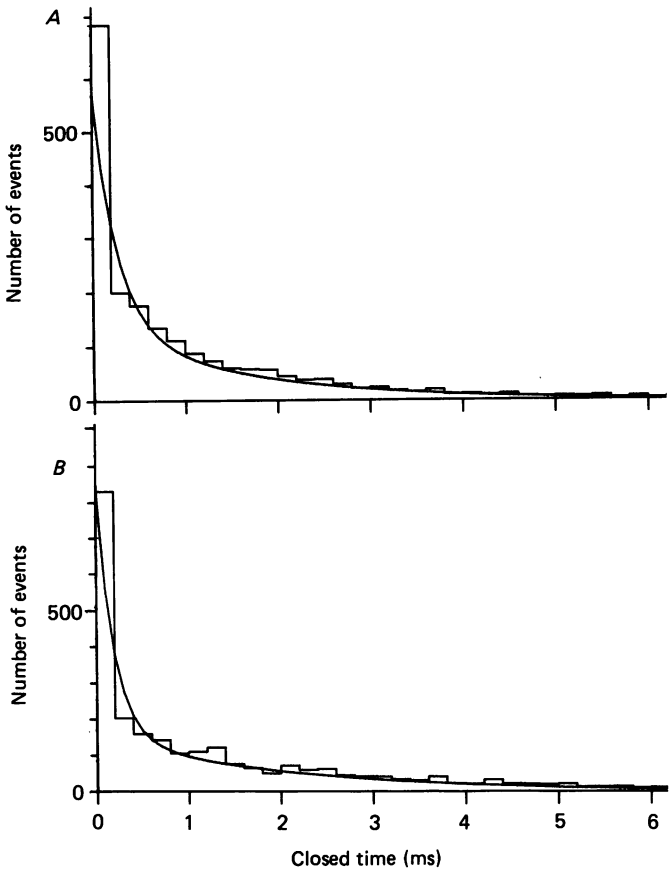

Fig. 13. Closed-time histograms for low- (*A*) and high- (*B*) conductance channel events. Continuous lines are combined curves of two exponentials. The time constants of the fast and slow component were 0·27 and 1·6 ms for *A* and 0·19 and 2·0 ms for *B*. 35% of the total events belonged to the fast component in *A* and 30% in *B*. Total number of events used to fit the curve was 1364 for *A* and 1725 for *B*. Data were obtained from a membrane patch of a 1-day-old muscle cell.

and last open transition in the cluster. We used this parameter as an empirical measure of channel kinetic behaviour to compare the two types of channels further. Usually channels were in the silent state for a prolonged period of time (seconds) and sporadically opened in a cluster of bursts. During this period P_0 was generally high. In other infrequent cases channels opened briefly in a cluster. Since the latter case was not frequent we measured P_0 values only in the former mode.

As reported by Auerbach & Lingle (1986*b*) in *Xenopus* myocytes and Ogden & Colquhoun (1983) in the frog neuromuscular junction, the value of P_0 was large at 20–100 μM (Fig. 15), that is, channels stayed in the open state most of the time during a cluster of bursts. P_0 was always larger in the low-conductance channel than in the high-conductance channel.

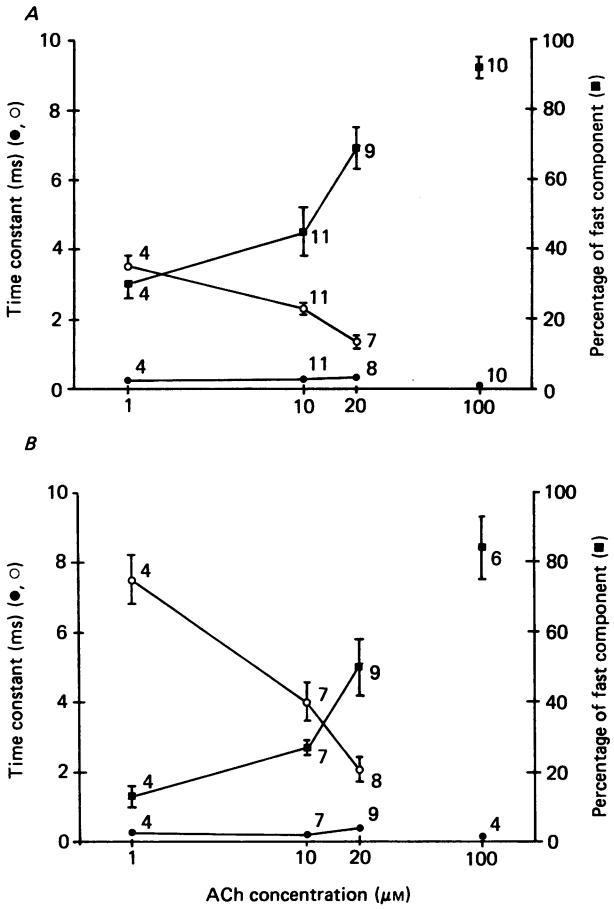

Fig. 14. Closed-time parameters *versus* ACh concentration for the low- (*A*) and high- (*B*) conductance channel event. Open circles represent the time-constant value of the slow component and filled circles that of the fast component. Squares represent percentage of the fast component in the total number of events. Bars attached to each point are the standard error of mean. Figures are the number of samples. At 100 μM-ACh no distinction could be made between the fast and slow component. As described in the text, at 100 μM the channel was frequently blocked by ACh molecules. Therefore, parameters in the closed-time histogram are not directly comparable to those in lower concentrations.

Channel blocking at 100 μM ACh

At first glance in Fig. 10*A* and *B* it is obvious that the noise is greater in the open state than in the closed state. This is particularly true for high-conductance channel events. In order to examine the nature of this extra noise, we made frequency histograms of individually sampled points as shown in Fig. 16. Each distribution has two peaks corresponding to two states of channels, i.e. open and closed. The left peak corresponds to the closed state, which in all cases was well fitted by a Gaussian distribution with standard deviation of $0{\cdot}72 \pm 0{\cdot}07$ pA ($n = 32$). The second peak on the right of each distribution represents the open state of the channel. At 10 μM-ACh the second peak was symmetrical and also fitted reasonably well with a Gaussian

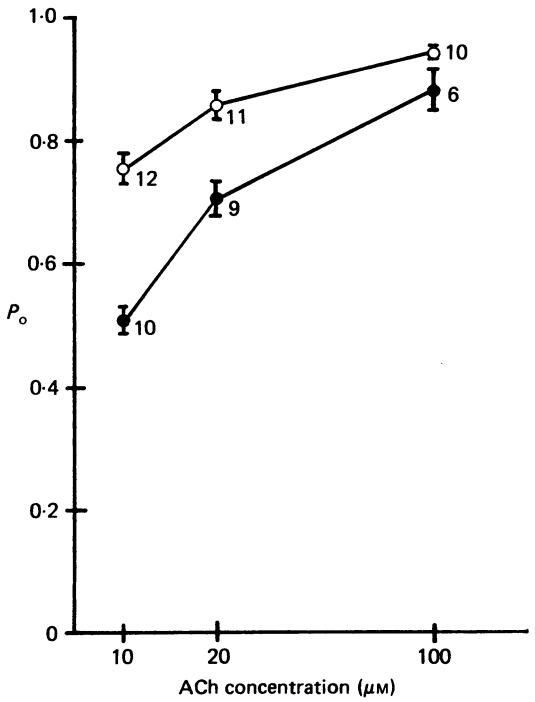

Fig. 15. Probability of a channel being open (P_o) *versus* ACh concentration for the low-conductance channel (open circles) and for the high-conductance channel (filled circles). Each point represents the mean of samples, the number of which is indicated at the side. The bar is the standard error of mean. P_o was measured as defined in the Methods.

distribution (Fig. 16*A* and *B*). The standard deviation was slightly larger for the open state. The ratio between the standard deviation of the open-channel state and the closed-channel state was $1{\cdot}08 \pm 0{\cdot}08$ ($n = 9$) for low-conductance channel events and $1{\cdot}16 \pm 0{\cdot}07$ ($n = 7$) for high-conductance channel events. Similar extra noise in the open state has been reported in chick (Auerbach & Sachs, 1984) and in rat myotubes (Sigworth, 1985). Sigworth (1985) examined possible sources of these open-channel fluctuations and suggested that they arise from conformational fluctuations in the channel protein.

In contrast to these symmetrical fluctuations, the second peak at 100 μM-ACh (Fig. 16*C* and *D*) was not fitted with a Gaussian distribution, particularly in the high-conductance channel event (Fig. 16*D*). The data were all skewed towards the left. The Gaussian curve on the second peak was generated using the portion of the histogram greater than the current value at the peak. It is known that at high ACh concentrations ACh molecules may enter ACh receptor channels and block them (Sine & Steinbach, 1984*b*). If the blockade of channels occurs frequently on a time scale faster than the time resolution of the recording system, we expect extra noise during open state and the histogram to be skewed (Yellen, 1985). We, therefore, attempted to estimate the rate of blocking and unblocking from the skewed histogram as shown in Fig. 16*D*, using the technique developed by Yellen (1985) for Ca^{2+}-activated K^+

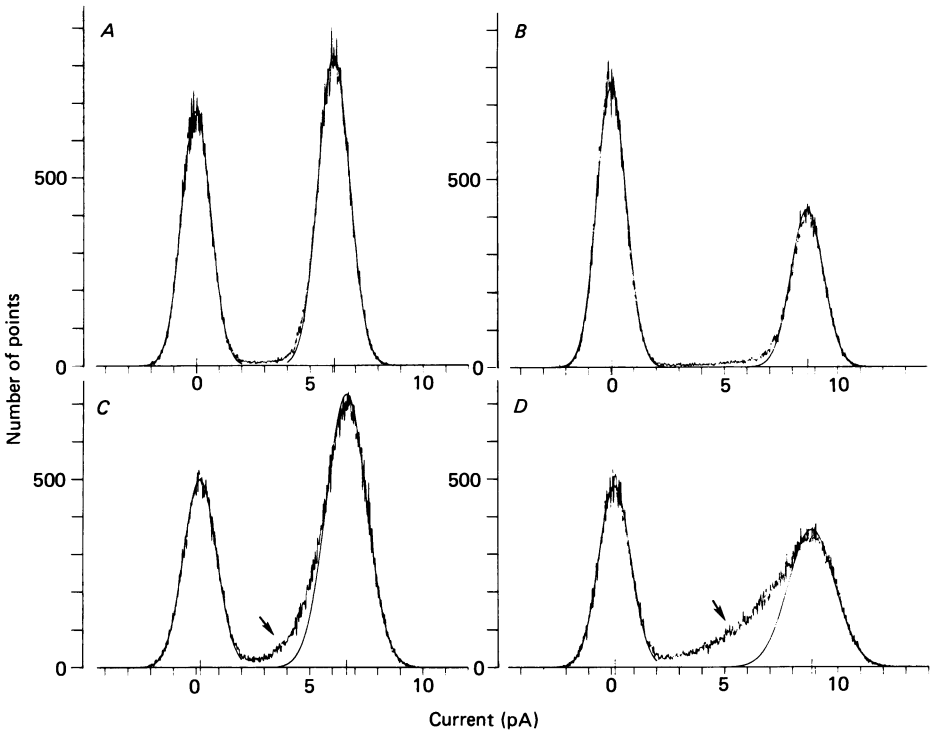

Fig. 16. Amplitude distribution of sampled individual points in the current recording. Number of points was plotted against the current amplitude of the point. *A* and *C* represent low-conductance channel events and *B* and *D* represent high-conductance channel events. Data in *A* and *B* are from the same patch of 5-day-old muscle at an ACh concentration of 10 μM. Data in *C* and *D* are from the same patch of 1-day-old muscle at an ACh concentration of 100 μM. Each distribution has two peaks. The first peak on the left represents closed-time noise and the second one on the right represents open-time noise. Fitted continuous lines are Gaussian curves. When the data distribution was obviously skewed as in the second peak in *D*, the Gaussian curve was fitted using the part of the curve greater than the value at the peak. An arrow in *C* and *D* indicates clear deviation from a Gaussian curve.

channels in the adrenal chromaffin cell. Since the open-state noise was much more prominent in the high-conductance channel event, we used these histograms for the curve fitting. The fit was excellent as shown in Fig. 17. In five patches the blocking and unblocking rates were estimated as $1{\cdot}16 \pm 0{\cdot}24 \times 10^4$ s^{-1} (the association rate constant = $1{\cdot}16 \pm 0{\cdot}24 \times 10^8$ M^{-1} s^{-1}) and $5{\cdot}04 \pm 1{\cdot}34 \times 10^4$ s^{-1}, respectively. The blocking rate found here is slightly greater but the unblocking rate is close to that reported by Ogden & Colquhoun (1985) for the frog neuromuscular junction (blocking rate $3{\cdot}7 \times 10^7$ M^{-1} s^{-1} and unblocking rate $5{\cdot}6 \times 10^4$ s^{-1}). This blockade by ACh molecules made the analyses of channel kinetics even more difficult at higher concentrations.

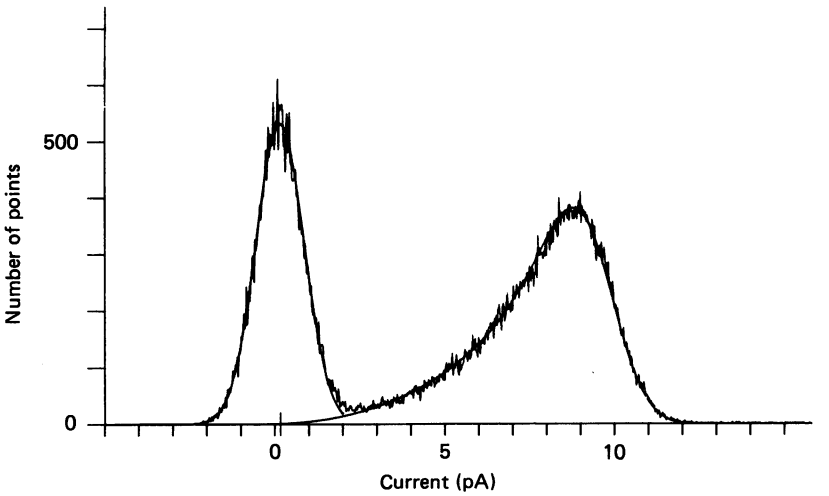

Fig. 17. The sample amplitude distribution as shown in Fig. 16*D*. Fitted continuous line is a Gaussian curve for the first peak and a beta function for the second peak. The procedures for fitting a beta function are described in the Methods.

DISCUSSION

Previously we have described changes in the relative proportion of two types of channel events during 6 days in culture. The low-conductance channel events were dominant in younger cultures but in older cultures the relative proportion of high-conductance channel events increased. There was no change in the apparent open time nor was there a change in the unitary conductance within each type of channel. We could not, however, conclude that this change indicates that the relative number of channel molecules was changing or simply that the high-conductance type of channel began to open more frequently even though the number of channel molecules was the same (Brehm *et al.* 1984*a*).

In this study we found that the aspects of channel kinetics that we have examined remain the same during culture. Therefore, the increase in the low-conductance channel events with time in culture is not likely to be due to an enhanced opening rate of this type of channel, although we still don't know whether binding characteristics change during development. It is more likely that the observed shift of channel-event population is the result of change in the number of channel molecules. Since the total number of α-bungarotoxin binding sites did not change during this period (Kidokoro & Gruener, 1982), an increase in the number of high-conductance channels must be approximately offset by a decrease in the low-conductance channel molecules.

Difference in kinetics between low- and high-conductance channels

Two types of ACh receptor channels in developing *Xenopus* muscle have been described as having different unitary conductances as well as different apparent mean open times (Clark & Adams, 1981; Greenberg *et al.* 1985; Brehm *et al.* 1984*a*). High-conductance channel events have a shorter mean apparent open time than

low-conductance channel events. This difference was confirmed in this study although the difference was smaller in this study than in the previous work. At least a part of the discrepancy originates from the difference in the measurement as pointed out earlier. We found that the low-conductance channel events are more likely to re-open after brief closures. Due to technical limitations some of the brief closures were undetected. We estimated the relative number of unresolved closures as described in the Methods section. Considering these unresolved closures the high-conductance channel events have a corrected mean open time of $1{\cdot}35 \pm 0{\cdot}29$ ms ($n = 24$). For the low-conductance channel event this value is $1{\cdot}99 \pm 0{\cdot}73$ ms ($n = 29$). These values correspond to a channel closing rate (α) of 740 s^{-1} for the high-conductance channel and 500 s^{-1} for the low-conductance channel. These values are admittedly tentative because we did not resolve many brief closures which were in the first bin of the closed-time histogram. Colquhoun & Sakmann (1985) estimated the value for the closing rate as 714 s^{-1} at 10·5–11·5 °C and at -130 mV in the frog neuromuscular junction and in a mouse cell line (BC3H–1) Sine & Steinbach (1986*b*) reported 23·5 s^{-1} at -100 mV and at 11 °C.

The difference in the number of gaps per burst between the high- and low-conductance channel event was prominent whereas the time constant for the fast component of the closed-time histogram was similar between these two types of channel events.

We will now try to interpret our results with the aid of the reaction scheme below.

$$\underset{\text{State I}}{2A+R} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \underset{\text{State II}}{A+AR} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \underset{\text{State III}}{A_2R} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \underset{\text{State IV}}{A_2R^*}. \qquad (1)$$

A represents ACh molecules and R represents receptors. * indicates the open state of the channel and all other states are closed.

The fast component in the closed-time histogram at 0·2 μM-ACh was quite clear in all cases. They were 0·36 ms for the high- and 0·31 ms for the low-conductance channel event. If we assume that 0·2 μM-ACh is the limiting low concentration and that the fast major component is representing state III in the scheme, we can calculate the opening rate (β) and agonist dissociation rate (k_{-2}) from the following equations (Colquhoun & Sakmann, 1981; Colquhoun & Hawkes, 1981).

$$1/(\beta + k_{-2}) = \tau_1,$$

where β/k_{-2} = number of gaps per burst and τ_1 is the time constant of the fast component. β and k_{-2} thus calculated are 480 and 2300 s^{-1}, respectively, for the high-conductance channel event and 1020 and 2210 s^{-1}, respectively, for the low-conductance channel event. $\beta/\alpha = 0{\cdot}66$ for the high-conductance channel and 2·0 for the low-conductance channel.

These values are similar to those reported by Dionne & Leibowitz (1982) for snake muscle (750–825 s^{-1} for β and 3400–6000 s^{-1} for k_{-2}). However, they are substantially slower than those published recently by Colquhoun & Sakmann (1985) ($\beta = 30600 \pm 1180$ s^{-1} and $k_{-2} = 16300 \pm 1200$ s^{-1}). (Note that their definition of k_{-2} is different from ours: twice their k_{-2} equals our k_{-2}.) The main reason for this discrepancy is in the interpretation of components in the closed-time histogram. Our

fast component most likely corresponds to the intermediate component seen by Colquhoun & Sakmann (1985). The briefest component in their case ('nachschlags') was not resolved in our experimental conditions. They interpreted this briefest component as dwell time in state III where two ligand molecules are bound to the receptor but the channel is still closed.

The major difficulty raised by Colquhoun & Sakmann (1985) in their interpretation of the intermediate component as representing state III is as follows. At high concentrations of ACh, channels undergo a desensitized state for prolonged periods of time. When a channel recovers from the desensitized state the channel opens very efficiently. The probability of being in the open state was around 0·98 with 100 μM-ACh in the frog neuromuscular junction (Ogden & Colquhoun, 1983; Ogden, 1985). This translates into a value for the ratio of the two rate constants, β/α, of around 50. If the closing rate is taken as 800 s^{-1}, then β must be 40000 s^{-1}. This is much larger than calculated from the intermediate component of Colquhoun & Sakmann (1985). In the case of *Xenopus* muscle, P_0 values at 20 μM-ACh were grouped into three different mean values of 0·9, 0·3 and less than 0·01 for the low-conductance channel. About 80 % of clusters of bursts were from the highest P_0 population. For the high-conductance channel the highest P_0 population accounted for more than 80 % of all clusters of bursts and had a mean P_0 of 0·6 at 20 μM-ACh (Auerbach & Lingle, 1986*b*). Similarly we measured P_0 values at the major mode as 0·86 for the low-conductance channel event and 0·71 for the high-conductance channel event at 20 μM, and at 100 μM they were 0·94 for the low- and 0·88 for the high-conductance channel event. If we estimate β from these numbers for P_0 at 100 μM and values for α it was about 7800 s^{-1} for the low-conductance channel and 5400 s^{-1} for the high-conductance channel. Thus it is difficult also in *Xenopus* muscle to reconcile this discrepancy in β calculated from low-ACh-concentration data and that from high-concentration data. It may be necessary to revise scheme (1) or to change the interpretation of data.

Variability of channel kinetics

Previously it was noticed that the variability of the apparent open time for low-conductance channel events was greater than that for high-conductance channel events (Brehm *et al.* 1984*a*). In this study we ruled out a possibility that this variability in the apparent open time stemmed from the variability in the membrane potential. A good correlation was found in the number of gaps per burst between high- and low-conductance channel events. Since it is most likely that high- and low-conductance channels are different molecules this suggests that some molecular environment is affecting these two types of ACh receptor channel molecules in a similar manner. Recently Sakmann, Methfessel, Mishina, Takahashi, Takai, Kurasaki, Fukuda & Numa (1985) discovered that when the γ subunit of the calf ACh receptor was combined with other remaining subunits of *Torpedo* receptors, the apparent open time was similar to that of the calf ACh receptor. They then suggested that the γ subunit is primarily responsible for determining the channel open time. It is, however, possible that various other factors also dictate the kinetics of the channel. If the difference in the kinetics between the high- and low-conductance channels was due to a difference in one of the subunits, say the γ subunit, then the

variability which we observed within each type of channel must have originated from other sources such as the level of phosphorylation or lipid environment. Furthermore, Leonard *et al.* (1984) have shown that the mean apparent open time of the low-conductance channel shortens quickly at the very early stage of development while that for the high-conductance channel remains unchanged. Modification of channels could also change the kinetics. It is reasonable to assume that there is more than one way to modify the channel kinetics.

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