Journal of Physiology (1992), **451**, *pp.* 145–158
With 7 figures
Printed in Great Britain

ACTIVATION AND DESENSITIZATION OF EMBRYONIC-LIKE RECEPTOR CHANNELS IN MOUSE MUSCLE BY ACETYLCHOLINE CONCENTRATION STEPS

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(*Received 24 January 1991*)

SUMMARY

1. Pulses of acetylcholine (ACh) in concentrations between 0·1 and 1000 μM were applied repetitively to outside-out patches of enzymatically denervated (14 days) mouse muscle with the liquid filament switch. Solutions superfusing the patch could be changed rapidly (within 0·2 ms).

2. Single-channel activity was studied under steady-state conditions in the outside-out and in the cell-attached mode. The single-channel conductance was 26 pS in outside-out patches, characteristic for embryonic-like channels. Apparent mean open time was about 2·5 ms, a shorter component of closed times was 800 μs and burst length was about 5 ms.

3. Channel currents elicited by pulses of ACh were averaged. The time-to-peak current was concentration dependent and decreased from a level of about 10 ms below 10 μM to about 400 μs at 100 μM-ACh.

4. For a typical experiment, the average peak current, i_{max}, increased from −0·4 pA with 0·1 μM to −82 pA with 1000 μM-ACh, close to the value at saturation. The half-maximal response was at 60 μM-ACh. The dose–response curves for i_{max} had double-logarithmic slopes of 1·1–1·3, consistent with two binding sites at the embryonic nicotinic acetylcholine receptor (nAChR).

5. The current elicited by ACh pulses decreased rapidly after the peak. The time constant of desensitization increased from 20–50 ms with 1000 μM-ACh to up to more than a second with 1 μM-ACh.

6. The current in steady state (fully desensitized) increased up to 10 μM-ACh, but decreased slightly to values of $i_{max}/100$ to $i_{max}/500$ when higher concentrations were applied.

7. In addition to the well-known differences between adult and embryonic nAChR concerning the apparent mean open time and burst length, we found differences in the slope of the dose–response curve for i_{max}, in the ratio of peak to steady-state response, and in the rise time of the response.

INTRODUCTION

Nicotinic acetylcholine receptor (nAChR) channels of vertebrate muscle are known to be present in two main types, one that appears in embryonic and denervated

muscle and another that is associated chiefly with adult endplates. There is an increase in nAChR channel conductance and a decrease of apparent open time when the muscle matures. First evidence of such changes came from the analysis of ACh-induced noise recorded from skeletal muscle of rats (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980). Employing single-channel recording it was discovered that two different classes of channels were expressed during development (Hamill & Sakmann, 1981; Brehm, Kidokoro & Moody-Korbett, 1984; Leonard, Nakajima, Nakajima & Carleson, 1988). A small-conductance class (40 pS) is present in embryonic muscle, while in older cells the majority of channels had a conductance of 60 pS. The two classes have different gating properties; the 40 pS channels open 2–5 times longer than the 60 pS channels (reviewed by Schuetze & Role, 1987). An elegant explanation for the varying functional properties is that the channels have different compositions of subunits: embryonic and adult AChRs share common α-, β- and γ-subunits but differ in the fourth component, having either a δ- or a ϵ-subunit (Mishina, Takai, Imoto, Noda, Takahashi, Numa, Methfessel & Sakmann, 1986; Gu & Hall, 1988).

The studies cited above were performed under steady-state conditions with long applications of ACh. We have studied adult muscle receptors in excised patches with pulses of ACh, imitating the non-equilibrium activation of receptors by transmitter quanta (Franke, Hatt & Dudel, 1991). The preparations were freshly dissociated enzymatically. Thus, unlike the case when cell lines in culture were used (Brett, Dilger, Adams & Lancaster, 1986; Sine & Steinbach, 1986; Maconochie & Knight, 1989; Dilger & Brett, 1990), we were sure that we were working on the adult receptor type. The main result of the study by Franke *et al.* 1991 was that the maximal ACh-elicited current increases exponentially by almost the power of 3 for ACh concentrations up to 10 μM. At large ACh concentrations (> 100 μM) channels desensitized with time constants of 15–50 ms to a steady-state level of $< 1/10000$ of the peak current. Since these channel characteristics were quite unexpected (compare Cachelin & Colquhoun, 1989) the present study repeats essentially the same experimental procedure as for adult muscle (Franke *et al.* 1991), but with denervated muscle. The aim is to find whether there are further differences between adult and denervated channels, in addition to the established ones.

METHODS

Preparation of denervated muscle fibres

Interossal muscles from the hindfeet of adult mice, killed by cervical dislocation, were excised under sterile conditions and placed in a dish containing physiological solution with 0·4 mg ml^{-1} collagenase (Sigma, type II C 6885). Muscles were then incubated for 2 h at 27 °C. Thereafter, the solution containing collagenase was replaced by 'Dulbecco's modified Eagle's medium' substituted with 5% horse serum and 5% fetal calf serum. The presynaptic nerve terminals are removed by this procedure (Költgen, Brinkmeier & Jockusch, 1990). The muscles were dissociated mechanically by trituration. The dissociated muscles were kept in a standard tissue culture incubator at 37 °C and 5% CO_2 for 14 days.

For measurements the medium in the dishes was replaced by physiological solution containing (mM): 162 NaCl, 5·3 KCl, 2 $CaCl_2$, 0·67 NaH_2PO_4, 0·22 KH_2PO_4, 15 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), 5·6 glucose, pH 7·4. The cells could be used for patch clamping for 1–2 h at room temperature. Measurements were taken in the cell-attached or outside-out mode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). In the cell-attached mode the

pipette was filled with solution containing acetylcholine in various concentrations. In the outside-out mode the pipette was filled with 'intracellular' solution containing (mM): 140 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 ethylene-bis-(oxonitrilo)-tetraacetate (EGTA), 10 HEPES buffer, 10 glucose, pH 7·2. Acetylcholine was applied to outside-out patches using the liquid filament switch in which the solution superfusing the patch can be changed within about 0·2 ms (Franke, Hatt & Dudel, 1987; Dudel, Franke & Hatt, 1990). Acetylcholine-containing solution was ejected by pressure from a polyethylene tube (40–80 μm diameter). The polyethylene tube was fixed in a steel tube (0·8 mm diameter). The solution ejected from the polyethylene tube forms a well-defined, thin filament of fluid in a bulk superfusion. The steel tube is fixed to a piezo crystal (minitranslator P810.10, Physik Instrumente, Waldbronn, FRG) which, on application of 100 V, moves the tube upward by 20 μm. The time course of displacement was tested with magnetic field plates. The patch pipette with the excised, outside-out patch at its tip is located about 10 μm above the liquid filament ejected by the polyethylene tube. Upon application of 100 V to the piezo crystal, the liquid filament containing agonist is shifted upwards and hits the patch. At the end of the voltage pulse, the liquid filament swings away from the patch and superfusate (control solution) washes the patch again.

Channel currents were recorded with a Neher–Sigworth (List EPC7) amplifier. Records were stored on a videotape (modified Sony PCM-501 ES). They were digitized for processing at 20–50 kHz and evaluated off-line on a series 300 Hewlett-Packard microcomputer (Franke, Hatt & Dude, 1986; Dudel & Franke, 1987).

RESULTS

Channel characteristics of receptors of denervated muscle

Patches were formed in the non-endplate regions of the muscle fibres to avoid recording from receptors possibly surviving in the endplate. Exposure of an outside-out patch to pulses of ACh-containing solutions elicited channel openings, within 400 ms with high ACh concentrations and more slowly with lower concentrations (Fig. 1). With up to 3 μM-ACh, single-channel openings of nearly 3 pA amplitude could be discerned. With 1000 μM-ACh, the peak response was about 100 pA, corresponding to the current through approximately thirty-five open channels. Peak responses of this order of magnitude were observed all along the surface of the denervated fibres. At all concentrations, the number of open channels decreased in the continued presence of ACh, reflecting desensitization. Desensitization became more rapid and complete on increasing ACh concentration. With 1000 μM-ACh, in which the response decayed to about 1/100 at the end of a 500 ms pulse, peak activation was achieved again on repeating the pulse 2·5 s later (not illustrated).

To compare the behaviour of our preparations with the well-known steady-state responses (see Schuetze & Role, 1987), ACh was applied continuously in long pulses (Fig. 2). The number of open channels in steady state increases steeply when the concentration of ACh is increased from 0·1 to 10 μM, but little more on increasing the ACh concentration further. With 1000 μM-ACh the number of open channels seems to be reduced compared to the activation with 100 μM. Also the temporal pattern changed: while at low concentration bursts of openings with few short gaps were mostly observed, high concentrations elicited longer bursts, or clusters of openings separated by long desensitized periods (Sakmann, Patlak & Neher, 1980). In the patch of Fig. 2, the responses to 1000 μM-ACh pulses were also measured (not illustrated). The peak responses were equivalent to the opening of forty-two channels. Maximally four channels, i.e. about 10% of the whole population opened simultaneously with steady-state application of ACh (see 1 μM-ACh).

The chemically denervated muscle fibres were expected to have expressed

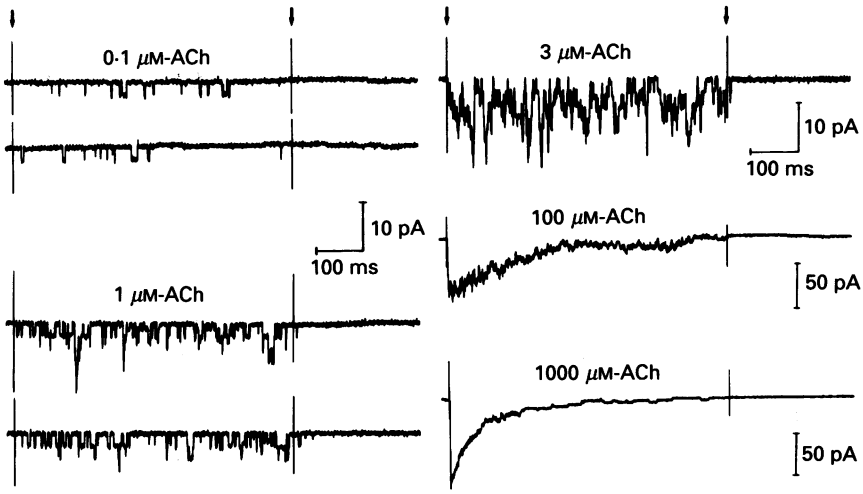

Fig. 1. Activations of single-channel openings by pulses of ACh (500 ms) at different concentrations. For 0·1 and 1 μM-ACh, two tracks are shown. Low-pass filter at 1·5 kHz. Note the different current scales for 100 and 1000 μM-ACh. Polarization was to −80 mV.

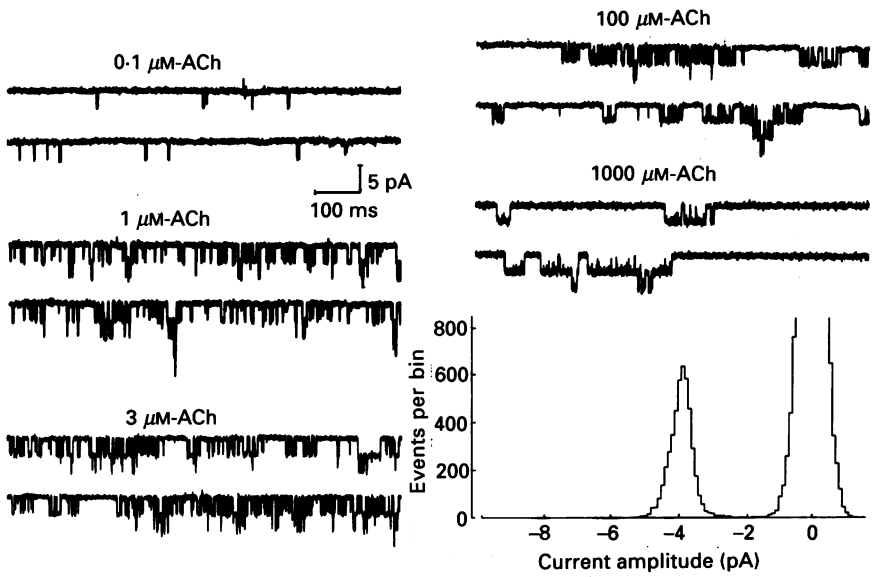

Fig. 2. Single-channel openings activated by different concentrations of ACh at one outside-out patch. ACh was applied via the liquid filament switch, and the pulse length was 1 min (beginning and end of pulse are not shown). Two traces are shown for each concentration. Polarization was to −80 mV. Low-pass filter was at 1·5 kHz. The histogram shows the distribution of single-channel current amplitudes measured with 0·1 μM-ACh.

embryonic-like receptors which should predominate, at least in the extrasynaptic regions from which we have recorded. Embryonic-like receptors had lower single-channel conductances than the adult ones, and therefore a distribution of single-

channel current amplitudes, which was measured in the presence of 0·1 μM-ACh when no superimposed openings were recorded, is included in Fig. 2. Figure 3 shows measurements of single-channel current amplitudes at different potentials for adult and denervated muscle, including cell-attached recordings, many examples of which

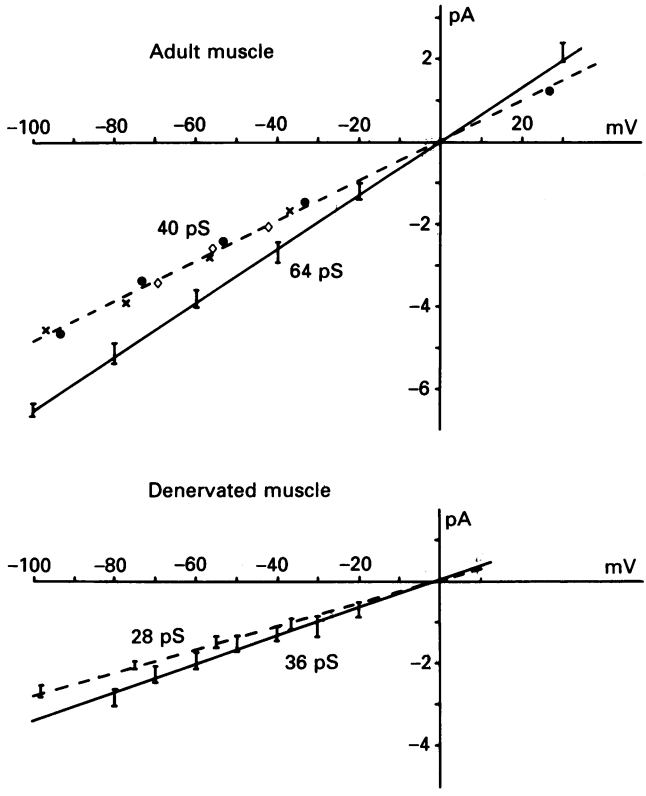

Fig. 3. Current–voltage relations for adult and embryonic channels. Experiments in the cell-attached mode are fitted with the dashed lines (different symbols indicate different experiments); those in the outside-out mode are fitted with continuous lines. At each membrane potential, amplitude histograms containing at least 200 single-channel openings were constructed.

have been given in the literature (see Schuetze & Role, 1987). For outside-out conditions, these current–voltage relations yield single-channel conductances of 64 pS in adult and 36 pS in denervated muscle. For cell-attached recordings, the lower values of 40 and 28 pS resulted. The slightly higher single-channel conductance under outside-out conditions is unexplained.

Channel openings in denervated muscle were further characterized in the distributions shown in Fig. 4, measured during long pulses of 1 μM-ACh, as in Fig. 3. Evaluation started 1 s after the beginning of the ACh pulse. The apparent mean open time, τ_o was 2·3 ms and the mean burst length, τ_b, was 5·2 ms. The critical gap length (minimal gap length separating bursts; Colquhoun & Sakmann, 1985) was taken as 4 ms. This choice is justified by the distributions of closed times shown in Fig. 4*C*,

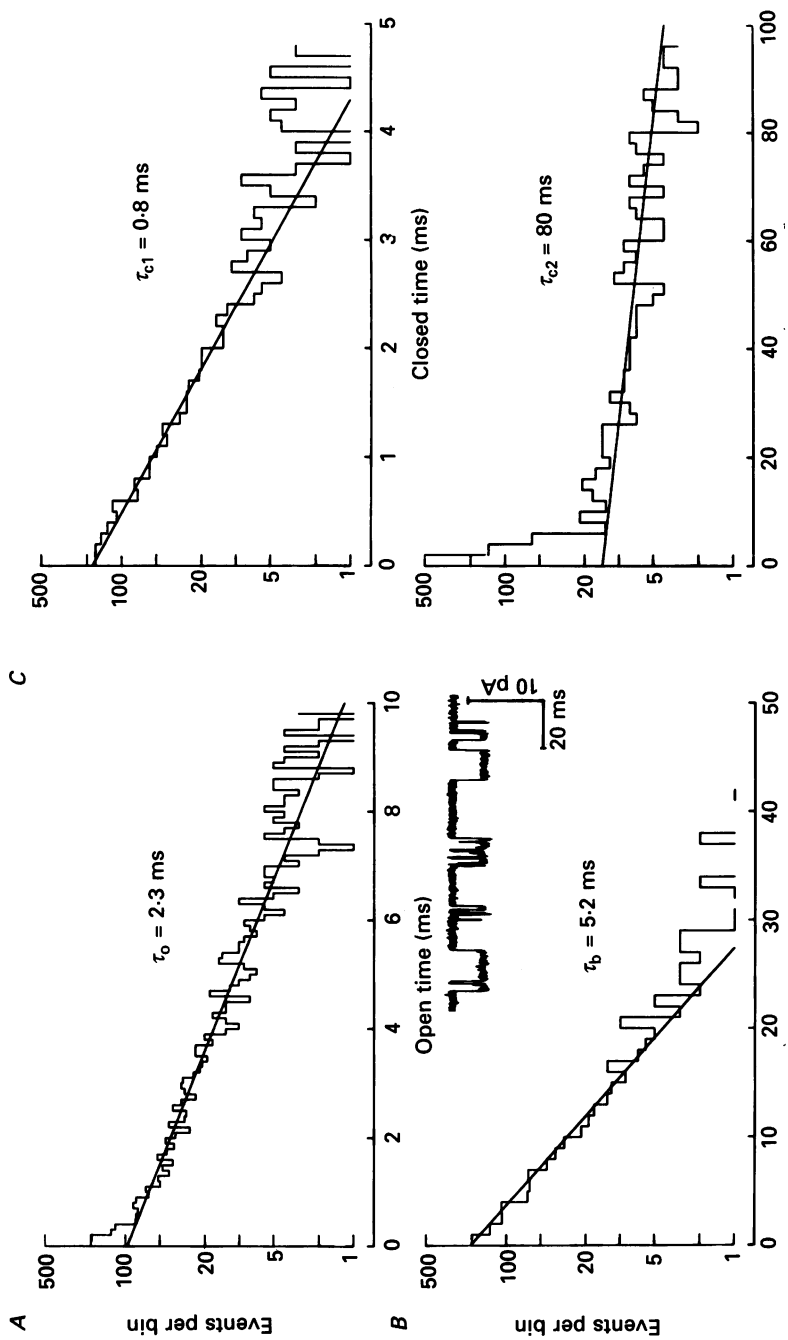

Fig. 4. Evaluation of single-channel openings elicited by long pulses of ACh (1 μM). Outside-out patch, −80 mV. Evaluation started 1 s after the beginning of the ACh pulse. Ordinates have logarithmic scales. In the inset, six bursts are shown with different numbers (between 0 and 8) of short gaps. *A*, distribution of apparent openings, fitted with a single exponential of time constant τ_o. Binwidth 100 ms. *B*, distribution of bursts, fitted with a single exponential of time constant τ_b. Binwidth 1 ms. Critical gap length 4 ms. *C*, distribution of closed times in different time resolution, fitted with two exponentials with time constants τ_{c1} and τ_{c2}. Binwidth 100 μs (above) and 2 ms (below).

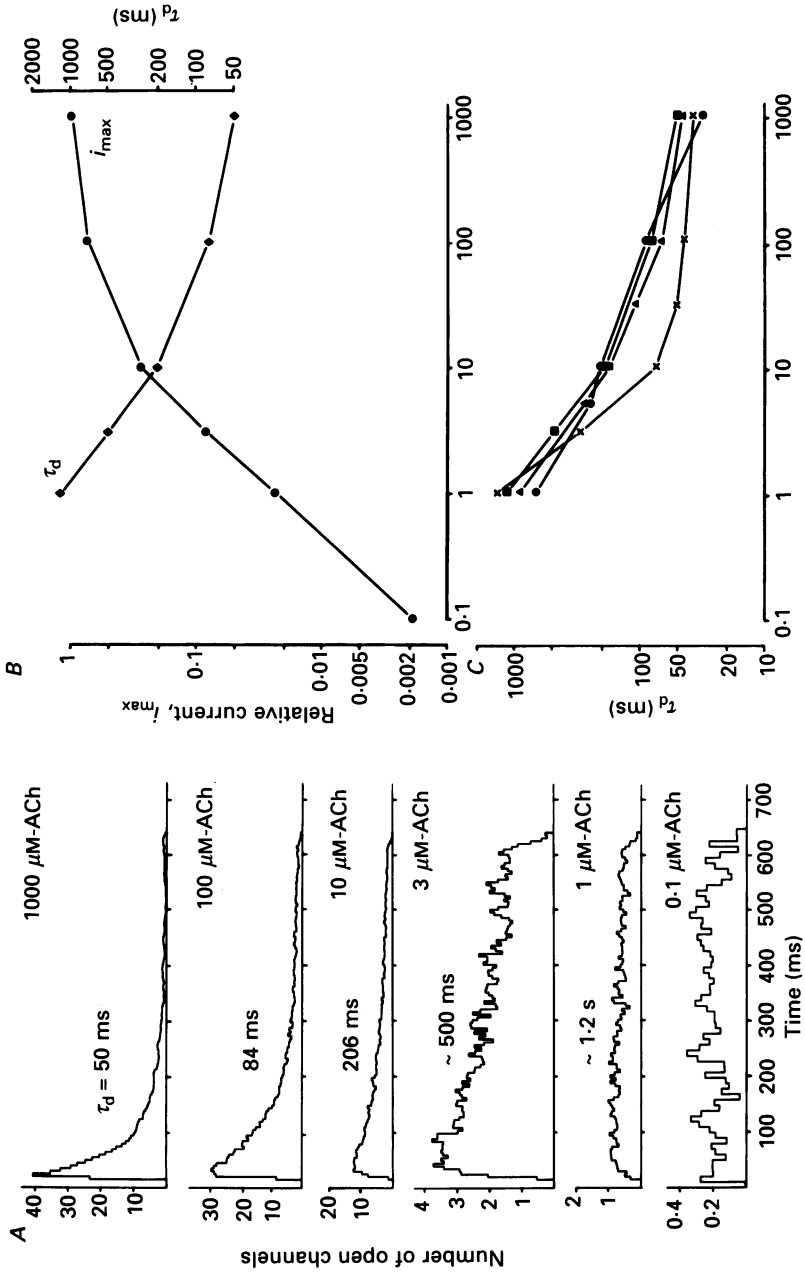

Fig. 5. Evaluation of single-channel openings elicited by pulses of ACh (600 ms) on outside-out patches in different concentrations. *A*, averages of 'idealized tracks'. Binwidth 1 ms for all concentration of ACh except 0·1 μM; 10 ms at 0·1 μM. The time constant of desensitization, τ_d, is given at each concentration. Note different ordinate scales. *B*, dependence of τ_d and of the maximal ACh-elicited current i_{max} on ACh concentration for the patch shown in *A*. Values of i_{max} were normalized to the value at 1000 μM. Ordinate for τ_d is located on the right-hand side. *C*, evaluation of τ_d for four outside-out patches. For comparison, the experiment shown in *A* and *B* is plotted again.

which can be fitted with the sum of two exponentials. The shortest component of the distribution of closed times, representing the gaps within bursts, could not be resolved in our measurements, as in adult muscle (Franke *et al.* 1991). An intermediate component of closed times had a time constant, τ_{c1}, of 0·8 ms, whereas

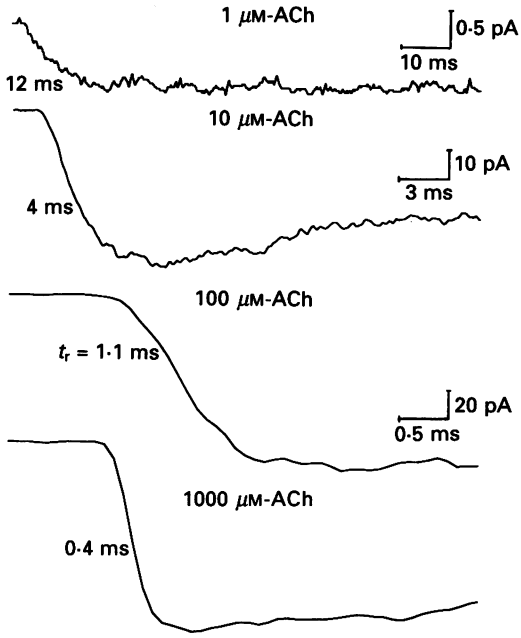

Fig. 6. Average currents obtained by summation of records shown in Fig. 1, elicited by pulses containing 1, 10, 100 and 1000 μM-ACh, respectively, in one patch. Only the beginnings of the elicited currents are shown. The values indicated are the rise times t_r from 10 to 90% of full amplitude. Membrane potential was −27 mV. Low-pass filter at 5 kHz.

the mean closed time between bursts, τ_{c2}, was 80 ms. Similar values for τ_0, τ_b and τ_{c1} were found in two other experiments in the outside-out mode and in three experiments in the cell-attached mode using the same concentration of ACh (1 μM). These values agree well with those reported in the literature for similar conditions (see Schuetze & Role, 1987).

The results of Fig. 4 were measured with application of ACh in long (1 min) pulses. The characteristics of single channels elicited at the onset of a phasic response to an ACh pulse can be recorded only when low numbers of receptor channels are contained in one patch. It is difficult to collect large numbers of events for good distributions under these conditions. However, such measurements gave results similar to those from recordings in the steady state.

Dose–response curve and desensitization of the channels

Figure 5*A* shows average responses of one patch activated by pulses of different ACh concentrations. The amplitude scales are given as numbers of open channels. With 1000 μM-ACh, forty channels opened within about 1 ms, while with 1 μM-ACh the peak average current was reached only after 10 ms (see also Fig. 6). After the peak

response, the currents decayed to lower levels with time constants of desensitization, τ_d, which are indicated in each panel of Fig. 5*A* and plotted in Fig. 5*B*. Figure 5*B* also contains a plot of the dose dependence of peak current i_{max}, which increased in

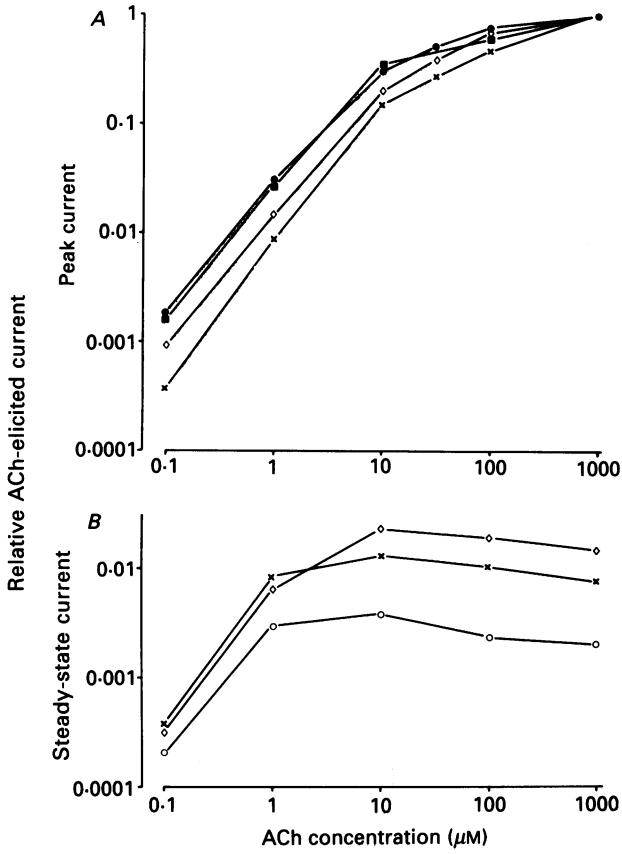

Fig. 7. Concentration dependence of ACh-elicited currents in four different outside-out patches. *A*, dependence of peak current i_{max} on ACh concentration. The ordinates are normalized to the values of i_{max} at 1000 μM. Polarization to: ●, −60 mV; ■ −65 mV; ◇ −80 mV; ×, −70 mV. *B*, dependence of the steady-state current on ACh concentration evaluated for two patches as shown in *A* plotted with the same symbols. Same ordinate scale and identical polarizations as in *A*.

direct proportion to the ACh concentration between 0·1 and 10 μM ACh, and less steeply for larger concentrations.

The rising phases of the average currents elicited by pulses of different ACh concentrations are shown in Fig. 6 with high time resolution. The rise times, t_r, of the currents were defined as the time between 10 and 90% of the full amplitude. With 1000 μM-ACh, this rise time t_r, was only 0·4 ms which is close to the limitations of the application system. With 100 μM-ACh, t_r, = 1·1 ms should not have been seriously distorted by the non-instantaneous ACh application. Lowering the ACh concentration further, t_r increased and reached a constant level of 10–15 ms for very low concentrations (see also Franke, Hatt, Parnas & Dudel, 1991, Fig. 3).

In Fig. 7, the dose–response curves for i_{max} are plotted for four experiments like that in Fig. 5*A*. The maximal double-logarithmic slopes of these curves were 1·1–1·3. The power law describing these curves should have an exponent of at least 2. The concentration at the half-maximal response, EC_{50}, of the dose–response curves for i_{max} was 30–100 μM ACh. Figure 7*B* shows the dose dependence of steady-state currents, i_s. This rises almost as steeply as i_{max} for concentrations of ACh up to 1 μM but little more for higher concentrations. EC_{50} values for the steady-state currents were between 1 and 10 μM-ACh. The attenuations ratio of desensitization (i_s/i_{max}) depended on the ACh concentration. The apparent attenuation was about 1/3 with 1 μM, 1/25 with 10 μM, 1/100 with 100 μM and 1/200 with 1000 μM-ACh. For ACh concentrations > 10 μM, this attenuation was stronger than the increase in i_{max}. In other words, the dose–response curve for the steady-state current i_s fell slightly when the ACh concentration was increased above 10 μM.

The time constants of desensitization are plotted in Fig. 5*C* for four patches. They decrease from about 1 ms with 1 μM-ACh to 30–46 ms with 1000 μM-ACh. These values are in the same range as those reported by us for adult fibres (Franke *et al.* 1991). If desensitization were a one-step reaction for binding ACh, an equilibrium at 1/200 of the peak response before desensitization would require a time constant of recovery from desensitization after removal of ACh of $200 \times \tau_d$, i.e. of about 4 s. However, as stated above, complete recovery from desensitization on removing ACh took only about 2·5 s. It follows that desensitization is a multi-step reaction.

DISCUSSION

On denervation of an adult muscle, the embryonic type of receptor appears just outside the end-plate region (Miledi, 1960; Brockes & Hall, 1975; Gu & Hall, 1988). The subunits δ and ϵ have also been identified with specific antibodies in embryonic and in adult muscle, respectively (Gu & Hall, 1988; Gu, Franco, Gardner, Lansman, Forsayeth & Hall, 1990).

Embryonic and adult receptors are best distinguished by their single-channel conductances of 36 and 65 pS, respectively (in outside-out patches). The chemically denervated preparation studied here showed only the 36 pS channel conductance (Figs 1 and 2) and therefore seems to comprise only the embryonic type of nAChRs. The recorded single-channel currents also displayed the relatively long open times and burst duration characteristics for the embryonic nAChR (see Schuetze & Role, 1987). Our measurements can be compared directly to those from the previous study which was performed under identical conditions (Franke *et al.* 1991). In a closely related preparation, rat myotubes, Haramillo & Schuetze (1988) also compared channels from embryonic and adult muscle. This preparation was held at about 10 °C, and therefore channel conductance was lower and duration of openings and closings longer than in our corresponding measurements. Also, in rat myotubes the open/closed channel kinetics were slower in embryonic than in adult muscle.

So far we have discussed single-channel characteristics obtained from steady-state measurements in the continuous presence of low ACh concentrations. What further information was obtained by applying various ACh concentrations in rapid steps? The first new finding concerns the dose–response curve for the peak currents i_{max}, which gives the concentration dependence of the reaction of the receptor with ACh

before desensitization. In double-logarithmic plots, this dose–response curve at low ACh had a slope of only 1·1–1·3, i.e. clearly lower than the respective slope of 2·3–2·7 in adult channels (Franke *et al.* 1991). While it seems necessary to assume three ACh binding steps for the opening of adult channels, two such steps may be sufficient in the case of the embryonic channels. The ACh concentration at half-maximal channel activation (EC_{50}) was about 100 μM in adult channels (Franke *et al.* 1991), while we found $EC_{50} = 60$ μM in embryonic channels. This difference in EC_{50}, and also the lower slope of the dose response for i_{max}, produces a higher ACh sensitivity for low concentrations of the embryonic nAChR compared to that of the adult nAChRs. The higher sensitivity of embryonic channels might have functional significance, since immature synapses may start to work with lower than normal ACh concentrations (Jaramillo, Vicini & Schuetze, 1988).

A new characteristic of the ACh receptor reaction obtained by step application of ACh is the rise time of the average channel current after the step. This rise time is a complicated function of the ACh concentration, as treated in detail elsewhere (Franke *et al.* 1991). At the same ACh concentration, the rise time of the elicited current in embryonic nAChRs was found to be about twice that in adult nAChRs. Similarly, the rise time of spontaneous quantal synaptic currents was longer for embryonic than for adult muscle (Jaramillo & Schuetze, 1988). These authors used a relation given by Land, Salpeter & Salpeter (1981) to deduce the rate of the conformational change to open the channel (β) from the rise times of the quantal currents. This deduction seems to be of limited value, since the rise time also depends on the rates of channel closing and of ACh binding and unbinding (Colquhoun & Hawkes, 1977; Parnas, Flashner & Spira, 1989; Franke *et al.* 1991).

Sine & Steinbach (1987) observed in cell-attached measurements on BCH3 cells rapid transitions between open and closed states with 1000 μM-ACh, representing open channel block by the agonist. The blockages observed by Sine & Steinbach (1987) had a mean duration of around 30 μs. Analogous events cannot be fully resolved with the frequency resolution possible under our conditions. However, an increase in open channel noise and a reduction of the apparent single-channel current amplitude are seen if openings of the channel are interrupted by a large number of short closings. We did not observe this behaviour in steady-state in the outside-out mode at -80 mV (Fig. 2). Additionally, the peak current i_{max} decreases for high concentrations of agonist if substantial open channel block is present. This was not the case in our experiments (see Figs 5 and 7) performed at membrane potentials around -50 mV. In control experiments (not shown here), we found a decrease of i_{max} at high ACh concentrations only at potentials more negative than -80 mV. Apparently, there are quantitative differences in the voltage dependence of open channel block between preparations (Sine & Steinbach 1987; Colquhoun & Ogden, 1988).

Dilger & Brett (1990) have also applied ACh in pulses to nicotinic receptors on patches from a BCH cell line. Their method of ACh application seems to be somewhat slower and less reproducible than ours. Rise time t_r can be evaluated from their published average currents. With 100 μM-ACh, t_r values of 0·9 or 2·4 ms result (Figs 1 and 4 of Dilger & Brett, 1990). On lowering the ACh concentration in the pulse to 3 μM, t_r increased to 15 ms. Sine & Steinbach (1986) have derived rate constants for activation reactions of receptors from the same cell line, using steady-state single-

channel recordings. The rate, β, of the change from the closed to the open channel conformation was 480 s^{-1}. This corresponds to a time constant of the opening reaction of about 2 ms, which seems much too long for a minimal rise time of 0·9 ms (which was measured at the non-saturating ACh concentration of 100 μM). If one simulates the complete activation reaction (Franke *et al.* 1991) using the rate constants of Sine & Steinbach (1986), values of t_r of 5·5 and 62 ms are expected for 100 μM and 3 μM-ACh. These t_r values are much higher than those obtained from the records of Dilger & Brett (1990). In the absence of simultaneous measurements of steady-state rate constants, and of rise times in response to ACh pulses the discrepancy must remain unexplained. This is one of the reasons why we also characterized the present preparation with respect to steady-state kinetics (Fig. 4).

Finally, the stepwise application of ACh revealed a rapid desensitization of the embryonic-like or denervated nAChR (see also Dilger & Brett, 1990). With time constants of desensitization of 40–60 ms on application of 1000 μM-ACh (Fig. 5*C*), this process was almost as rapid as in adult muscle. In adult muscle, in steady state with 1000 μM-ACh the number of open channels is desensitized to 10^{-4}–10^{-5} of the initial peak (C. Franke, H. Hatt & J. Dudel, unpublished). However, in embryonic-like channels, after desensitization to a steady state a considerable average channel current, i_s, remained, even with high ACh concentrations, amounting to 1/100–1/500 of the peak current, i_{max}, at the respective ACh concentration. The presence of an additional, slower component of desensitization (Cachelin & Colquhoun, 1989) may explain the decline of steady-state current at higher concentrations. However, this component, if it exists, depresses the current by a factor of only 2–5 whereas the fast component depresses the current by a factor of 100–500. As mentioned above, the i_s/i_{max} ratio was much smaller than the quotients τ_d/τ_{rec} (the latter being the time constant of recovery from desensitization after removing ACh) at a certain ACh concentration. Desensitization thus seems to be a complex process which needs further study (see Feltz & Trautmann, 1982; Cachelin & Colquhoun, 1989). Functionally, the less complete steady-state desensitization of embryonic-like nAChR channels as compared to those of the adult increases the relative sensitivity of the embryonic-like nAChRs to high concentrations of ACh.

The authors wish to thank Dr Dean O. Smith, Madison, for critical reading of the manuscript and Miss B. Preibisch and M. Hammel for technical assistance. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 220).

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