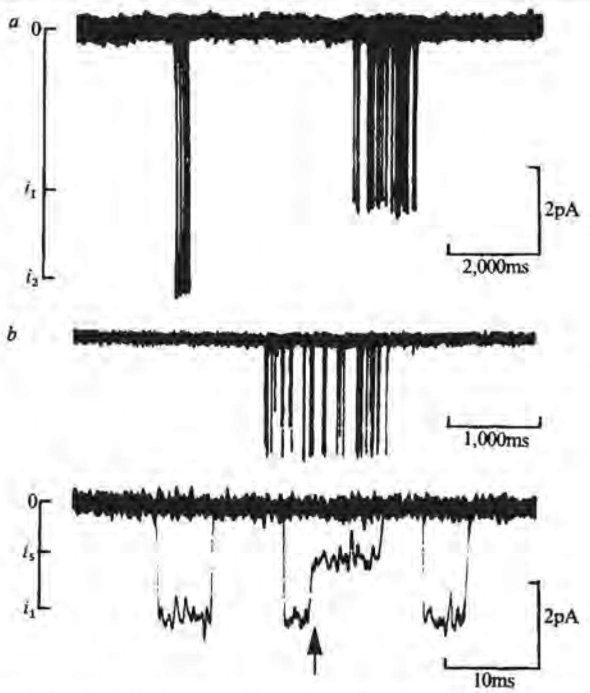

Fig. 3 AChR channel currents activated by desensitizing concentrations of ACh. *a*, Current recording from a sarcolemma patch activated by 10 μM ACh. Two distinct non-interacting bursts of currents were evident that corresponded in their amplitudes to the two main classes evident at low ACh concentrations. Membrane potential was −130 mV, temperature 18 °C. Day 12 myoball, 2 days after colchicine treatment; 400 Hz filtering. *b*, Current recording from another patch. In the top trace a burst of currents corresponding in amplitude to the smaller main level is shown at low time resolution. The bottom trace gives two superimposed traces of the same burst shown at higher time resolution. One distinct transition between the main level and a sublevel was evident and is indicated by the arrow. Membrane potential −100 mV, temperature 18 °C. Day 14 myoball, 4 days after colchicine treatment; 400 and 2,000 Hz filtering.

replaced by Li^+ or Cs^+, the conductance of all three states (Fig. 2*c*) decreased or increased proportionately. The various conductance states of AChR channels however differ in their average lifetime: for the patch described in Fig. 1 the average duration of the larger and smaller main levels was 12 and 36 ms, respectively, whereas the sublevel measured from the same patch had an average duration of 24 ms. However, these durations represent only apparent lifetimes because the fast fluctuations of the single channel current in the time range <1 ms (see accompanying paper[5]) were not taken into account. The ratio of the time the patch current is at the sublevel to the time it is at either the main or the sublevel varied between 0.02 and 0.12 in six different patches, indicating that the probability of the open channel adopting the substate is low. It is further reduced when the temperature is increased: at 18 °C current sublevels are only measurable as short notches (<5 ms) in the channel's closing time course. On the other hand, increasing the membrane potential from −70 mV to −120 mV (temperature 5 °C) increased the probability of the open channel adopting the substate from 0.05 to 0.15 (one patch). When channels have adopted the substate, the probability that they either close completely or open fully was similar. For example, in the patch illustrated in Fig. 1 the ratio of transitions back to the main state to transitions to the closed state was 1.3. Apparent transitions between the two main current levels are also observed, but their average number is not significantly different from that expected by unresolved random coincidence of two main unit current events.

These findings suggest that ACh activates two independent classes of channels in embryonic rat muscle that are distinguished by a slightly different main conductance. Both can adopt, with low probability, a similar substate of lower conductance.

This view is further supported by experiments where channels were activated by ACh at concentrations >2 μM where single channel currents appear in bursts. Bursts reflect sequential open–close transitions of the same AChR channel[6]. In embryonic muscle they fall into two main classes with respect to the size of their current pulses, corresponding to the two classes observed at low ACh concentration (Fig. 3*a*). Transitions between these two main current levels were never observed during a burst. However, transitions between the main level and the sublevel do occur, as shown in Fig. 3*b*. The upper trace shows a burst of current pulses at low time resolution, consisting of 25 well separated current events. At high time resolution two events have a complex shape characterized by a transition from the main level to the sublevel. One of these events is illustrated in the lower trace of Fig. 3*b*. The average probability of this channel being in the substate (given the channel is in the open state), as determined from eight consecutive bursts, was 0.08. This experiment shows directly that a single AChR channel complex, in the presence of constant ACh concentrations, fluctuates between at least three states: a main open state, a substate which is adopted at low probability, and the closed state(s).

The AChR complex purified from *Torpedo* electroplaque is a pentameric protein complex comprised of five homologous subunits[7] which span the membrane[8]. In lipid bilayers it can form a unit conductance channel which has a similar conductance to that of the AChR channel in rat muscle[9]. The observation that ACh-activated channels in embryonic muscle cells can adopt several conductance states could indicate that these subunits rearrange themselves to form the different open states of a channel. Similarly the two independent classes of channels may represent two different aggregation states of the same set of subunits.

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Fluctuations in the microsecond time range of the current through single acetylcholine receptor ion channels

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Acetylcholine-like drugs cause ion channels in the skeletal muscle endplate to open briefly[1], producing, at random intervals, rectangular pulses of current with constant amplitude but random duration, that can be recorded by the patch clamp method[2,3]. However, even when the agonist concentration is so low that channel activations are very well separated, we have observed, with high resolution methods[4], that openings may be interrupted by shut periods (gaps) so brief that they are very unlikely to arise from two independent channel activations. This sort of behaviour has been predicted on the basis that two or

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more openings might occur during the time for which the receptor remains occupied by agonist[5,6]. If this were correct, important new information about agonist activation of ion channels could be obtained from measurements of the gaps between openings. However, short gaps could arise in other ways: for example from brief blockage of the ion channel[7], perhaps by the agonist itself. We now present results obtained with the acetylcholine-like agonist, suberyldicholine (SubCh, 20–100 nM), which suggest that the brief gaps do not result from ion channel block by the agonist itself, but which are consistent with a mechanism in which the channel opens and closes several times during a single agonist receptor occupancy. We have also observed that the number of short (<1 ms) current pulses is greater than we expected.

Single ion channel currents from the perisynaptic region of normal frog (*Rana temporaria*) cutaneus pectoris muscle were recorded at 10–13 °C (ref. 4). The Ringer solution contained (mM) NaCl 115, KCl 2.5, $CaCl_2$ 1.8 and HEPES buffer 3, *p*H 7.2

Figure 1*a* shows the typically low frequency of single channel currents (always $<3\ s^{-1}$). Individual currents are shown, with high time resolution, in Fig. 1*b*,*c*; they are clearly interrupted by brief gaps. During most of these gaps the current does not reach the resting value; however, the frequency response of the system is such that the channel would have to be shut for nearly 300 μs for this to be attained. We assume that the gaps represent brief, but complete, closures of the ion channel, and Fig. 1*d*–*g* show how the duration of such closures was estimated. In good records, events with a duration of 50–70 μs could be clearly resolved.

The entire record was digitized so that the duration of all resolvable gaps, as well as openings, could be measured. After this measurement, a safe value was chosen for the minimum resolvable duration, and the record was revised by concatenation of adjacent gaps and openings separated by intervals less than this chosen resolution. Thus an idealized record, with consistent resolution throughout, was obtained for the construction of histograms. All distributions of time intervals were fitted with the sum of one or more exponential functions by the method of maximum likelihood (that is, the actual measured durations were used, not the histogram frequencies).

The distribution of all gaps is exemplified in Fig. 2. In Fig. 2*a*, the time scale extends up to 2 s, and the gap durations are fitted well by an exponential component with a mean of 352 ms. This is presumably the mean interval between independent activations of ion channels. However, the first bin (up to 50 ms) extends a long way off the graph; the distribution of short gaps is shown in more detail in Fig. 2*b*, in which the time scale extends up to only 500 μs. There is a clear exponential component of the gap distribution with a mean, in this example, of 45 μs, which corresponds to the short gaps illustrated in Fig. 1. Values of 45–70 μs were consistently observed in nine other experiments. This fast component represented, in this case, 77 per cent of the total area of the gap duration distribution, corresponding to a total number of 2,231 short gaps (although many of these would

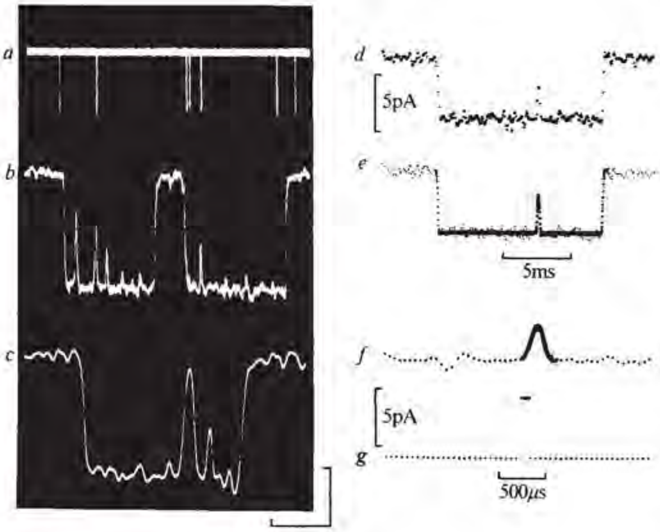

Fig. 1 Examples of single channel currents (*a*, *b*, *c*) and the method used to fit their time course (*d*, *e*, *f*). Low pass filter set at 4 kHz. *a*, Illustration of low frequency of events (seven in 5 s in this case) with 100 nM SubCh at −123 mV. Calibration bars: 4 pA and 1 s. *b*, Several brief closures interrupting an open channel current; taken from the experiment illustrated in *a*. Calibration bars: 2 pA and 10 ms. *c*, Another group of three openings, at higher time resolution than in *b*; 20 nM SubCh at −128 mV. Calibration bars: 2 pA and 2 ms. *d*, A burst with one resolvable gap, digitized at 16 kHz; 50 nM SubCh at −171 mV. *e*, The same data as in *d* with a fitted line (heavy line) superimposed on it. The response of the recording system to a step input was measured experimentally, and four such response functions, with alternating direction, were convolved to generate the fitted line. *f*, The gap shown in *d* and *e*, with greatly expanded time scale, but the same amplitude scale. A fitted line is superimposed on the data to illustrate the fitting of brief gaps. This fitted line was generated, as explained above, as the expected response of the system to the input shown in g. This fit estimates that the burst shown in *d* consists of two openings of length 7.69 and 4.58 ms, separated by a gap of 104 μs, so the total length of the burst is 12.37 ms.

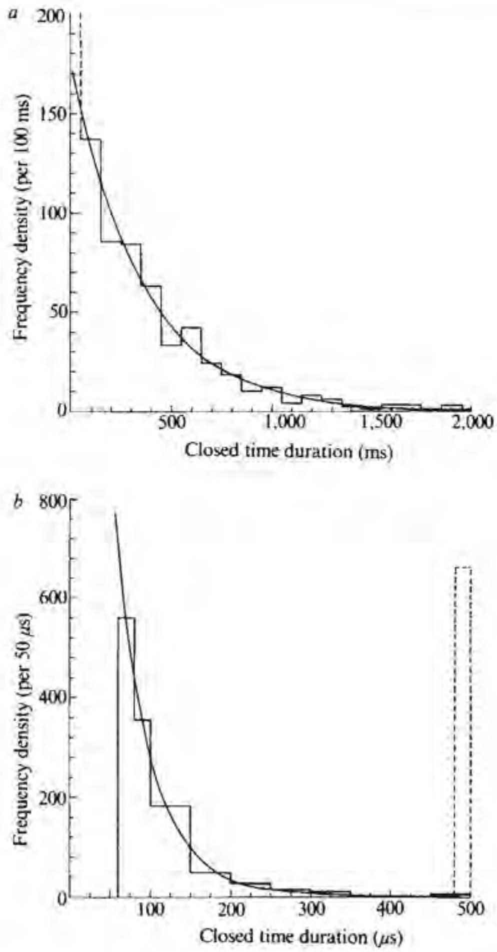

Fig. 2 Distribution of the durations of all closed times (gaps) between open channel currents. Experiment with 20 nM SubCh at −131 mV. Ordinate is frequency density (number of observations per time interval of the specified length). All 1,308 gaps that were measured were simultaneously fitted (as described in the text) with the sum of three exponentials, and the fitted curve was superimposed on the histogram of the observations which is shown with two different time scales in *a* and *b*. In this case we found components with time constants of 352 ms (representing 20.5% of total area under the distribution), 45 μs (76.7% of area) and 0.66 ms (2.8% of area). *a*, Distribution of gaps, shown up to 2,000 ms, with fitted curve. The leftmost bin (dashed line) contains many short gaps, and extends well off the graph. *b*, The same distribution, shown up to 500 μs, only, with fitted curve. The resolution was fixed (see text) at 60 μs in this experiment. The rightmost bin (dashed line) represents all gaps longer than 500 μs. This includes all those shown in *a*.

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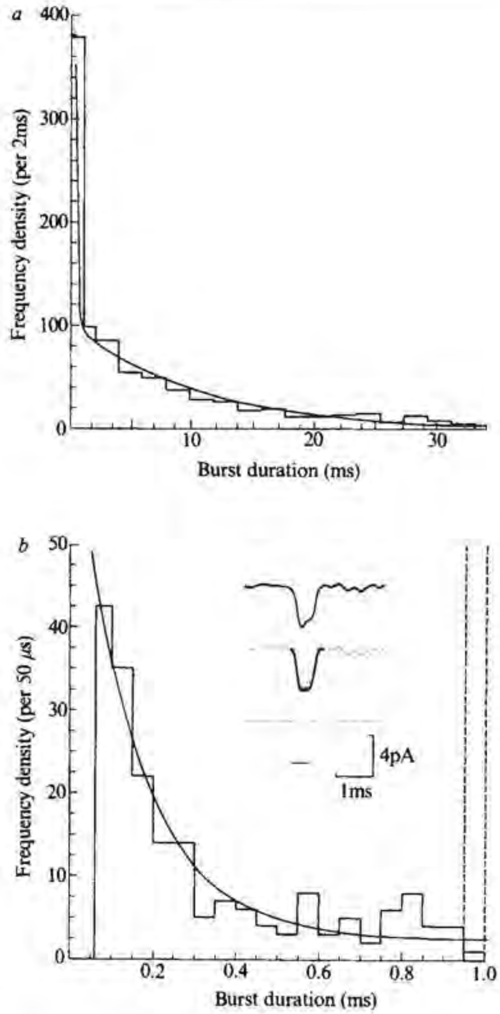

Fig. 3 Distribution of the burst duration, as defined in the text, for the same experiment as shown in Fig. 2. With a resolution of 60 μs and critical gap length of 1.5 ms, there were 683 bursts altogether. The durations of these bursts were fitted (see text) with the sum of two exponentials. In this case we found components with time constants of 10.2 ms, which is similar to the value expected from a noise analysis in the conditions of this experiment, and 0.15 ms. In 12 experiments the average faster time constant was 0.35 ± 0.12 ms. *a*, The distribution of burst length, and fitted curve, shown up to 35 ms. *b*, The same distribution and fitted curve, but bursts up to 1 ms duration only are shown. The rightmost bin (dashed line) represents all bursts longer than 1 ms. The inset shows (top) an example of a brief opening. This is fitted (see Fig. 1, right column) with a superimposed line (middle) calculated as the response to the step input shown (bottom). The amplitude (3.95 pA) was taken as the average of amplitudes of all full size openings previously fitted in this experiment. The duration is 495 μs.

be too short to resolve). In addition we have consistently found a much smaller component (about 2% of total area) with an intermediate time constant of the order of 1 ms, but this will not be considered further here.

The evidence presented above suggests that the 'openings' will contain many unresolved gaps, and are thus not well defined. We therefore define a burst of openings as any series of 'openings' interrupted by gaps that are all less than some critical length (usually 0.5–3 ms). Insofar as this length is very much smaller than the mean time between independent events (352 ms in Fig. 2), the burst duration is a well defined quantity. This occurrence of openings in quick succession was called the Nachschlag phenomenon when it was first observed, and this term seems appropriate, whatever the mechanism responsible for the phenomenon. Figure 3 shows the distribution of burst durations; in Fig. 3*a* the time scale extends to 35 ms, and there are obviously too many short bursts to be fitted by a single exponential distribution. The double exponential distribution shown has a slow component with mean of 10.2 ms, and a fast component with mean of 0.15 ms. The total area under the distribution represents 717 bursts and the slower component corresponds to 73% of the total area in this example, with 20 nM SubCh. Thus we infer, for the experiment illustrated in Figs 2 and 3, that there are, on average 2,231/717 = 3.1 gaps per burst. Similar values (2–4) were found in 10 experiments.

The origin of the short openings illustrated in Fig. 3 is obscure. They may represent a separate type of channel altogether, or perhaps brief openings by channels with only one agonist molecule bound. We shall explore these hypotheses elsewhere.

The most important question that arises is whether the brief gaps result from ion channel block by the agonist, SubCh, itself. Decamethonium (in much higher concentrations) is known to do this[8]. If this were the mechanism, the number of gaps (blockages in this case) per burst should be directly proportional to the agonist concentration. However, the number of gaps per burst with 100 nM SubCh, relative to that with 20 nM, was 0.97 ± 0.11 (three experiments at each concentration), and showed little sensitivity to membrane potential. Therefore, ion channel block by SubCh, in the low concentrations used, cannot explain the brief gaps that we observe. Although it is conceivable that brief closures of the channel might arise from block by some endogenous muscle constituent, or from a mechanism connected with ion permeation, the most plausible alternative to ion channel block is that the closures arise from multiple openings during a single receptor occupancy[5,6]. Suppose, for example, that two agonist molecules must bind sequentially before the channel can open (see, for example, ref. 9). Then (see ref. 6), at low agonist concentration, the mean length of a gap should be approximately $(\beta + 2k_{-2})^{-1}$, and the number of gaps per burst should be approximately $\beta/2k_{-2}$, where β is the rate constant for opening of a doubly occupied receptor–channel complex, and k_{-2} is the microscopic rate constant for dissociation of an agonist molecule. Our results, if interpreted in this way, thus suggest preliminary estimates for β of the order of 10,000–15,000 s^{-1}, with k_{-2} roughly 2,000 s^{-1}. This interpretation of our results with SubCh is not compatible with the common assumption that the agonist binding step is very fast, and that the open–shut conformation change is rate-limiting[10]. We are now investigating whether or not the same can be said of agonists other than SubCh. The noise spectrum expected if this interpretation were correct would consist predominantly of a single component, with a time constant close to the mean length of the burst (slow component), that is, 10 ms in the experiment shown in Fig. 3. But this time constant would not correspond, as is often assumed, to the true lifetime of the open state. In the above example the average burst consists of roughly four openings in quick succession, so the mean open lifetime, $1/\alpha$ (where α is the channel closing rate constant) would be only about 2.5 ms.

Results similar, in some respects, to ours have recently been found (S. G. Cull-Candy and I. Parker, in preparation) for glutamate-operated channels in locust muscle.

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