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# A PATCH-CLAMP STUDY OF ACETYLCHOLINE-ACTIVATED ION CHANNELS IN *ASCARIS SUUM* MUSCLE

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## Summary

Acetylcholine-activated single-channel currents were recorded from cell-attached and inside-out patches of isolated muscle vesicles from *Ascaris suum*. Acetylcholine (1–10 $\mu$mol $l^{-1}$) activated cation-selective channels of two amplitudes: 40–50 pS and 25–35 pS. Both channels had linear I/V relationships and mean open durations independent of voltage. The larger conductance was analysed in detail to determine its open-, closed- and burst-time kinetics; the open and burst durations were composed of two components (short and long), while closed durations had at least three components (short, intermediate and long). The data were then corrected to allow for missing short events in order to estimate various parameters including corrected mean open time (1.26+0.11 ms, mean±S.E.). Values were also derived for the efficacy ($\beta/\alpha$=4.9) and affinity [$1/K_D$=147×$10^3$ (mol $l^{-1}$)$^{-1}$] of acetylcholine at this receptor. Larger concentrations of acetylcholine (25–100 $\mu$mol $l^{-1}$) were shown to produce desensitization and caused single-channel currents to occur in clusters with long closed times (mean 171 s) between clusters. It was concluded that the extrasynaptic muscle of *Ascaris suum* contains two types of acetylcholine-activated ion channel and these are possible sites of action of various anthelmintic drugs. This paper is the first to describe acetylcholine-activated single-channel currents in invertebrate muscle.

## Introduction

*Ascaris suum* is a parasitic nematode which lives in the intestine of the pig and is a target species for a variety of anthelmintic drugs, including pyrantel and levamisole, which are thought to be acetylcholine agonists. Acetylcholine has long been known to have effects on the muscle cells of *Ascaris suum* (Del Castillo *et al.* 1963); application of acetylcholine to whole worms or muscle-flaps will cause the muscle to contract. It is now believed that acetylcholine is the excitatory neuromuscular transmitter in nematodes (Willett, 1980; Johnson and Stretton, 1985; Gration *et al.* 1986). It has also been shown that there are extrasynaptic acetylcholine receptors on the bag region of *Ascaris suum* muscle, which respond to ionophoretically applied acetylcholine (Martin, 1982). Acetylcholine acts on

Key words: acetylcholine channel, patch-clamp, *Ascaris suum*, muscle.

these receptors to increase input conductance and to produce depolarization. The effect of acetylcholine on *Ascaris* muscle is blocked by tubocurarine but not by atropine, hence these muscle receptors may be classified pharmacologically as nicotinic (Natoff, 1969; Rozhkova *et al.* 1980; Martin, 1982).

The pharmacology of the nicotinic receptors on *Ascaris* muscle differs from that of any type of vertebrate nicotinic receptor. Mecamylamine is a very potent blocker of *Ascaris* nicotinic receptors; this normally blocks the nicotinic receptors of autonomic ganglia in vertebrates, but not skeletal muscle receptors (Natoff, 1969; Rozhkova *et al.* 1980). These differences are exploited therapeutically by various nicotinic anthelmintic compounds which act selectively on the acetylcholine receptor of this parasite (Aubry *et al.* 1970; Coles *et al.* 1975; Harrow and Gration, 1985).

Acetylcholine acts on nicotinic receptors and has been shown to activate cation-selective single-channel currents in vertebrate preparations (for a review, see Schuetze and Role, 1987) and in certain insect neurones (Sattelle, 1986; Beadle *et al.* 1989). The aim of the present study was to record acetylcholine-activated single-channel currents from nematode muscle. This was done using isolated muscle vesicles formed from the bag region of the muscle cells of *Ascaris suum*. Recordings obtained were analysed: (a) to characterise this ion channel, (b) to compare it with other nicotinic channels, and (c) as a basis for future study of anthelmintic compounds on this channel. A preliminary account of some of this work has appeared (Martin and Pennington, 1990).

## Materials and methods

In general the methods of Hamill *et al.* (1981) were followed. The application of these methods to *Ascaris* has been described previously (Martin, 1985), as has the development of the method for the use of isolated muscle vesicles (Martin *et al.* 1990).

### *The vesicle preparation*

*Ascaris suum* were collected from the local abattoir and maintained in Locke solution (replaced daily) at 32°C in a water bath. The *Ascaris* were used for experiments within 4 days. Muscle-flap preparations were treated with collagenase (Sigma type 1A) $1\,mg\,ml^{-1}$ for 10–20 min at 37°C to produce isolated muscle vesicles. The vesicles were harvested using a Pasteur pipette and maintained at 37°C until they were transferred to the experimental chamber for patch recording. They were always used within a few hours of formation. Recordings were made at room temperature (15–22°C). The experimental chamber was mounted on the stage of a Reichert–Jung Biostar inverted microscope, and vesicles were viewed at ×200 magnification.

### *Solutions*

Muscle-flap preparations and vesicles were maintained in a solution containing (in $mmol\,l^{-1}$): NaCl, 35; sodium acetate, 105; KCl, 2; $MgCl_2$, 2; Hepes, 10;

glucose, 3; ascorbic acid, 2; and EGTA, 1; pH adjusted to 7.2 with NaOH. Collagenase was applied to the muscle flap in this solution but without the EGTA. In the experimental chamber vesicles were bathed in the following solution (in $mmol\,l^{-1}$): CsCl, 35; caesium acetate, 105; $MgCl_2$, 2; Hepes, 10; and EGTA, 1; pH adjusted to 7.2 with CsOH. Electrodes for cell-attached or inside-out patches were filled with (in $mmol\,l^{-1}$): CsCl, 140; $MgCl_2$, 2; Hepes, 10; and EGTA, 1; pH adjusted to 7.2 with CsOH. In experiments to study the ion-selectivity of the channel, the bath solution was diluted 50:50 with distilled water giving a solution with $70\,mmol\,l^{-1}$ $Cs^+$ and $19.5\,mmol\,l^{-1}$ $Cl^-$. Most of the recordings were made with $Cs^+$ as the main cation to block the $K^+$ channels known to occur in this membrane. All solutions used during recordings were $Ca^{2+}$-free, to avoid recording from $Ca^{2+}$-activated $Cl^-$ channels (Thorn and Martin, 1987).

*Recording*

Recordings were made from cell-attached or inside-out patches. Patch electrodes, made from micro-haematocrit capillary tubes (Garner Glass 7052), had a resistance of 1–3 MΩ and were coated with Sylgard to improve frequency responses. Currents were recorded using a List EPC-7 and a Racal Thermionic Store Four FM tape recorder.

*Data processing*

Records were analysed using a CED 1401 interface and DCS 286 PC computer, with software from Dr J. Dempster, Strathclyde University. Filtering was by an eight-pole Bessel filter, 3 dB, 2.5 kHZ, sampling time 40 μs and minimum detectable interval of 0.16 ms. Threshold for channel opening was set at 70 % to exclude the smaller-amplitude channel when a patch contained both amplitudes.

*Exponential curve fitting*

In our experiments individual channel activations were interrupted by short closed periods (the Nachschlag phenomenon, Colquhoun and Sakmann, 1985), so bursts of openings, as well as single openings, can be considered. Bursts are defined as groups of openings separated by gaps that are all shorter than a specified length, $T_{crit}$. The mean length of the gaps between bursts was several 100-fold greater than the mean length of gaps within bursts, so the choice of $T_{crit}$ was not critical and a value of 1 ms was chosen from examination of the data.

The next stage of the analysis was exponential fitting of the open, closed and burst durations by the method of maximum likelihood (Colquhoun and Sigworth, 1983; Martin, 1985), to a probability density function (p.d.f.) of the form:

$$\text{p.d.f.} = \sum_{i=1}^{K} \frac{a_i e^{(-t/T_i)}}{T_i}, \qquad (1)$$

where $a_i$ represents the area of the $i$th component ($\Sigma a_i = 1$), $T_i$ is the fitted time constant, $t$ represents time and $K$ equals the number of exponential terms fitted. A burst distribution, for example, may be fitted by two exponentials; in general it is

not correct to identify the components with particular physical events or to use 'short bursts' or 'long bursts' as though there were two quite separate types of phenomena. Nevertheless, such terms are generally used (Colquhoun and Sakmann, 1985) as a way of discussing the mathematical components that have been identified; in some cases the components do have an approximate physical significance (Colquhoun and Hawkes, 1982).

*Correction for missing events*

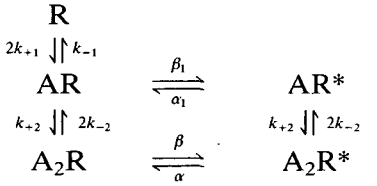
Once exponential components had been fitted to the burst and closed durations, it was possible to correct the data for missing events, using the method of Colquhoun and Sakmann (1985). The assumptions are that very short closings and very short openings will be missed in a standard analysis of open and closed durations, because of limitations of the resolution of the recording technique, and that, using the kinetic information about the data that have been recorded, it is possible to extrapolate back to calculate the number of short events that are likely to have been missed.

The first stage in this correction process is to estimate the total number of events from the observed number of events; this is done separately for gaps and for bursts. $N_{\text{bursts}}$ is the estimated total number of bursts and $N_{\text{gaps}}$ is the estimated total number of gaps. The next stage in the correction process is to calculate the total time spent in gaps within bursts, ($m_{\text{TS}}$), the corrected number of short gaps per long burst ($N_{\text{o}}$) and the corrected mean burst length ($MB$). Then the corrected mean open time in long bursts ($E$) and the corrected total open time ($CTOT$) are calculated, allowing for the missed short gaps.

The corrected open probability ($COP$) equals $CTOT$ divided by $t_{\text{max}}$; this makes the assumption that in each sample analysed there was only one of the main state conductance channels opening. This is believed to be correct, as double openings were only observed very occasionally and such samples were not analysed; however, if the number of large channels per patch is greater than 1, the values given for open probability are overestimates. Corrected mean open time is $CTOT/N_{\text{gaps}}$. The corrected mean open time is shorter than the mean open time calculated in any other way, because allowing for missed short events reduces the mean value.

*Rate constants*

This analysis can be extended if we adopt the same model of acetylcholine interacting with its receptor as Colquhoun and Sakmann (1985):



where R denotes the closed receptor-channel molecule, R* the open state and A the agonist (two molecules of agonist are believed to bind to each receptor). Oscillations between the two open states are believed to be very slow and this pathway could be omitted. Oscillations between $A_2R^*$ and $A_2R$ would cause short gaps within bursts and AR* possibly represents brief openings. This allows us to calculate a value for the rate constant for channel closing ($\alpha$), the rate constant for channel opening ($\beta$) and the dissociation rate constant ($k_{-2}$) (Colquhoun and Sakmann, 1985). Then a value can be obtained for efficacy (this equals $\beta/\alpha$), and a value proportional to the potency may be obtained from $(\beta/\alpha)^{1/2}/k_{-2}$.

If we then assume $k_{-1}=k_{-2}$ and $K_1=K_2$ (so $k_{+1}=k_{+2}$), and that the association rate constant $k_{+2}=1.0\times10^8\,m^{-1}s^{-1}$, as do Colquhoun and Sakmann (1985), because anything greater than $10^8\,m^{-1}s^{-1}$ is physically implausible (Gutfreund, 1972) and a value for $k_{+2}$ of less than $7\times10^7\,m^{-1}s^{-1}$ is very unlikely (Colquhoun and Ogden, 1988), then, from our own values of $k_{-2}$, we can derive a value for the equilibrium constant ($K_D=k_{-2}/k_{+2}$) and for affinity ($=1/K_D$).

### *Stationarity*

Five samples of data were tested for stationarity using the QSUM method, which has been described in detail previously (Glasbey and Martin, 1986). The data tested were shown to be stationary and were subsequently used for the detailed analysis of open-, closed- and burst-time kinetics. The maximum length of these samples was 40 s.

The results were also studied for any changes occurring with time by analysing sequential samples from the same recording using the full analysis described above. This showed no significant change in the open-, closed- or burst-time kinetics for up to 15 min, with 1, 3 or 10 $\mu mol\,l^{-1}$ acetylcholine. Open probability also did not change with time, so no observable desensitization was occurring at these concentrations over the time course of an experiment. This does not exclude the possibility of some very fast desensitization occurring in the 5–10 s between patch formation and the commencement of recording.

## Results

### *Single-channel currents*

The results reported for 1–10 $\mu mol\,l^{-1}$ acetylcholine were obtained from 57 patches (see Table 1); the recordings were from cell-attached or inside-out patches of membrane, with acetylcholine present in the pipette from the beginning of the recording. In addition to these, a few ($N=3$) recordings were made from outside-out patches, where application of acetylcholine to the bath solution was seen to activate channels in otherwise quiet patches.

### *Acetylcholine activates two conductances*

Acetylcholine (1–10 $\mu mol\,l^{-1}$) present on the outside surface of the muscle membrane of *Ascaris suum* activated single-channel currents with a main state

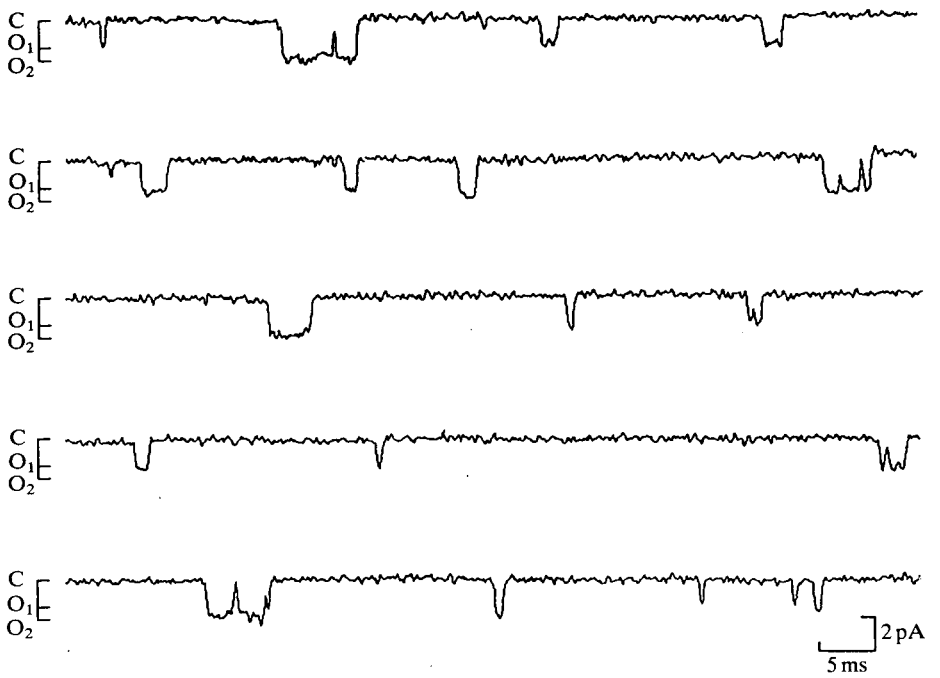



Fig. 1. Acetylcholine-activated channels. A cell-attached patch from a muscle vesicle. C, closed state; $O_1$, smaller conductance channel (34 pS); $O_2$, larger conductance channel (48 pS). Estimated patch potential: −50 mV, 10 $\mu$mol $l^{-1}$ acetylcholine in pipette solution.

conductance of 40–50 pS and a smaller conductance of 25–35 pS (Fig. 1 and Table 1). These channels had very short mean open times in the range of a few milliseconds (Fig. 1) and both types of channel had a linear I/V relationship (Fig. 2).

The currents activated by acetylcholine were of two amplitudes (Fig. 3). The amplitude histogram has been fitted by two Gaussian curves, showing that there are two types of event in this patch with conductances of 42 and 24 pS (means of the Gaussian curves). Some of our recordings ($N$=3) showed just the smaller conductance state, and some patches ($N$=5) showed just the larger conductance, but most patches ($N$=13) contained both types of conductance. When both conductance states were present in a patch we never observed transitions from one amplitude to the other (which would have been expected if the smaller conductance was a sub-conductance state of the larger one). We only saw transitions from the closed state to one or other of the conductance states, which argues strongly for there being two types of ion channel in the *Ascaris suum* muscle membrane, both activated by acetylcholine.

Table 1. *Experimental details and channel conductances*

| [Acetylcholine] ($\mu$mol l$^{-1}$) | Total no. of patches | Inside-out patches | Cell-attached patches | Channels observed | | Main-state conductance (pS) (mean±S.E.) |
|---|---|---|---|---|---|---|
| | | | | Number | % | |
| 1 | 24 | 5 | 19 | 5 | 21 % | 46.4±2.87 |
| 3 | 19 | 4 | 15 | 12 | 63 % | 52.1±1.63 |
| 10 | 14 | 1 | 13 | 4 | 29 % | 44.6±5.00 |
| All | 57 | 10 | 47 | 21 | 37 % | 48.1±1.94 |

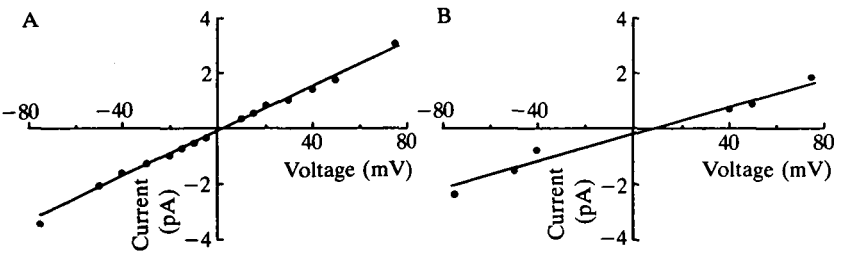



Fig. 2. I/V plots for acetylcholine-activated channels in a cell-attached patch with 10 $\mu$mol l$^{-1}$ acetylcholine in the pipette. A and B are from the same patch. (A) Larger channel: slope conductance 41 pS; reversal potential 2.9 mV; correlation=0.998, $N$=15. (B) Smaller channel: slope conductance 25 pS; reversal potential 7.9 mV; correlation=0.989, $N$=6.

### *Voltage-sensitivity of open times*

To examine the voltage-sensitivity of channel open times, apparent mean open times (obtained directly from the PAT software, without correction for missed short events) were examined at different patch potentials. The apparent mean open durations of the large-conductance channel were measured in this way in five experiments, for between nine and 16 different potentials in each experiment. The mean open time was found to be insensitive to voltage (e.g. for six of 14 potentials for one cell-attached patch with 10 $\mu$mol l$^{-1}$ acetylcholine: −75 mV, 2.38 ms; −50 mV, 2.44 ms; −40 mV, 2.25 ms; +40 mV, 2.13 ms; +50 mV, 2.27 ms; +75 mV, 2.01 ms. In each experiment all the values were tested for a correlation between potential and open time and none was found). In agreement with this, samples of data from the same experiment but at different patch potentials (±50 mV, ±75 mV) showed no differences in open-, closed- or burst-time kinetics with changes in potential.

### *Acetylcholine-activated channels are cation selective*

Both types of channel activated by acetylcholine in these experiments were cation selective. In eight experiments with solutions containing symmetrical cation

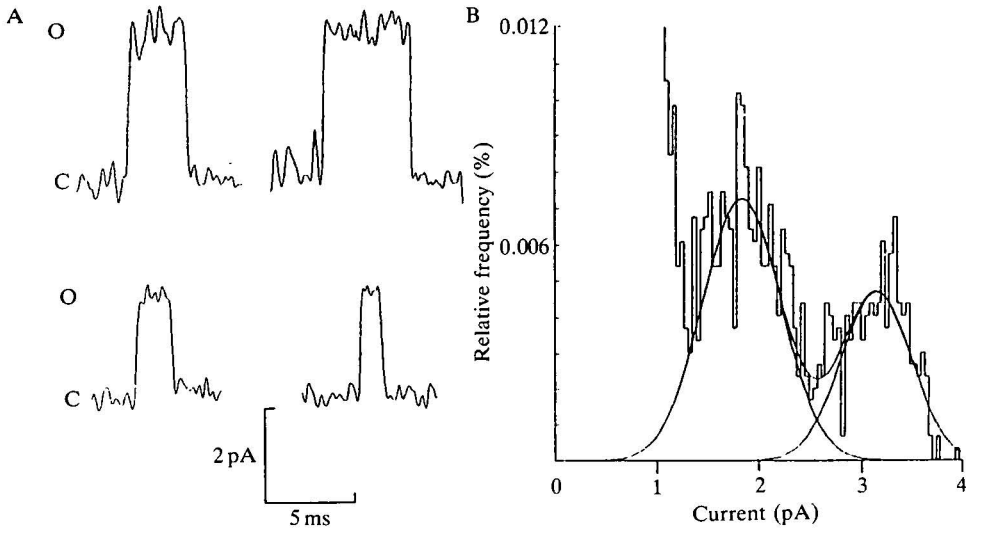



Fig. 3. (A) Individual single-channel openings of two different conductances recorded from a cell-attached patch with 10 $\mu$mol $l^{-1}$ acetylcholine in the pipette. Patch potential: +75 mV. Top traces show the larger channel: 3.14 pA (42 pS), lower traces show the smaller channel: 1.83 pA (24 pS). (B) Amplitude histogram showing the distribution of these two channel amplitudes. The histogram has two distinct peaks fitted by two Gaussian curves; the means of the curves are 3.14 pA and 1.83 pA and these correspond to the single-channel events shown in A. (% is the relative frequency of each amplitude.)

concentrations but asymmetrical anion concentrations the reversal potential was found to be near zero and to be consistent with a cation-selective channel (Fig. 2). In four experiments with isolated inside-out patches, this was further tested by changing the cation and anion concentrations in the bathing solutions. Observed shifts in reversal potential were consistent with those predicted by the Nernst equation for a cation conductance (Fig. 4). In the experiment illustrated, the reversal potential shifted from 5 to 21 mV for a change in $[Cs^+]$ from 140 to 70 mmol $l^{-1}$; the predicted values for a cation channel in these solutions would be 0 and 18 mV, respectively. In these experiments an anion conductance would have shifted the reversal potential in the opposite direction to that observed. For the experiment illustrated (Fig. 4) the predicted reversal potentials for an anion channel were −33 and −51 mV, respectively.

In most experiments the conducting cation was caesium, but acetylcholine channel currents were also observed in two experiments (with outside-out patches) when $Cs^+$ was replaced by $Na^+$, indicating that the channels were also permeable to $Na^+$. The channel was less permeable to $Na^+$ than to $Cs^+$, as the conductance of the channel was less when it was conducting $Na^+$ than when it was conducting $Cs^+$.

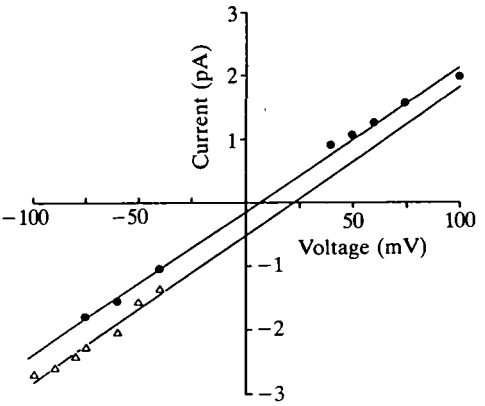



Fig. 4. I/V plot for acetylcholine-activated cation channels in an inside-out patch with 3 $\mu$mol $l^{-1}$ acetylcholine in the pipette. Initial solutions (●) were as in Materials and methods: predicted reversal potentials, $Cl^-$ −33 mV, $Cs^+$ 0 mV. △, bath solutions changed to 70 mmol $l^{-1}$ $Cs^+$ and 19.5 mmol $l^{-1}$ $Cl^-$; predicted reversal potentials, $Cl^-$ −51 mV, $Cs^+$ 18 mV. The actual reversal potentials were, 5 mV (●) and 21 mV (△) which is consistent with this being a cation channel. Lines were fitted by linear regression as in Fig. 2.

### *Exponential curve fitting*

Only the larger channel conductance was analysed in detail to determine its open-, closed- and burst-time kinetics (as described in Materials and methods). Data files used for this analysis were from 12 experiments, from cell-attached and inside-out patches exposed to 1, 3 or 10 $\mu$mol $l^{-1}$ acetylcholine. The files contained apparent open and closed times (obtained directly from the PAT software, without correction for missed short events). The number of events analysed varied between 198 and 2000, but was generally greater than 350. Bursts were made up of either single openings or multiple openings separated by short gaps (<1 ms); a typical burst with multiple openings is illustrated in Fig. 5.

The open times and burst times were best fitted by two exponentials and the closed times by three exponentials (Fig. 6). The results obtained are given in Table 2, which presents the kinetic details for 1, 3 and 10 $\mu$mol $l^{-1}$ acetylcholine. The results were pooled as there were no significant changes with concentration.

### *Open-time distribution*

The open times were best fitted by two exponentials (Table 2). The two components will be referred to as short openings and long openings. $T_1$ for short openings had a mean value of 1.18 ms, which is very short but comparable to values obtained for other acetylcholine-activated channels. $T_2$ for long openings had a mean value of 4.89 ms, again consistent with results for other acetylcholine-activated channels (Numa, 1986; Schuetze and Role, 1987). The short component

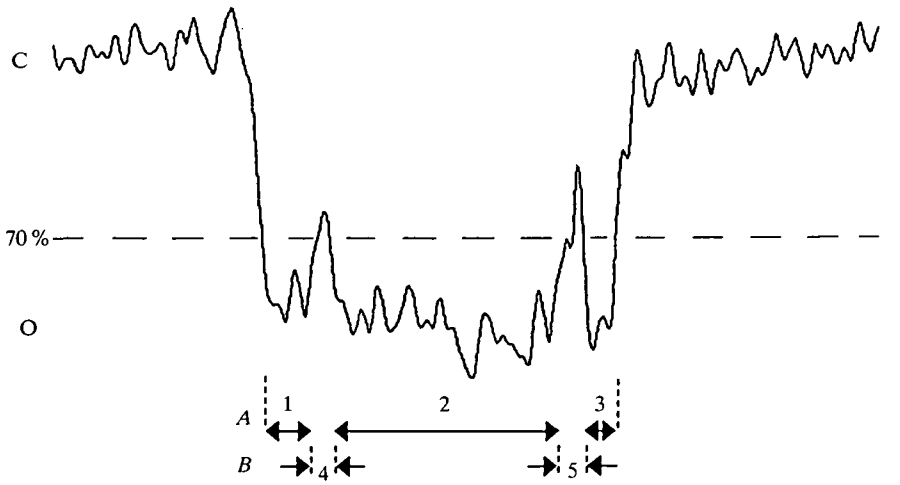



Fig. 5. Typical burst. C: closed state, O: open state (2.42pA). The 70% line shows 70% of unit current (1.68 pA) level. The trace was from a cell-attached patch, with 1 $\mu$mol $l^{-1}$ acetylcholine at −50 mV. *A*, open durations within the burst: 1=1.44 ms, 2=6.08 ms, 3=0.84 ms. *B*, short gaps in the burst, 4=0.24 ms, 5=0.20 ms.

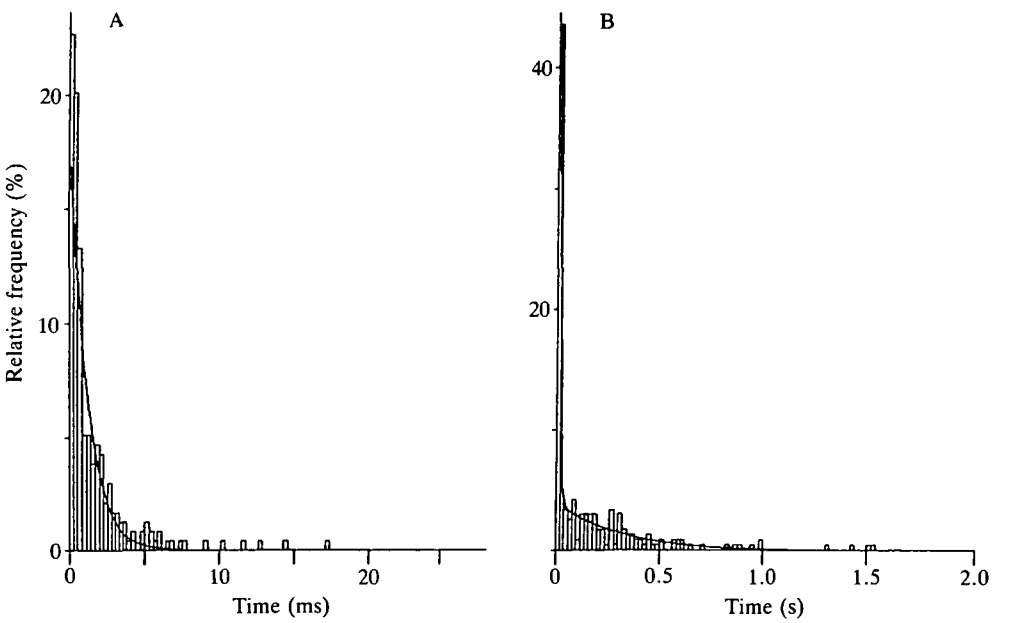



Fig. 6. (A) Open-time histogram. (B) Closed-time histogram. Results from a cell-attached patch, with 1 $\mu$mol $l^{-1}$ acetylcholine at −50 mV. (% is the relative frequency of the open and closed durations.)

Table 2. *Open-, burst- and closed-time kinetics*

| [Acetylcholine] ($\mu$mol l$^{-1}$) | $N$ | $T_1$ (ms) | $T_2$ (ms) | $T_3$ (ms) | $A_1$ | $A_2$ |
|---|---|---|---|---|---|---|
| Open times | | | | | | |
| 1–10 | 12 | 1.18±0.08 | 4.89±0.60 | – | 0.80±0.05 | – |
| 25 | 2 | 0.48±0.18 | 1.38±0.09 | – | 0.61±0.21 | – |
| Burst times | | | | | | |
| 1–10 | 12 | 1.17±0.21 | 7.62±2.71 | – | 0.66±0.07 | – |
| 25 | 2 | 0.28±0.02 | 1.44±0.30 | – | 0.20±0.01 | – |
| Closed times | | | | | | |
| 1–10 | 12 | 0.20±0.03 | 196.6±85.8 | 549.7±165.4 | 0.48±0.07 | 0.31±0.07 |
| 25 | 2 | 0.17±0.02 | 300.8±129.8 | 1142.0±930.9 | 0.34±0.03 | 0.61±0.03 |

$T_1$, $T_2$ and $T_3$ are the time constants and $A_1$ and $A_2$ the areas of the components of the p.d.f.
For openings and bursts, two exponentials were fitted and $A_2=1-A_1$.
For closings, three exponentials were fitted and $A_3=1-(A_1+A_2)$.
Values are means±1 S.E.M.; $N$ is the number of experiments.

comprised 80% of the openings and the long component 20%, hence the proportion of charge carried by the two types of opening was approximately 1:1.

The number of open states of the channel must be at least the number of exponential components that are needed to fit the open-time distribution (Colquhoun and Hawkes, 1982), suggesting that the *Ascaris* acetylcholine-activated channel has at least two open states.

### *Burst-time distribution*

The burst times were also best fitted by two exponentials (Table 2). The two components will be referred to as short bursts and long bursts. $T_1$ for short bursts (mean 1.17 ms) is nearly the same as $T_1$ for short openings (mean 1.18 ms), showing that these shorter bursts were made up of single openings, although $T_2$ for long bursts (mean 7.62 ms) is longer than $T_2$ for long openings (mean 4.89 ms), as these bursts are multiple openings.

Short bursts account for 66% of events and long bursts for 34%. Thus, long bursts are most important for carrying current with only one-quarter of the current carried by short bursts.

### *Closed-time distribution*

The closed times were best fitted by three exponentials (Table 2). The three components will be referred to as short, intermediate and long gaps. This result shows that the channel has at least three closed states. The short gaps were very consistent, with a $T_1$ of 0.20±0.03 ms (mean±S.E., $N$=12). The time constants for intermediate and long gaps showed greater variability and, when comparing the results of individual experiments, the values of $T_2$ and $T_3$ did not always seem to refer to the same components. For example, in one experiment with 10 $\mu$mol l$^{-1}$ acetylcholine $T_2$ was 30.9 ms and $T_3$ was 102.6 ms, while in another experiment

with 10 $\mu$mol $l^{-1}$ acetylcholine $T_2$ was 291.4 ms and $T_3$ was 666.9 ms. This suggests there may be more closed states occurring, but only three are detectable in any one experiment.

We were not successful in our attempts to fit more than three exponentials to the closed times in this analysis; there may be some long infrequent closed states contributing a very small percentage to the total area. It might be necessary to fit the closed times with up to six exponentials to describe fully the close time distribution, since it has been reported in other preparations that desensitizing concentrations of acetylcholine are best fitted by six exponentials: the usual three exponentials, two for putative desensitized closed states and a sixth exponential for channel block (Sakmann *et al.* 1980; Colquhoun and Sakmann, 1983; Sine and Steinbach, 1984; Ogden and Colquhoun, 1985). In our experiments it is unclear whether any desensitization has occurred with 1–10 $\mu$mol $l^{-1}$ acetylcholine, so we proceed on the assumption of three closed states. If there is any desensitization at these concentrations it occurs quickly in the few seconds before recording begins.

$T_1$ for short gaps was 0.2 ms, and this represented the very short closings which occur within bursts. $T_2$ for intermediate gaps had a mean value of 196.6 ms, and $T_3$ for long gaps had a mean value of 549.7 ms. The mean values for areas (Table 2) indicate that 48 % of gaps were short, 31 % were intermediate and 21 % were long. Hence, the proportions of time occupied by short, intermediate and long gaps were in the ratio 1:635:1202. Thus, short gaps contribute very little to the total closed time, although there were a relatively large number of them.

All the kinetic details quoted have been for the larger-conductance channel. So far we have only recorded from one patch in which the smaller-conductance channel occurred alone and frequently enough for detailed analysis. The results of this analysis showed no significant difference from the parameters quoted in Table 2, but further experimentation and analysis are necessary before any conclusions can be made about the kinetics of the smaller channel.

*Correction for missing events*

The next stage in the analysis was to correct the data for missing events using parameters obtained from fitting multiple exponentials. Some of the corrected values which best describe the channel characteristics are given in Table 3. The analysis calculated numbers of long and short bursts, numbers of long, intermediate and short gaps and values for the total time spent in gaps within bursts and for corrected total open time, which were subsequently used to generate the other corrected values given in Table 3.

The corrected mean open time at 1–10 $\mu$mol $l^{-1}$ acetylcholine was very consistent in all 12 experiments analysed, with a value of 1.26±0.11 ms (mean±S.E.). The uncorrected values, obtained from the PAT program, of approximately 2 ms were larger because they contained undetected short gaps. The results showed that the corrected mean open time did not change with the concentration of acetylcholine (Table 3).

The corrected open probability had a mean value of 0.029±0.013 (Table 3), but

Table 3. *Channel characteristics corrected for missing short events*

| [Acetylcholine] ($\mu$mol l$^{-1}$) | $N$ | Corrected mean open time (ms) | Corrected open probability | Corrected mean burst length (ms) | Corrected short gaps/ long burst | Corrected mean open time in long bursts (ms) |
|---|---|---|---|---|---|---|
| 1 | 3 | 1.25±0.13 | 0.017±0.009 | 2.83±0.48 | 2.04±0.54 | 1.33±0.06 |
| 3 | 5 | 1.28±0.22 | 0.030±0.027 | 3.98±2.08 | 6.60±2.54 | 1.10±0.26 |
| 10 | 4 | 1.26±0.23 | 0.037±0.022 | 3.05±0.74 | 3.57±0.83 | 1.09±0.17 |
| 1–10 | 12 | 1.26±0.11 | 0.029±0.013 | 3.38±0.86 | 4.45±1.18 | 1.15±0.12 |
| 25 | 2 | 0.86±0.14 | 0.0052±0.0037 | 1.25±0.23 | 0.73±0.10 | 0.71±0.30 |

Each of these characteristics is defined in Materials and methods.
Values are means±1 S.E.M.; $N$ is the number of experiments.

was concentration-dependent, with the open probability increasing with increasing concentration from 1 to 10 $\mu$mol l$^{-1}$ acetylcholine. The mean corrected open probability for 1 $\mu$mol l$^{-1}$ acetylcholine was 1.7%, for 3 $\mu$mol l$^{-1}$ it was 3% (a 71% increase over 1 $\mu$mol l$^{-1}$), and for 10 $\mu$mol l$^{-1}$ it was 3.7% (a 117% increase over 1 $\mu$mol l$^{-1}$). The corrected mean burst length (3.38±0.86 ms, mean±S.E., $N$=12) and corrected short gaps per long burst (4.45±1.18, mean±S.E., $N$=12) were more variable than the corrected mean open time. The results suggest that the bursts were longest at 3 $\mu$mol l$^{-1}$ acetylcholine and that bursts activated by this concentration have the largest number of short gaps per long burst.

The corrected mean open time in long bursts (1.15±0.12 ms, mean±S.E., $N$=12) was a consistent parameter and was not concentration-dependent (Table 3). The corrected values for this parameter are shorter than the uncorrected values, again because the correction allows for unresolved short gaps.

The range of parameters in Table 3 characterises the behaviour of this ligand-activated cation channel when activated by acetylcholine; future results obtained by activating this channel with other agonists may be compared with these parameters.

### *Desensitization at higher agonist concentrations*

Experiments at 25 $\mu$mol l$^{-1}$ acetylcholine ($N$=5) produced a different pattern of channel opening to that observed at lower concentrations; single-channel currents occurred in clusters with long closed times (mean 171 s) between each cluster (Fig. 7). The effect became more pronounced at 50 or 100 $\mu$mol l$^{-1}$ acetylcholine ($N$=8), where no channel openings were observed.

At 25 $\mu$mol l$^{-1}$ acetylcholine, two of the experiments on cell-attached patches were analysed in detail; the channel kinetics was studied in the first cluster of openings to occur in each recording. The open times and burst times were best fitted by two exponentials and the closed times by three exponentials, as with lower concentrations (1–10 $\mu$mol l$^{-1}$) of acetylcholine (Table 2). The data from

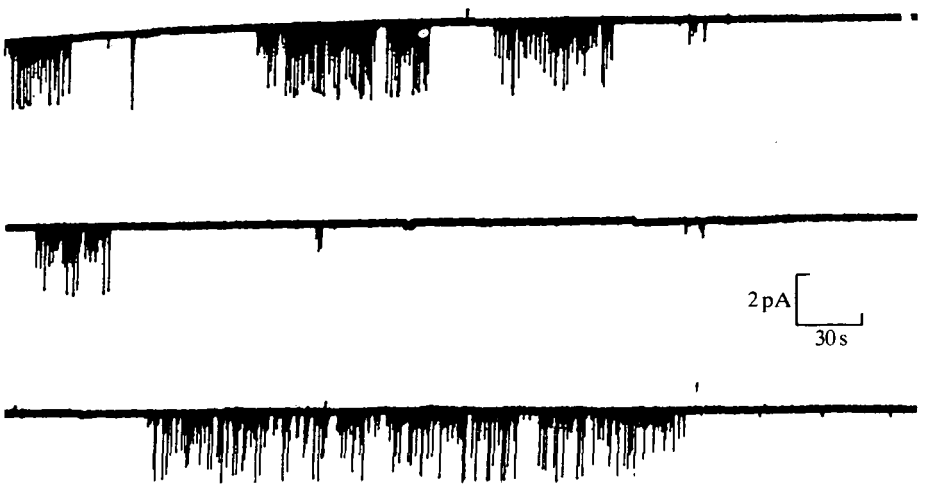



Fig. 7. Desensitisation at higher agonist concentrations in a cell-attached patch with 25 $\mu$mol $l^{-1}$ acetylcholine in pipette. Patch potential −50 mV. Traces are continuous and show the very long closed periods that occur at this concentration between groups of openings. The duration of clusters of openings was 60.7±30.2 s (mean±S.E., $N$=10) in one experiment and 59.6±15.0 s (mean±S.E., $N$=10) in the other. The durations of the very long closings between these clusters were 221.6±49.3 s and 120.2±46.3 s (mean±S.E., $N$=10) for the two experiments.

these two experiments were corrected for missing events (Table 3). Examination of these results showed that there were three components to this desensitization.

*Very long closed states*

Clusters were separated by very long closed times (mean 170 s) (see Fig. 7). These represent a desensitized state or states that did not occur at lower agonist concentrations. Such long closed times have been described before and fitted with two exponential components (Sakmann *et al.* 1980; Colquhoun and Sakmann, 1983; Colquhoun and Ogden, 1988). It has also been reported that acetylcholine desensitization occurs as a biphasic process, with both fast and slow processes. The very long closed times are considered to correspond to slow desensitization processes (Cachelin and Colquhoun, 1989). It is also possible that a rapid type of desensitization occurred during patch formation and before the commencement of recording; this would not have been detected by the present experiments (Brett *et al.* 1986).

*Open probability decreases with time*

When several samples ($N$=4) were analysed from successive clusters of openings in the same experiment, it was found that some of the kinetic parameters changed with time. The open probability was found to be lower within each subsequent group of openings, as there was an increase in $T_2$ for closings from 430.6 ms to 829.8 ms in 22 min (these closed times had only been fitted with two

exponentials). Despite this, the open- and burst-time kinetics and the corrected mean open time were unchanged even after 25 min. Hence, the reduction in open probability that occurred over 20–30 min might represent the appearance of an additional, long closed state occurring within clusters (rather than the observed ones increasing in length). This would represent another component of desensitization.

*Mean open times are reduced*

The results show that mean open times and burst times were shorter at 25 $\mu$mol $l^{-1}$ than at lower acetylcholine concentrations: at 25 $\mu$mol $l^{-1}$ $T_1$ and $T_2$ for openings and for bursts are reduced and the corrected mean open time is reduced to 0.86 ms compared with 1.26 ms for 1, 3 or 10 $\mu$mol $l^{-1}$ acetylcholine. Closed times at 25 $\mu$mol $l^{-1}$ acetylcholine are longer than for lower concentrations. The reduced corrected mean open time suggests that channel block may be occurring. However, if channel block is occurring at 25 $\mu$mol $l^{-1}$ acetylcholine in the *Ascaris* muscle it is of a type different from the 'classical' fast channel block observed with acetylcholine at the frog end-plate (Ogden and Colquhoun, 1985). At the frog end-plate, fast flickering shut of the channel causes an increase in the number of short gaps per long burst and a reduced channel amplitude. Our experiments showed a reduction in the number of short gaps per long burst and no change in channel amplitude, suggesting a slowly acting type of channel block (Hille, 1984).

## Discussion

*Conductance of acetylcholine-activated channels*

Acetylcholine-activated single-channel currents have been recorded with conductances in the range 20–60 pS from vertebrate muscle (see reviews by Schuetze and Role, 1987; Numa, 1986), from vertebrate nervous tissue (Cull-Candy and Mathie, 1986; Derkach *et al.* 1987; Mathie *et al.* 1987) and from invertebrate neurones (Sattelle, 1986; Sattelle *et al.* 1986; Beadle *et al.* 1989) in the cockroach and (Wu *et al.* 1983) in *Drosophila*. Our report is the first to describe these channels in invertebrate muscle.

In most preparations, the conductance of the acetylcholine-activated channel is greater with $Cs^+$ than with $Na^+$ as the conducting cation (the sequence is usually $K^+ > Cs^+ > Na^+ > Li^+$, Sakmann *et al.* 1985), but the conductance may be reduced in the presence of 1 mmol $l^{-1}$ $Ca^{2+}$ or $Mg^{2+}$ (Mishina *et al.* 1986). Our conductances were measured with $Cs^+$ as the conducting cation, in the absence of $Ca^{2+}$, but with 2 mmol $l^{-1}$ $Mg^{2+}$ present. The effects of $Ca^{2+}$ and $Mg^{2+}$ on our channel were not investigated, but the conductance of the acetylcholine-activated channel might be greater in the absence of $Mg^{2+}$.

Our channels were of two amplitudes: 40–50 pS and 25–35 pS. One possible explanation for obtaining these ranges of conductance (rather than the channels having exactly the same conductance in each patch) is that our recordings were made at room temperature, which varied from 15 to 22 °C. It has been shown that

the conductance of acetylcholine channels may increase with temperature, from 40 pS at 13 °C to 55–60 pS at 20–25 °C and 70–75 pS at 37 °C (Mulrine and Ogden, 1989).

*Ascaris* muscle is like that of many other species, e.g. rat, mouse, human, *Xenopus* and calf (Schuetze and Role, 1987), in having channels of two different conductances, both activated by acetylcholine. The two types of channel in vertebrate muscle are described in three ways: as 'embryonic' and 'adult' channels; as 'fast' and 'slow' channels; or as 'junctional' and 'extrajunctional' channels (Schuetze and Role, 1987). The only one of these descriptions that might fit the two channels in the *Ascaris* is that of 'fast' and 'slow', but so far we have insufficient data on the mean open times of the smaller channel so we refer to them simply as the larger- and smaller-conductance channels.

In bovine muscle, the two types of channel conductance recorded have been shown to be due to two separate ion channels with differences in protein structure (Mishina *et al.* 1986). No information is available about the protein structure of the channels in *Ascaris suum*, but our results strongly suggest that there will be two types of channel with differences in the protein structures. In the rest of the discussion we will consider the larger channel only.

*Voltage-sensitivity*

Another feature of vertebrate acetylcholine-activated channels is that single-channel I/V plots are almost always linear (as ours was) while the whole-cell currents may show very marked rectification (Sakmann *et al.* 1985). This is believed to be due to the mean open time of such channels being voltage dependent. Preparations in which the whole-cell current is linear have a mean open time independent of voltage (Sakmann *et al.* 1985).

Some of the features of the *Ascaris* channel are compared with those of other preparations in Table 4. Our open time durations were not voltage-sensitive nor did the amplitude of the channel currents show rectification. In this respect the *Ascaris* channel resembles the channel from *Torpedo* and is unlike the many mammalian muscle channels (e.g. Sakmann *et al.* 1985) or the channels of the frog or *Xenopus* (Colquhoun and Sakmann, 1985; Brehm *et al.* 1984). The mean open times can be calculated in various ways, but comparable values for *Ascaris* and *Torpedo* using the time constant $T_2$ having fitted two exponentials to the open times, show that the *Ascaris* channel has much longer open times ($T_2$=4.9 ms) than the *Torpedo* channel ($T_2$=0.6 ms), and is much closer to the channel in the frog end-plate ($T_2$=4.2 ms) in this respect.

A great deal of work has been done on the protein structure of nicotinic acetylcholine receptors, especially from *Torpedo* electroplax and calf muscle (Numa, 1986). This has included the production of hybrids such as the one described in Table 4 formed from the $\alpha$-subunit of calf and the $\beta$, $\gamma$ and $\delta$ subunits of *Torpedo*. This particular hybrid is of interest because its properties are closer to those of the *Ascaris* channel than to those of either the calf or the *Torpedo* channel from which it was formed.

Table 4. *Acetylcholine-activated channels in various preparations*

| Preparation | Conductance (pS) | Mean open time (ms) | Corrected mean open time (ms) | Voltage-dependence of open times | Reference |
|---|---|---|---|---|---|
| *Ascaris suum* | 48 | 4.9 | 1.26 | No | Present paper |
| *Torpedo* | 42 | 0.6 | – | No | Sakmann *et al.* (1985) |
| Calf muscle | 40 | 7.6 | – | Yes | |
| Hybrid | 42 | 3.2 | – | No | |
| Frog muscle (end-plate) | 44 | 4.2 | 1.40 | Yes | Colquhoun and Sakmann (1985) |
| *Xenopus* muscle | 64 | <1 | – | Yes | Brehm *et al.* (1984) |

Mean open time (ms) is the time constant for the longer of two exponentials fitted to the open times.

Corrected mean open times (ms) is as defined in Materials and methods.

Each study used a range of acetylcholine concentrations.

The hybrid listed contained the $\alpha$-subunit of calf and the $\beta$, $\gamma$ and $\delta$ subunits of *Torpedo*.

### *Rate constants*

Our data were corrected for missing short events and the results obtained are summarised in Table 3. The analysis was extended, using the model of Colquhoun and Sakmann (1985) of acetylcholine interacting with its receptor, outlined in Materials and methods. The rate constants for channel closing ($\alpha$) and channel opening ($\beta$) and the dissociation rate constant ($k_{-2}$) can be calculated from our corrected data (Table 5).

The highest mean values for $\alpha$ and $\beta$ and the lowest mean value for $k_{-2}$ were obtained at $3\,\mu\text{mol}\,l^{-1}$ acetylcholine. The results were tested using *t*-tests, but a combination of small sample sizes and relatively large variation in the results for each concentration meant that the differences were not statistically significant.

Table 5. *Estimates of rate constants and efficacy*

| [Acetylcholine] ($\mu$mol l$^{-1}$) | *N* | $\alpha$ ($s^{-1}$) | $\beta$ ($s^{-1}$) | $k_{-2}$ ($s^{-1}$) | $\beta/\alpha$ |
|---|---|---|---|---|---|
| 1 | 3 | 753±36 | 3649±884 | 1044±298 | 4.85 |
| 3 | 5 | 1082±190 | 5957±1761 | 504±60 | 5.51 |
| 10 | 4 | 998±160 | 3988±631 | 635±138 | 4.00 |
| 1–10 | 12 | 972±97 | 4724±803 | 682±103 | 4.86 |
| 25 | 2 | 1705±715 | 2539±91 | 1771±305 | 1.49 |

Derivation of rate constants is described in detail in Materials and methods.

$\beta/\alpha$=efficacy.

Values are means±1 S.E.M.; *N* is the number of experiments.

This is what would be expected, as $\alpha$, $\beta$ and $k_{-2}$ would not normally vary with low agonist concentrations (i.e. concentrations too low to cause significant desensitization or channel block (Sakmann *et al.* 1980; Colquhoun and Sakmann, 1985; Colquhoun and Ogden, 1988).

The combined values for 1, 3 and 10 $\mu$mol $l^{-1}$ acetylcholine were compared with those of Colquhoun and Sakmann (1985) for 0.1 $\mu$mol $l^{-1}$ acetylcholine. Their value of $\alpha$ for acetylcholine is 714 $s^{-1}$, ours is 972 $s^{-1}$; their value for $\beta$ is 30 600 $s^{-1}$, ours is 4724 $s^{-1}$; their value for $k_{-2}$ is 8150 $s^{-1}$ and ours is 682 $s^{-1}$, showing very similar values for $\alpha$, but our values of $\beta$ and $k_{-2}$ are approximately 10-fold less than theirs. Thus, the behaviour of acetylcholine at the receptor on *Ascaris* extrasynaptic muscle differs significantly from that reported for the frog muscle end-plate. This is not surprising, as responses to acetylcholine at an end-plate would be much faster than for extrasynaptic muscle (this is reflected in the rate constant for channel opening ($\beta$) which was 10 times faster at the frog end-plate than in our system).

The results at 25 $\mu$mol $l^{-1}$ acetylcholine (Table 5) were tested, using *t*-tests, with the results obtained for 1, 3 and 10 $\mu$mol $l^{-1}$ acetylcholine combined. This showed that $\alpha$ was significantly higher ($P$=0.02) at 25 $\mu$mol $l^{-1}$ than at 1, 3 and 10 $\mu$mol $l^{-1}$ and hence that the rate of channel closing increased with increasing agonist concentration, while $\beta$ was not significantly different at 25 $\mu$mol $l^{-1}$ compared with 1–10 $\mu$mol $l^{-1}$, indicating that the rate of channel opening remained unchanged. These results are consistent with the occurrence of channel block at 25 $\mu$mol $l^{-1}$ acetylcholine. It has been predicted that, with desensitization, $\alpha$ should be independent of agonist concentration while $\beta$ should increase with increasing agonist concentration but, with channel block, $\alpha$ should increase with increasing agonist concentration while $\beta$ should be independent of agonist concentration (Sakmann *et al.* 1980). Our results also show that the dissociation rate constant, $k_{-2}$, was significantly higher ($P$=0.001) at 25 $\mu$mol $l^{-1}$ than at 1–10 $\mu$mol $l^{-1}$. This, together with the increased rate of channel closing ($\alpha$), is responsible for the reduction observed in corrected mean open time 25 $\mu$mol $l^{-1}$ compared with 1–10 $\mu$mol $l^{-1}$.

### *Efficacy, affinity and potency*

The final step in the analysis of our data was to estimate values for efficacy, affinity and potency of acetylcholine at the *Ascaris* receptor and compare these estimates with the values obtained by Colquhoun and Sakmann (1985) for the frog end-plate receptor.

Values for efficacy ($\beta/\alpha$) are given in Table 5. The values for 1, 3 and 10 $\mu$mol $l^{-1}$ acetylcholine are very similar and were combined, giving a mean value of 4.9; this compares to Colquhoun and Sakmann's (1985) value of 43 for 0.1 $\mu$mol $l^{-1}$ acetylcholine and shows that acetylcholine has a much lower (1/10) efficacy at the *Ascaris* receptor than at the frog receptor. The efficacy at 25 $\mu$mol $l^{-1}$ on our receptor was 1.49, which is lower than for the other concentrations and reflects the higher value of $\alpha$ for 25 $\mu$mol $l^{-1}$ acetylcholine.

We estimated values for the equilibrium constant ($K_D$) and the affinity ($1/K_D$). For 1, 3 and 10 $\mu$mol $l^{-1}$ acetylcholine, $K_D$ was 6.8 $\mu$mol $l^{-1}$ [affinity= $147\times10^3$ (mol $l^{-1}$)$^{-1}$] compared to Colquhoun and Sakmann's values of $K_D$=80 $\mu$mol $l^{-1}$ [affinity=$12.5\times10^3$ (mol $l^{-1}$)$^{-1}$]. Hence, affinity is 10 times higher at the *Ascaris* receptor than at the frog receptor. The values at 25 $\mu$mol $l^{-1}$ acetylcholine for our receptor were: $K_D$=17.7 $\mu$mol $l^{-1}$ [affinity= $56\times10^3$ (mol $l^{-1}$)$^{-1}$]. The affinity at 25 $\mu$mol $l^{-1}$ acetylcholine is lower than at the other concentrations, which reflects the finding that the dissociation rate constant $k_{-2}$ was higher at 25 $\mu$mol $l^{-1}$ than at the lower concentrations.

Potencies were estimated relative to the potency of acetylcholine at the frog end-plate (Colquhoun and Sakmann, 1985). This gave a potency at 1, 3 and 10 $\mu$mol $l^{-1}$ acetylcholine of 4.04, suggesting that acetylcholine is four times as potent on *Ascaris* muscle as at the frog end-plate. The potency at 25 $\mu$mol $l^{-1}$ acetylcholine on *Ascaris* muscle was 0.86, reflecting the much lower efficacy and the higher value of $k_{-2}$ at 25 $\mu$mol $l^{-1}$ acetylcholine compared with the other concentrations.

### *Desensitization*

Nothing is known about the physical nature of desensitized states of the acetylcholine receptor, but desensitization can be effected by calcium (Chemeris *et al.* 1982; Oswald, 1983) and phosphorylation of the receptor may accelerate desensitization (Huganir *et al.* 1986). The role these play in the normal mechanism of desensitization is unclear. Three different reaction schemes have been fitted to observations of desensitization by Cachelin and Colquhoun (1989) and all three require two distinct desensitized conformations of the receptor. Desensitization probably results from the receptor-channel molecule adopting long-lived shut conformations in which the agonist binding sites are occupied, with the onset of desensitization being slower at lower agonist concentrations (Cachelin and Colquhoun, 1989).

The occurrence of desensitization at the *Ascaris* acetylcholine receptor is of particular interest, because it may be relevant therapeutically. It may be that giving too high a dose of certain anthelmintics is less effective than using a lower dose. Various nicotinic anthelmintics, such as pyrantel and levamisole, have a selective action on acetylcholine receptors in nematodes, but not on those of the vertebrate host (Aubry *et al.* 1970; Coles *et al.* 1975; Harrow and Gration, 1985).

Harrow and Gration (1985), using intracellular current-clamp on muscle cells of *Ascaris suum*, showed that high doses of morantel and pyrantel (>100 $\mu$mol $l^{-1}$) produced smaller responses than lower concentrations, while no such effect was reported for levamisole or acetylcholine (up to 10 mmol $l^{-1}$). The lack of desensitization with acetylcholine at concentrations up to 10 mmol $l^{-1}$ is in sharp contrast to our finding that the response was completely abolished at a concentration of 50 $\mu$mol $l^{-1}$. Another report on the actions of pyrantel and levamisole (Coles *et al.* 1975) contradicts the results of Harrow and Gration (1985) and it

would be very interesting to study them at a range of concentrations at the single-channel level.

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