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Levamisole-activated single-channel currents from muscle of the nematode parasite *Ascaris suum*

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1 The patch-clamp technique was used to examine levamisole-activated channels in muscle vesicles from *Ascaris suum*. Cell-attached and isolated inside-out patches were used.

2 Levamisole (1–90 μM), applied to the extracellular surface, activated channels which had apparent mean open-times in the range 0.80–2.85 ms and linear I/V relationships with conductances in the range 19–46 pS. Ion-replacement experiments showed the channels to be cation selective.

3 The kinetics of the channels were analysed. Generally open- and closed-time distributions were best fitted by two, and three expotentials respectively, indicating the presence of at least two open states and at least three closed states. The distributions of burst-times were best-fitted by two exponentials.

4 Channel open- and burst-times were voltage-sensitive: at low levamisole concentrations (1–10 μM), they increased with hyperpolarization. At higher concentrations of levamisole (30 μM and 90 μM) flickering channel-block was observed at hyperpolarized potentials. Using a simple channel-block model, values for the blocking dissociation constant, K_B were determined as 123 μM at − 50 mV, 46 μM at − 75 mV and 9.4 μM at − 100 mV.

5 At the higher concentration of levamisole (30 μM and 90 μM) long closed-times separating 'clusters' of bursts were observed, at both hyperpolarized and depolarized membrane potentials and this was interpreted as desensitization.

Keywords: Levamisole; *Ascaris*; nicotinic-channels

Introduction

Nematode parasites inflict much suffering on human and animal hosts. Approximately one in five of the world's human population is infected with intestinal nematodes (Standen, 1975), while infestation in domestic animals is a major source of economic loss. Antihelminthic drugs, such as levamisole, are used to control these infestations.

Studies on the mode of action of levamisole indicate it is a selective agonist at acetylcholine receptors present on the muscle cells of parasitic nematodes. One such nematode is *Ascaris suum*. Levamisole and acetylcholine treatment of *Ascaris suum* results in depolarization and spastic paralysis of the muscle (Aceves *et al.*, 1970; Van Neuten, 1972; Coles *et al.*, 1975; Martin, 1982; Harrow & Gration, 1985; Colquhoun *et al.*, 1991).

The acetylcholine receptors of *Ascaris* muscle have been classified as nicotinic, as nicotinic agonists produce contractions antagonised by tubocurarine, whereas muscarinic agonists and antagonists have little effect on this preparation (Natoff, 1969; Rozkova *et al.*, 1980; Martin, 1982, Colquhoun *et al.*, 1991). The pharmacology of the acetycholine receptors on *Ascaris* muscle is most like that of the nicotinic ganglionic receptors of vertebrates, most notably mecamylamine is a potent antagonist at both receptor sites (Natoff, 1969; Rozkova *et al.*, 1980).

The aim of the present study was to investigate the action of levamisole at the single-channel level in *Ascaris* by use of the extrasynaptic acetylcholine receptors present in isolated muscle vesicles (Martin *et al.*, 1990; Pennington & Martin, 1990). Our results show that levamisole opens cation selective channels, but in addition, produces open channel-block and desensitization.

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Methods

Single-channel recordings of levamisole-activated currents were made from *Ascaris* muscle vesicles. In general the data were analysed by the same methods as used in previous studies with acetylcholine (Pennington & Martin, 1990).

The vesicle preparation

Ascaris suum were collected from the local abattoir, maintained in Locke solution (replaced daily) at 32°C and used within 4 days. To prepare the vesicles, a 2 cm section from the anterior region of the worm was taken, cut along one of the lateral lines and pinned out to form a muscle-flap preparation. This muscle flap was treated with collagenase solution for about 10 min at 37°C, (composition as follows): mM NaCl 35, Na acetate 105, KCl 2, $MgCl_2$ 2, HEPES 10, glucose 3, ascorbic acid 2 and collagenase (1 mg ml^{-1}) pH adjusted to 7.2 with NaOH. The preparation was then kept in a maintenance solution at 37°C (composition as follows): mM NaCl 35, Na acetate 105, KCl 2, $MgCl_2$ 2, HEPES 10, glucose 3, ascorbic acid 2 and EGTA 1; pH adjusted to 7.2 with NaOH. The vesicles formed over the next hour, as outgrowths from the bag region of the muscle cells. The vesicles were used within 5 h.

Electrical recordings

The vesicles were transferred to the experimental chamber where 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 viewed at × 200 magnification. Vesicles were bathed in the experimental chamber in the following solution (composition mM): CsCl 35, Cs acetate 105, $MgCl_2$ 2, HEPES 10 and EGTA 1; pH adjusted to 7.2 with CsOH.

Recordings were made from either isolated inside-out pat-

ches or cell-attached patches. Patch-electrodes were made from micro-haematocrit capillary glass (Garner Glass 7052) with a resistance of 1–3 MΩ. The pipettes were coated with-Sylgard to improve frequency responses. Electrodes were filled with pipette solution (composition, mM) CsCl 140, $MgCl_2$ 2, HEPES 10 and EGTA 1; pH 7.2 with CsOH. To examine the effects of levamisole, different concentrations of levamisole were placed in the pipette solution. All of the recordings were made with Cs^+ as the main cation to avoid contamination of patch recordings with K^+ channels known to be present in the membrane. The solutions were also Ca^{2+}-free to avoid contamination of patch recordings with Ca^{2+}-activated Cl^- channels known to occur in this membrane (Thorn & Martin, 1987).

In experiments to determine ion-selectivity of the channel, the ionic composition of the bath solution was altered by diluting 50:50 with distilled H_2O. Inside-out patches were used for these experiments to ensure changes in ionic concentration at the cytoplasmic membrane surface.

Currents were recorded with a List EPC-7 and a modified Sony digital audio processor with a Betamax video recorder.

Data processing

Records were analysed with a CED 1401 interface and a DCS286 PC Computer, with PAT software kindly supplied by John Dempster, Strathclyde University. The records were filtered by an eight-pole Bessel filter, 3 dB, 1.5 kHz; the sampling time was 70 μs and the minimum detectable interval was 0.3 ms. The threshold for channel opening was set at 70% of the single channel amplitude determined from channel amplitude histograms so as to include only full openings. In some experiments a 50% threshold was also used to examine the effect on open times. Little practical difference was observed. It is pointed out that corrected P_{open} values were determined (Colquhoun & Sakmann, 1985) for levamisole concentrations between 2 and 10 μM and found to be not significantly different from apparent P_{open} values obtained from the Dempster software. Subsequently in this manuscript only apparent P_{open} values are quoted.

In these experiments, individual channel activations were interrupted with short close periods (the 'Nachschlag' phenomenon, Colquhoun & Sakmann, 1985), so bursts of openings as well as single openings were considered. Bursts were defined as groups of openings separated by gaps that are shorter than a specified length, T_{crit}. T_{crit} was determined by numerically solving the following equation:

$$1 - e^{-T_{crit}/T1} = e^{-T_{crit}/T2}$$

where T1 represents the brief closed state T1, and T2 represents the slightly longer closed state T2. The equation finds a value for T_{crit} which balances the proportion of gaps within a burst misclassified (as gaps between bursts) and gaps between a burst misclassified (as gaps within a burst) (Colquhuon & Sakmann, 1985). In general a value of 1 ms was used in these experiments. This particular value was not so important since gaps between bursts were several hundred fold larger than gaps within bursts.

Exponential curve fitting

Exponential curves were fitted to the open-, closed-, and burst-time by the method of maximum likelihood (Colquhoun & Sigworth, 1983; Martin, 1985) to a probability density function (p.d.f) of the form:

$$\text{p.d.f.} = \sum_{i=1}^{k} \frac{A_i e(^{-t}/T_i)}{T_i}$$

where A_i = the area of the ith component; T_i = the fitted time constant; t = time and K = number of exponentials fitted.

After exponential curve fitting, the data were corrected for missing events (Colquhoun & Sakmann, 1985; Pennington & Martin, 1990). This was carried out to compensate for brief events missed due to limited resolution of the recording apparatus.

The flickering channel-block seen at high levamisole concentrations was analysed by the simple channel-block scheme described by Adams (1976) and Colquhoun & Sakmann (1985):

$$C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O \underset{k_{-B}}{\overset{k_{+B}.X_B}{\rightleftharpoons}} B$$

where C denotes the closed state; O denotes the open state; B denotes the blocked state; X_B is the drug concentration; k_{+B} is the blocking rate constant; k_{-B} is the unblocking rate constant; α is channel closing-rate and β is the channel opening-rate. The model predicts that:

$$\text{mean open-time} = 1/(\alpha + k_{+B}.X_B),$$

$$\text{mean block-time} = 1/k_{-B},$$

$$\text{channel-block dissociation constant, } K_B = k_{-B}/k_{+B}$$

The slope of the reciprocal plot of mean durations of long openings (T2), against levamisole concentration was used to determine values for k_{+B}.

The mean block durations were determined by selecting the distinctive blocked times seen during flickering bursts greater than 0.3 ms (our limit of resolution). The method of maximum likelihood was then used to fit a single exponential to obtain a value for the mean block duration. It is pointed out that there was inevitably some overlap between the blocked time and the 'Nachschlag' closings, however this error is minimal as the interruptions due to channel block far exceed the number of interruptions due to the 'Nachschlag' closings.

Drugs

Levamisole ((−)-(5)-2,3,5,6-tetrahydro-6-phenylimadaz[2,1-b]thiazole]) was obtained as levamisole hydrochloride from Sigma chemical company, Fancy Road, Poole, Dorset, BH17 7NH. Collagenase, Sigma type 1A, was also obtained from Sigma chemical company.

Statistical analysis

The method of least squares was used to obtain the best fits for the exponential equations used to described the voltage-sensitivity of the rate constants k_{+B} and k_{-B} and the dissociation constant K_B, a Spearman correlation coefficient (r) was used to test the quality of the fits. Values are given as mean ± s.e.mean. Levels of significance were obtained by use of Student's t tests, a significance level of 0.05 was used.

Results

With levamisole in concentrations between 1–90 μM, in the patch-pipette, channel currents in 43% of 143 cell-attached and isolated inside-out patches were recorded. These channels had apparent mean open-times in the millisecond range and amplitudes of around 2.5 pA at −75 mV. Detailed analysis of channel data from a single patch recorded from both the cell-attached and the isolated inside-out configuration showed no significant differences in channel kinetics. In 20 experiments with levamisole omitted from the

patch pipette solution no such channel openings were observed. It was therefore concluded that levamisole activated these currents.

Channel conductance and current/voltage relationships

The pipette and bath-solutions had symmetrical cation concentrations but non-symmetrical anion concentrations (pipette main-ions: Cs^+ 140 mM, Cl^- 144 mM: bath main-ions: Cs^+ 140 mM, Cl^- 39 mM). Under these conditions with isolated inside-out patches, the predicted Nernst reversal potential for a cation selective channel is 0 mV, and for an anion channel is − 33 mV. In 26 out of 31 experiments the *I/V* plots of levamisole-activated channels were linear with reversal near 0 mV (Figure 1) indicating that these channels were cation-selective and could conduct Cs^+. The slopes had an average conductance of 32.9 ± 1.23 pS (mean ± s.e., $n = 26$) and ranged between 19–46 pS.

As a further test of the permeability of the channel the bath-solution was diluted 50:50 with distilled water. The predicted effect of this is to produce a shift in the Nernst reversal potential from 0 mV to + 18 mV for a cation selective channel and from − 33 mV to − 51 mV for an anion selective channel. In the experiment illustrated in Figure 1, the reversal potential shifted from 0 mV to + 13 mV.

Calculation of the relative permeability of Cs^+ and Cl^- with the Goldman constant field equation (Hille, 1984), revealed that Cs^+ was ten times more permeable than Cl^-. Similar results were obtained in three other experiments.

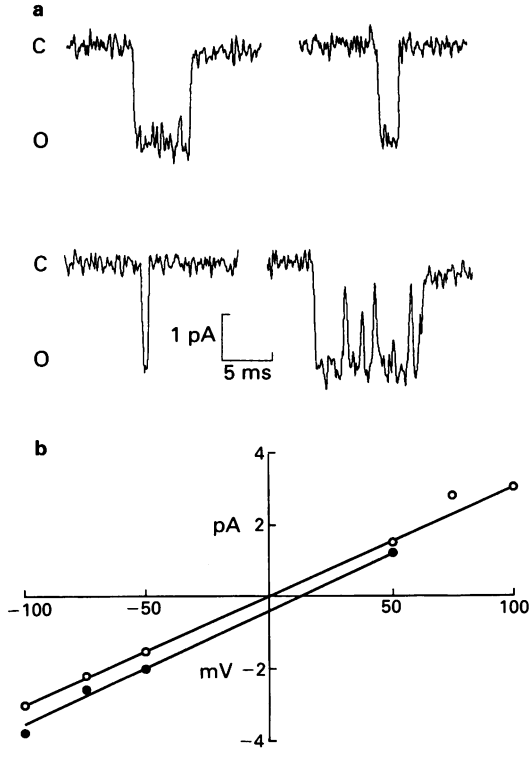

Figure 1 (a) Individual channel openings recorded from an isolated inside-out patch, at a membrane potential of − 75 mV and with levamisole 3 μM. The conductance of these channel currents was 26 pS. C: denotes the closed state. O: denotes the open state. (b) *I/V* plot of channel currents activated by 3 μM levamisole in an isolated inside-out patch. The initial plot (○) has a conductance of 31 pS and a 0 mV reversal potential. Dilution of the bath solution (●) produced a shift in the reversal potential to + 13 mV. The predicted Nernst potentials during the initial *I/V* plot were: for Cs^+, 0 mV; for Cl^- − 33 mV. The predicted Nernst potentials for the *I/V* plot obtained with the diluted solution were: for Cs^+, 18 mV; for Cl^-, − 51 mV.

Exponential curve-fitting

Channel data from 31 patches were analysed at various membrane potentials, ranging from + 150 mV to − 150 mV. The data were best-fitted by the sum of exponentials (see Methods). Files contained between 200 and 10,000 apparent open-, burst- and closed-times.

With 2–10 μM levamisole in the pipette the open- and burst-time distributions were best-fitted by two exponentials, while closed-time distributions were best-fitted by three exponentials (Table 1,2,3). Since the number of open-, burst- and closed- states must at least equal the number of exponential components fitted (Colquhoun & Hawkes, 1982), levamisole-activated channels in *Ascaris* have at least two open-states, at least two burst-states and at least three closed-states. Higher concentrations of levamisole (30 μM and 90 μM) produced substantial changes in the distribution of both the open- and closed-times, these changes are considered in the sections on channel-block and desensitisation.

Voltage-sensitivity of open- and burst-time durations

At lower concentrations of levamisole (2–10 μM) the corrected mean open-times (Figure 2) and corrected mean burst-times exhibited a degree of voltage-sensitivity, decreasing with depolarization. For example the value at − 75 mV for the corrected mean open-time was 1.47 ± 0.11 ms (mean ± s.e., $n = 10$), this value was significantly greater ($P < 0.03$) than the value for the corrected mean open-time at + 75 mV of 1.10 ± 0.25 ms ($n = 4$). The corrected mean burst duration at − 75 mV was 2.50 ± 0.28 ms ($n = 16$), this compared to a value of 1.50 ± 0.25 ms ($n = 4$) at + 75 mV where the corrected mean burst durations were also significantly reduced ($P < 0.05$).

Probability of channel opening

The probability of the channel being open (P_{open}) was concentration-dependent at depolarized potentials where P_{open} increased with concentration. However at hyperpolarized potentials concentration had no significant effect on P_{open}. Figure 3 illustrates the effect of concentration on P_{open} at ± 75 mV. Similar results were observed at ± 100 mV. It is suggested subsequently that the explanation for the failure of P_{open} to increase at − 75 mV and − 100 mV is due to the blocked state of the channel, which occurs with high concentrations of levamisole at hyperpolarized potentials, directly closing without re-entering the open-state.

At low agonist concentrations the membrane potential had little affect on P_{open}. For example, in one experiment with 3 μM levamisole, P_{open} was 0.0061 at + 75 mV compared to 0.0078 at − 75 mV; similar results were obtained in 8 other experiments with 2–10 μM levamisole. This lack of effect of membrane potential on P_{open} contrasts with the effect of membrane potential on open-times; the lack of effect on P_{open} is explained by the compensating increase in the rate of channel opening at depolarized potentials. For example in one experiment with 3 μM levamisole, the channel opening rate at − 75 mV was 8.6 channel openings per second compared to 13.6 channel openings per second at + 75 mV.

Evidence of channel block

At higher concentrations of levamisole (30 μM and 90 μM), there was a recognisable change in the kinetics of channel opening (Figure 4). At hyperpolarized potentials, channel openings were briefer than at lower concentrations and appeared as sequences of openings separated by brief closings characteristic of a 'flickering' channel-block (see, Neher & Steinbach, 1978). This behaviour may be explained by levamisole acting to produce a voltage-sensitive open channel-block, in addition to its action as an agonist. The cationic charge and large size of levamisole is consistent with

Table 1 Open-time kinetics for levamisole-activated channels

Levamisole conc. (μM)		*Open-times* (ms) A1	A2	T1	T2
Membrane potential − 75 mV					
2–10	(n = 17)	0.66 ± 0.06	0.34 ± 0.06	0.88 ± 0.10	2.30 ± 0.24
30	(n = 9)	0.78 ± 0.09	0.22 ± 0.09	0.37 ± 0.09	0.69 ± 0.08
90	(n = 12)		1.00		0.57 ± 0.06
Membrane potential − 100 mV					
2–10	(n = 13)	0.59 ± 0.08	0.41 ± 0.08	0.86 ± 0.12	2.54 ± 0.36
90	(n = 5)		1.0		0.39 ± 0.05
Membrane potential + 75 mV					
3–10	(n = 4)	0.62 ± 0.10	0.38 ± 0.10	0.71 ± 0.10	1.60 ± 0.14
30	(n = 5)	0.66 ± 0.07	0.34 ± 0.07	0.48 ± 0.07	1.22 ± 0.29
90	(n = 8)	0.66 ± 0.08	0.34 ± 0.08	0.64 ± 0.10	2.02 ± 0.28
Membrane potential + 100 mV					
3–10	(n = 4)	0.79 ± 0.12	0.21 ± 0.12	0.71 ± 0.08	2.35 ± 0.50
30	(n = 4)	0.81 ± 0.04	0.19 ± 0.04	0.50 ± 0.10	2.03 ± 0.47
90	(n = 4)	0.67 ± 0.06	0.33 ± 0.06	0.65 ± 0.07	1.88 ± 0.24

T1 and T2 are the time constants representing the two open states. The proportion of brief openings (T1) and long openings (T2) are represented by the areas of A1 and A2. Values are mean ± s.e..

Table 2 Closed time kinetics for levamisole-activated channels

Levamisole conc. (μM)		*Closed times* (ms) A1	A2	A3	A4	T1	T2	T3	T4
Membrane potential − 75 mV									
2–10	(n = 18)	0.46 ± 0.04	0.26 ± 0.05	0.28 ± 0.05		0.35 ± 0.04	237 ± 41	821 ± 127	
30	(n = 9)	0.46 ± 0.06	0.20 ± 0.04	0.28 ± 0.04	0.06 ± 0.02	0.76 ± 0.16	24 ± 10	228 ± 80	1586 ± 446
90	(n = 11)	0.55 ± 0.04	0.17 ± 0.02	0.22 ± 0.03	0.06 ± 0.02	0.63 ± 0.08	12 ± 3	251 ± 107	2528 ± 714
Membrane potential − 100 mV									
2–10	(n = 14)	0.38 ± 0.05	0.26 ± 0.04	0.34 ± 0.08		0.37 ± 0.04	224 ± 52	1081 ± 139	
90	(n = 6)	0.51 ± 0.07	0.25 ± 0.03	0.18 ± 0.06	0.07 ± 0.02	1.57 ± 0.32	20 ± 12	223 ± 131	3684 ± 2579
Membrane potential + 75 mV									
3–10	(n = 5)	0.22 ± 0.05	0.56 ± 0.09	0.21 ± 0.09		0.24 ± 0.04	132 ± 38	595 ± 115	
30	(n = 5)	0.21 ± 0.09	0.09 ± 0.05	0.57 ± 0.08	0.12 ± 0.03	0.38 ± 0.23	8 ± 4	137 ± 73	675 ± 261
90	(n = 8)	0.22 ± 0.38	0.38 ± 0.05	0.34 ± 0.06	0.05 ± 0.02	0.56 ± 0.15	32 ± 11	127 ± 30	961 ± 380
Membrane potential + 100 mV									
2–10	(n = 4)	0.31 ± 0.04	0.52 ± 0.08	0.15 ± 0.05		023 ± 0.06	146 ± 22	672 ± 189	
30	(n = 4)	0.10 ± 0.02	0.42 ± 0.26	0.34 ± 0.14	0.13 ± 0.10	0.44 ± 0.01	51 ± 38	172 ± 74	526 ± 247
90	(n = 5)	0.10 ± 0.03	0.40 ± 0.07	0.46 ± 0.65	0.04 ± 0.02	0.38 ± 0.08	16 ± 4	87 ± 36	427 ± 128

Closed states are represented by three exponentials at 2–10 μM levamisole. Brief closings (T1), intermediate closings (T2) and long closings (T3), their relative proportions are represented by the areas A1, A2 and A3. At 30–90 μM there are four closed states represented by the time constants T1, T2, T3 and T4. Their relative proportions are represented by the areas A1, A2 and A3 and A4. All values are the mean ± s.e., n is the number of experiments.

Table 3 Burst-time kinetics for levamisole-activated channels

Levamisole conc. (μM)		*Burst-times* (ms) A1	A2	T1	T2
Membrane potential − 75 mV					
2–10	(n = 19)	0.49 ± 0.07	0.51 ± 0.07	1.24 ± 0.19	3.07 ± 0.35
30	(n = 8)	0.61 ± 0.09	0.39 ± 0.09	0.59 ± 0.15	2.06 ± 0.42
90	(n = 9)	0.59 ± 0.07	0.41 ± 0.07	0.74 ± 0.19	2.23 ± 0.32
Membrane potential − 100 mV					
2–10	(n = 12)	0.56 ± 0.07	0.44 ± 0.07	1.29 ± 0.22	3.78 ± 0.50
90	(n = 4)	0.44 ± 0.08	0.56 ± 0.08	0.30 ± 0.15	2.93 ± 0.96
Membrane potential + 75 mV					
3–10	(n = 5)	0.46 ± 0.08	0.54 ± 0.08	0.69 ± 0.03	1.89 ± 0.29
30	(n = 5)	0.77 ± 0.08	0.23 ± 0.08	0.66 ± 0.19	2.33 ± 0.79
90	(n = 7)	0.73 ± 0.05	0.27 ± 0.05	0.93 ± 0.23	2.73 ± 0.64
Membrane potential + 100 mV					
2–10	(n = 5)	0.71 ± 0.06	0.29 ± 0.06	1.06 ± 0.21	2.54 ± 0.48
30	(n = 3)	0.82 ± 0.08	0.18 ± 0.08	0.69 ± 0.21	4.01 ± 1.52
90	(n = 5)	0.69 ± 0.06	0.31 ± 0.06	0.80 ± 0.22	2.33 ± 0.52

T1 and T2 are the time constants representing the two burst states. The proportion of brief bursts (T1) and the long bursts (T2) are represented by the areas A1 and A2. Values are mean ± s.e..

the direction of the voltage-sensitivity of the block. In order to describe the effect further, a simple channel-block model (Adams 1976; Colquhoun & Sakmann, 1985) was used.

The presence of open channel-block reduced the duration of the open-states at hyperpolarized potentials. Table 1 shows that this reduction in open-times at 30 μM levamisole was associated with a reduction of both the brief and longer components of the open-time distribution. At − 75 mV with 90 μM levamisole where the open-durations were also reduced, only one open-state could be detected. The loss of one component at this concentration is most likely due to the brief openings reducing to a level below the limit of resolution of the recording apparatus, as a result of channel-block. At 90 μM the corrected mean open-time at −75 mV was 0.63 ± 0.06 ms (mean ± s.e., $n = 11$), a value significantly lower than that at + 75 mV which was 1.30 ± 0.26 ms (mean ± s.e., $n = 8$), ($P < 0.02$). Thus mean open-times showed a reversed direction of voltage-sensitivity at the higher levamisole concentrations. At + 75 mV where no channel-block was observed, there was no significant reduction in the mean duration of the two open-time components compared to values at lower concentrations (2–10 μM).

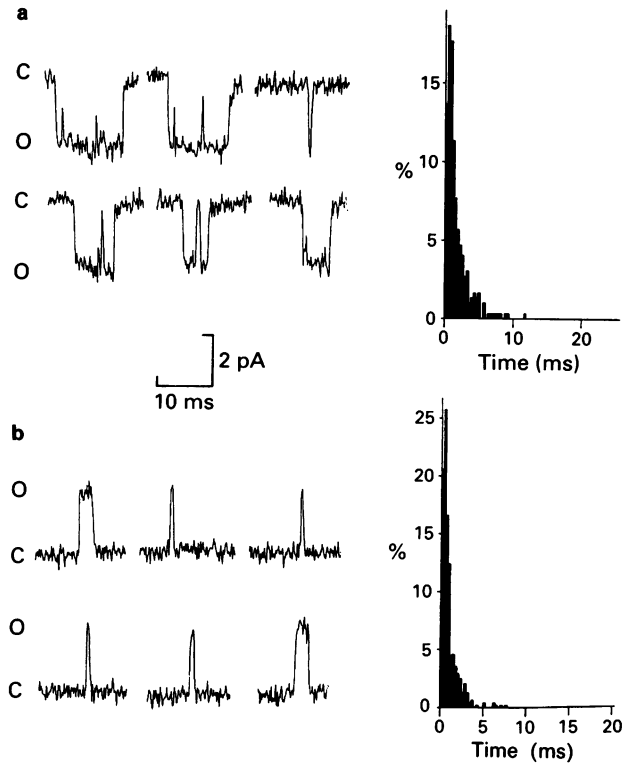

Figure 2 Levamisole (3 μM)-activated channels from a cell-attached patch illustrating the voltage-sensitivity of open-time durations. C: denotes the closed state. O: denotes the open state. (a) Individual channels recorded at − 75 mV and histogram of open-times at this potential. The open-times were fitted by two exponentials (not shown) with time constants of 0.92 ms and 2.14 ms. The corrected mean open-time was 1.59 ms. (b) Individual channels recorded at + 75 mV and histogram of open-times at this potential. The open-times were fitted by two exponentials (not shown) with time constants of 0.53 ms and 1.45 ms. The corrected mean open-time was 0.91 ms.

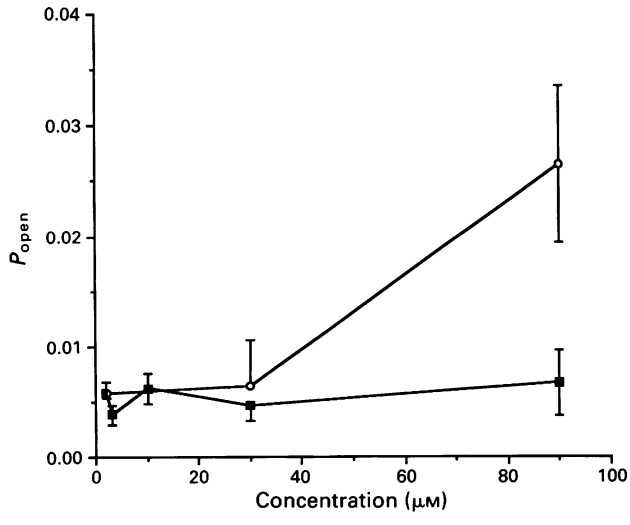

Figure 3 Graph showing P_{open} versus levamisole concentration; (■) − 75 mV membrane potential; (○) + 75 mV membrane potential. All values are mean (with vertical bars showing s.e.). $n = 4$ to $n = 9$.

Estimation of blocked-times

Mean block durations at membrane potentials of − 35 to − 100 mV for both 30 μM and 90 μM are shown in Table 4. Comparison of values at both 30 μM and 90 μM showed concentration did not affect mean block-times. The lack of effect of concentration on blocked-times is consistent with predictions of the simple channel-block model. Values obtained for mean block durations at both 30 μM and 90 μM were subsequently combined and their reciprocals used to estimate k_{-B} values (Figure 5).

Estimation of k_{+B}

The forward blocking-rate constants k_{+B} were determined from plots of the reciprocals of the mean duration of long open-times (T2) against drug concentration (Figure 6). Long open-times were used in preference to the brief open-times because they are more accurately determined, the short open durations are close to the limit of resolution of the setup and at 90 μM value for T1 appears to fall below the limit of resolution of the recording apparatus. In calculating k_{+B}, results obtained with 90 μM levamisole were not included. These points deviated from the straight line obtained with results at lower concentrations (2–30 μM). This may be due to the fact that at higher concentrations the mechanism of block may be more complex than the simple channel-block mechanism. However it is more likely that at the the higher concentration (90 μM) some of the open-time durations are lower than the limit of resolution of the recording apparatus, making the open-time duration an overestimation at this concentration. This interpretation is further supported by the fact that at 90 μM with hyperpolarized potentials open-times distributions could only be fitted by a single exponential.

Values for the forward blocking-rate constant, k_{+B} at − 50 mV, was $2.06 \times 10^7\ M^{-1}\ s^{-1}$; at −75 mV it was $3.82 \times 10^7\ M^{-1}\ s^{-1}$; and at − 100 mV it was $6.58 \times 10^7\ M^{-1}\ s^{-1}$. As predicted by the simple channel block model, k_{+B} increased with membrane hyperpolarization. The voltage sensitivity of k_{+B} was described by the equation: $k_{+B} = k_{+BO} \cdot \exp^{(E/Vk)}$. Where k_{+B} is the forward blocking rate constant at the membrane potential E, k_{+BO} is the value for k_{+B} at 0 mV and Vk is a constant. The value for k_{+BO} was $0.83 \times 10^7\ M^{-1}\ s^{-1}$ and Vk was − 35.5 mV. The correlation coefficient (r) was 0.97. Therefore k_{+B} showed an e-fold change every 36 mV. It was also possible to estimate the closing rate constants (α_2) for the long openings. This was the intercept of the plot of 1/T2 versus concentration. At − 50 mV the value for α_2 was $447\ s^{-1}$, at − 75mV it was $396\ s^{-1}$; and at − 100 mV it was $143\ s^{-1}$. As can be observed the closing rate decreases with membrane hyperpolarization, this supports earlier observations where open durations were longer at hyperpolarized potentials.

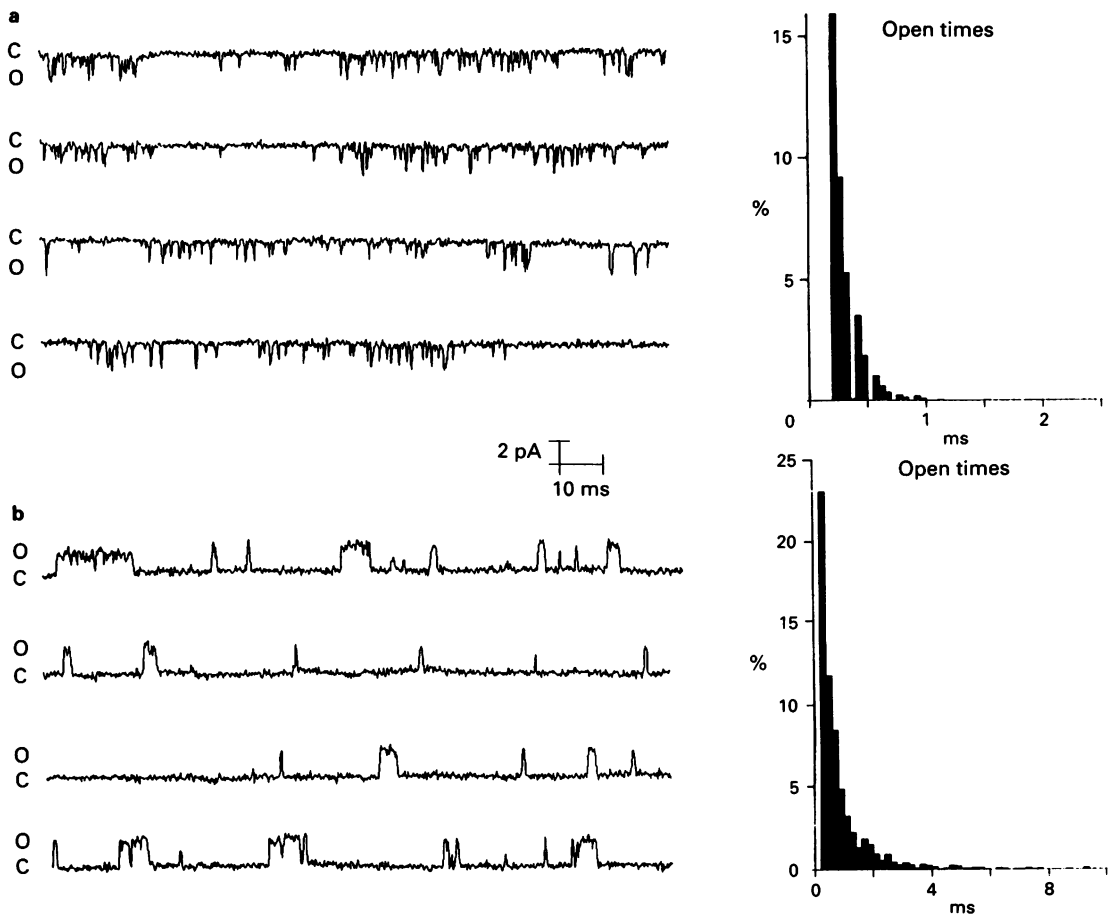

Figure 4 Levamisole-(30 μM) activated channels and open-time histograms from an isolated inside-out patch. (a) Membrane potential − 75 mV; (b) membrane potential + 75 mV. The open-times in this patch were fitted with two exponentials (not shown). (a) TI = 0.14 ms T2 = 0.69 ms; (b) T1 = 0.41 ms T2 = 1.91 ms. Note the presence of flickering open channel-block in (a).

Table 4 The effect of membrane potential on mean block time

	Mean block time (ms) *Membrane potential*			
Levamisole conc. (μM)	*− 35 mV*	*− 50 mV*	*− 75 mV*	*− 100 mV*
30		0.38 ± 0.05 (n = 5)	0.87 ± 0.18 (n = 9)	2.07 ± 0.34 (n = 4)
90	0.27 ± 0.05 (n = 4)	0.50 ± 0.07 (n = 6)	0.71 ± 0.09 (n = 11)	1.55 ± 0.18 (n = 6)
30 + 90	0.27 ± 0.05 (n = 4)	0.44 ± 0.05 (n = 11)	0.78 ± 0.10 (n = 20)	1.76 ± 0.18 (n = 10)

Values are mean ± s.e.

Determination of K_B

The values for k_{+B} and k_{-B} determined above were used to calculate the channel-block dissociation constant, K_B. At − 50 mV, K_B was $2.55 \times 10^3\ s^{-1}/2.06 \times 10^7\ M^{-1}\ s^{-1}$ or 123 μM; at − 75 mV, K_B was $1.75 \times 10^3\ s^{-1}/3.82 \times 10^7\ M^{-1}\ s^{-1}$ or 46 μM; and at − 100 mV, K_B was $0.62 \times 10^3\ s^{-1}/6.58 \times 10^7\ M^{-1}\ s^{-1}$ or 9.4 μM. The semi-log plot of K_B versus membrane potential is shown in Figure 7, this shows an e-fold change in K_B every 19.4 mV.

Burst durations

As stated earlier, the burst durations exhibit a degree of voltage-sensitivity. The bursts consisted of two components; the brief bursts (mean T1) and the longer bursts (mean T2). Values for T1 and T2, with their respective proportions A1 and A2 are listed in Table 3. The simple channel-block model predicts that the mean burst-length should increase with concentration. In order to examine this prediction the corrected mean burst lengths were determined at different levamisole concentrations. For example at − 75 mV with 2–10 μM levamisole the corrected mean burst length was 2.50 ± 0.28 ms (n = 16); with 30 μM levamisole it was 1.14 ± 0.32 ms (n = 6); and with 90 μM levamisole it was 1.40 ± 0.21 (n = 9). Thus there was no significant increase in the corrected mean burst duration. Similar results were obtained at − 100 mV. It was concluded that the mean burst durations did not increase with increasing levamisole concentration, a result not predicted by the simple channel-block model.

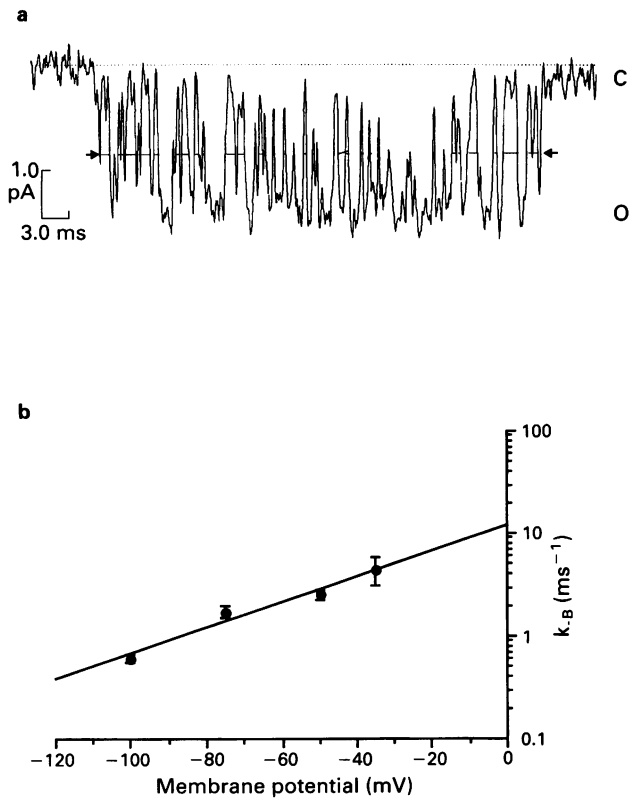

Figure 5 (a) Illustration of channel activity with 30 μM levamisole; isolated inside-out patch, − 75 mV. Fast flickering channel-block can be clearly observed. C: denotes the closed state. O: denotes the open state. The mean blocked-times were calculated from the measurements of blocked durations (indicated by the line between the arrows). In this experiment the mean blocked-time was 0.43 ms. (b) Semi-log plot of K_{-B} against membrane potential, all values are mean ± s.e.. The voltage sensitivity of K_{-B} was described by the equation: $K_{-b} = K_{-BO}.\exp^{(E/Vk)}$. Where K_{-B} is the unblocking rate constant at the membrane potential E, K_{-BO} is the value for K_{-B} at O m V and Vk is a constant. The value for K_{-BO} was 11.99 ms^{-1} and Vk was 35.02 mV.

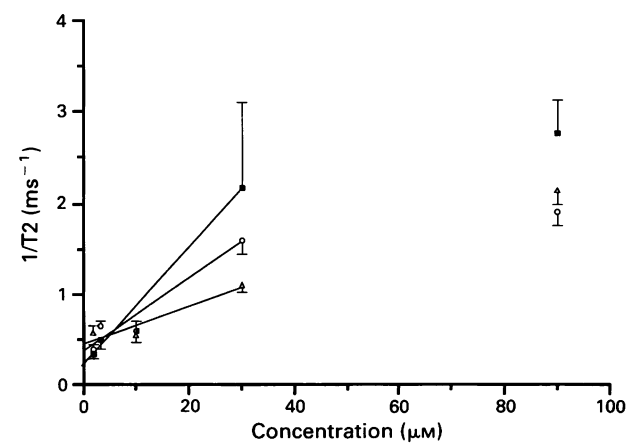

Figure 6 Reciprocal plot of open-time constant T2 against concentration, at − 50 mV (Δ), − 75 mV (O) and − 100 mV (■). The intercept represents the closing rate constant (α_2) and the slope represents the forward blocking rate (K_{+B}). Each point is the mean ± s.e. (vertical bars). Lines were fitted to 2–30 μM by least square regression without including the results at 90 μM. Values for K_{+B} were determined as − 50 mV, 2.06×10^7 M^{-1} s^{-1}; − 75 mV, 3.82×10^7 M^{-1} s^{-1}; and − 100 mV, 6.58×10^7 M^{-1} s^{-1}. The closing rate constant α_2 was also determined: − 50 mV, 447s^{-1}; − 75 mV, 396 s^{-1}; and − 100 mV, 143 s^{-1} from the intercept on the ordinate scale.

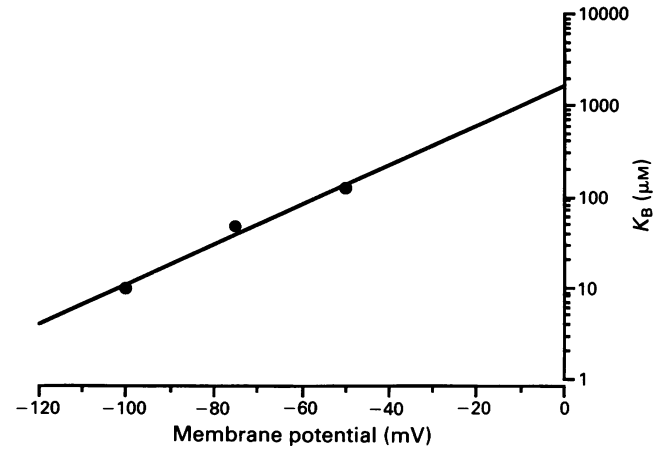

Figure 7 Semi-log plot of K_B versus membrane potential. The voltage-sensitivity of K_B was described by the equation: $K_B = K_{BO}.\exp^{(E/Vk)}$. Where K_B is the dissociation rate constant at the membrane potential E, K_{BO} is the value for K_B at 0 mV and Vk is a constant. The value for K_{BO} was 1780 μM and Vk was 19.4 mV.

Desensitization

At higher concentrations of levamisole (30 μM and 90 μM) long closed-times were seen in some patches between clusters of channel opening (Figure 8). These closed-times lasted many seconds and were observed at both depolarized and hyperpolarized potentials. The details of the closed-time components observed at higher concentrations are summarized in Table 2. These very long closed periods appear to correspond to a slow desensitization process (Cachelin & Colquhoun, 1989) and are similar to the very long closed-times observed with high concentrations of acetycholine in *Ascaris* (Pennington & Martin, 1990). In addition to the long closed durations, shorter closed times (between 10 and 50 ms) were detected at high levamisole concentrations. These might also be part of a desensitized state as they occur at both depolarized and hyperpolarized potentials but only with high concentrations.

Discussion

The simple block model

The simple channel-block model was used in this paper in order to describe quantitatively some of the effects observed

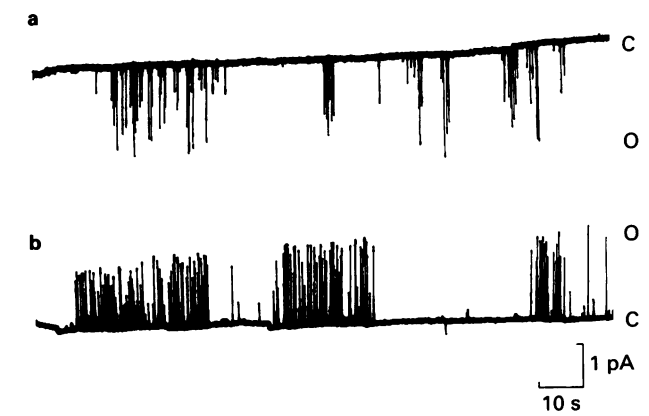

Figure 8 Channel current showing long (seconds) closed-times between clusters of openings. C: denotes closed state, O: denotes open state; 90 μM levamisole, cell attached patches. (a) − 75 mV; (b) + 75 mV. The long closed periods were interpreted as desensitization.

with levamisole. The predictions of this model, for a charged blocker, include the expectations that: the corrected mean open-times decrease with increasing levamisole concentration; burst durations increase with increasing drug concentration; the forward blocking rate constant (k_{+B}), the unblocking rate (k_{-B}), and the dissociation constant K_B all vary with membrane potential (k_{+B} and k_{-B} should have equal but opposite voltage-dependences); and the mean block duration and therefore k_{-B} are independent of drug concentration. The data presented in this report are consistent with the above predictions except that burst durations did not increase with concentration. This implies that the model used is a useful approximation but not a completely sufficient model.

We did not observe the predicted increase in the average duration of bursts, although there were frequent examples of long flickering bursts (see Figure 5). One possible explanation for the absence of increased mean burst durations could be that the channel might enter a closed state directly from the blocked state without reopening, i.e. the channel closes trapping levamisole or the blocked channel enters into a desensitised state. Limitations of the simple channel-block model are discussed further by Neher (1983).

One further difficulty in using the simple channel-block model is the presence of two open-states (O1 and O2). In our analysis it seems probable at 90 μM that the brief open-state (O1) fell below the limit of resolution of the recording set up. Thus the values for k_{-B} would be appropriate for the open-state O2 at 90 μM. Since the values for k_{-B} were not significantly different from the values for k_{-B} at 30 μM where the open-state O1 could still be resolved, it implies that the presence of O1 did not affect our estimation of k_{-B} for the open-state O2. Since the values for k_{+B} were determined from the values of the open-state O2 (see methods), our determination of K_B is likely to be an estimate of the dissociation constant for the block of the open-state O2.

Comparison with acetylcholine-activated channels in Ascaris

At the single-channel level both acetylcholine (Pennington & Martin, 1990) and levamisole activate channels in the same concentration range: acetylcholine 1–100 μM; levamisole 1–90 μM. The *I/V* relationship with both levamisole and acetylcholine are linear, both agonists showed evidence of desensitization with long closed periods appearing at higher concentrations.

Although acetylcholine and levamisole appear to activate the same channels there are some differences in the behaviour of the single-channel currents. Acetylcholine activates channels with two distinguishable conductance levels (25–35 pS and 40–50 pS) (Pennington & Martin, 1990); in contrast single-channel currents activated by levamisole had a range of detectable conductance levels between 19–46 pS with a mean of 32.9 ± 1.23 pS ($n = 26$).

The open-, closed-, and burst-time durations for levamisole or acetylcholine activated channels in this preparation show that both compounds produced two open-states and two burst-states and at least three closed-states. However the mean duration of the apparent open-times and burst-times for levamisole were shorter than for acetylcholine. The mean duration of apparent brief and long openings produced by acetylcholine (1–10 μM) was 1.18 ms and 4.89 ms, which compares with 0.80 ms and 2.30 ms for 2–10 μM levamisole under similar conditions. The mean brief and long burst durations for 1–10 μM acetylcholine were 1.17 ms and 7.62 ms compared to 1.24 ms and 3.70 ms for 2–10 μM levamisole.

A further difference between the effects of acetylcholine and levamisole is related to channel-block. Pennington & Martin (1990) did not find significant evidence for channel-block with acetylcholine on this preparation, although they did observe a reduction in the mean open-time at higher concentrations (25 μM) which might be interpreted as signs of block. Our results clearly show that levamisole blocks the channel at hyperpolarized potentials.

Comparison with previous voltage-clamp experiments in Ascaris

Harrow & Gration (1985) examined the effects, in *Ascaris*, of both acetylcholine and levamisole using two-microelectrode current-clamp and voltage-clamp. They found that the *I/V* relationship obtained from two-microelectrode voltage-clamp experiments obtained by applying levamisole by microperfusion or iontophoresis to *Ascaris* muscle had both linear (0 to −30 mV) and non-linear sections (> −30 mV). They suggested that the non linearity was due to a voltage-sensitive channel-block and/or desensitization. In our experiments we observed some desensitization at higher concentrations of levamisole, but this appeared at both depolarized and hyperpolarized membrane potentials, not just at membrane potentials greater than −30 mV. Channel-block was clearly observed in our experiments, the degree of block increased on membrane hyperpolarization. This is consistent with the voltage-sensitivity of the non linearity observed by Harrow & Gration (1985) and suggests that their observation reflects channel-block but not desensitization.

Harrow & Gration (1985) have shown that levamisole is an agonist at all concentrations (1 μM–10 mM) producing a sigmoid dose-response curve obtained under current-clamp conditions with a membrane potential of −30 mV. Our results clearly show that channel-block occurs at −35 mV and at this potential K_B was calculated at 285 μM. It is interesting to note that Harrow & Gration (1985) did not observe a reduction in the maximum conductance response with high concentrations (10 mM). This observation of Harrow & Gration (1985) is consistent with our observations at hyperpolarized potentials which showed that P_{open} failed to decrease at higher concentrations, despite the presence of channel block.

Possible therapeutic significance

Receptor desensitization produced by levamisole may have significant effects therapeutically; some *Ascaris* parasites continuously treated with higher doses of levamisole recover from the levamisole-induced paralysis (Aceves *et al.*, 1970; Coles *et al.*, 1974; 1975). This recovery may be due to desensitization since like desensitization, recovery occurs only at higher doses. Consequently it is suggested that lower doses which do not produce desensitization, potentially have a greater therapeutic effect. Also the fact that P_{open} in these experiments did not increase at hyperpolarized potentials when 2–90 μM levamisole was used suggests that high concentrations would not be therapeutically advantageous.

Therefore in conclusion, these experiments confirm that levamisole acts at the acetylcholine receptor on *Ascaris suum* muscle, both opening and blocking the channel.

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