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The anthelmintic pyrantel acts as a low efficacious agonist and an open-channel blocker of mammalian acetylcholine receptors

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Abstract

Pyrantel is an anthelmintic which acts as an agonist of nicotinic receptors (AChRs) of nematodes and exerts its therapeutic effects by depolarizing their muscle membranes. Here we explore at the single-channel level the action of pyrantel at mammalian muscle AChR. AChR currents are elicited by pyrantel. However, openings do not appear in clearly identifiable clusters over a range of pyrantel concentrations (1–300 μM). The mean open time decreases as a function of concentration, indicating an additional open-channel block. Single-channel recordings in the presence of high ACh concentrations and pyrantel demonstrate that the anthelmintic acts as a high-affinity open-channel blocker. When analyzed in terms of a sequential blocking scheme, the calculated forward rate constant for the blocking process is 8×10^7 M^{-1} s^{-1}, the apparent dissociation constant is 8 μM at a membrane potential of −70 mV and the process is voltage dependent. Pyrantel displaces α-bungarotoxin binding but the concentration dependence of equilibrium binding is shifted towards higher concentrations with respect to that of ACh binding. Thus, by acting at the binding site pyrantel activates mammalian AChRs with low efficacy, and by sterical blockade of the pore, the activated channels are then rapidly inhibited.

Keywords: Acetylcholine; Pyrantel; Nicotinic receptor; Patch clamp

1. Introduction

At the neuromuscular junction, acetylcholine (ACh) mediates fast neurotransmission by activating nicotinic receptors (AChR). The essential function of the AChR is to transduce the binding of nerve-released acetylcholine (ACh) into a depolarization of the muscle membrane. Three types of ligands interact with the AChR: agonists, competitive antagonists, and non-competitive inhibitors (NCIs). NCIs comprise a wide range of compounds with different chemical structures, mechanisms of action and sites of interaction. Two main types of NCIs have been proposed on the basis of their mechanisms of action: open-channel blockers, which sterically occlude the ion channel, and allosteric blockers, which decrease the open channel probability. The relationship between chemical scaffolds of NCIs and the mechanisms by which they exert their inhibitory action has been particularly difficult to elucidate. A given NCI may act at different sites or by different mechanisms depending on its concentration or AChR subtype (Benoit and Changeux, 1993; Spitzmaul et al., 1999). In addition, agonists have been shown to act as NCIs at high concentrations (Sine and Steinbach, 1984; Prince and Sine, 1998).

AChRs in nematode muscles are targets for anthelmintic chemotherapy. Pyrantel is an anthelmintic which acts by disrupting neuromuscular transmission in nematodes. Its efficacy is based on its ability to act as a full agonist of AChRs in these parasites (see review in Martin et al., 1997). Nematode muscle AChRs are structurally different from mammalian muscle AChRs. Several α and non-α subunits have been identified in the nematode *Caenorhabditis elegans* (Fleming et al., 1997; Martin et al., 1997). By contractility and membrane potential measurements it has been shown that the nematode axial muscle is 10–100 times more sensitive than the rat muscle to the acute action of pyrantel and levamisole (Atchison et al., 1992).

To gain new insights into structure–function relation-

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ships of the AChR we explore in detail at the single-channel level the activation of mammalian muscle AChR by pyrantel. The comparison of kinetics of activation of AChRs by pyrantel in mammals and helminths will pinpoint determinants of function. In addition, evaluation of the kinetics of activation of mammalian AChRs by anthelmintics will contribute to the development of more selective therapies.

We determine that pyrantel acts as a low efficacious agonist and a high-affinity open-channel blocker of the AChR. In contrast to what has been reported about the nematode AChR (Robertson et al., 1994), kinetics of activation and blockade of mammalian AChRs by pyrantel and by the endogenous neurotransmitter ACh are strikingly different.

2. Methods

2.1. Expression of AChR

Mouse cDNAs were subcloned into the cytomegalovirus based expression vector pRBG4 (Bouzat et al., 1994). HEK293 cells were transfected with α, β, δ, and ε cDNAs using calcium phosphate precipitation at a subunit ratio of 2:1:1:1 for α:β:δ:ε, respectively, essentially as described previously (Bouzat et al. 1994, 1998). For transfections, cells at 40–50% confluence were incubated for 8–12 h at 37°C with the calcium phosphate precipitate containing the cDNAs in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum. Cells were used for patch clamp recordings and binding assays 48 h after transfection.

2.2. Patch-clamp recordings and analysis

Recordings were obtained in the cell-attached configuration (Hamill et al., 1981) at 20°C and at a membrane potential of −70 mV (Bouzat et al., 1994). For studying the voltage dependence of the blocking process, channels were also recorded at −40, −90 mV and 70 mV. Patch pipettes were pulled from 7052 capillary tubes (Garner Glass, Claremont, CA) and coated with Sylgard (Dow Corning, Midland, MI). The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). Acetylcholine (ACh) at final concentrations of 1 μM and 300 μM, pyrantel (SIGMA, MO) at final concentrations ranging from 1 to 300 μM or both drugs were added to the pipette solution.

Single channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, Inc., CA), digitized at 5 μs intervals with the PCI-611E interface (National Instruments, Austin, TX), recorded to the hard disk of a computer using the program Acquire (Bruxton Corporation, Seattle, WA), and detected by the half-amplitude threshold criterion using the program TAC 4.0.10 (Bruxton Corporation, Seattle, WA) at a final bandwidth of 10 kHz. Open and closed time histograms were plotted using a logarithmic abscissa and a square root ordinate and fitted to the sum of exponentials by maximum likelihood using the program TACFit (Bruxton Corporation, Seattle, WA). For each patch, the number of opening events analyzed ranged from 1000 to 5000.

Clusters of openings corresponding to a single channel were identified as a series of closely spaced events preceded and followed by closed intervals greater than a specified duration (τ_{crit}); this duration was taken as the point of intersection of the predominant closed time component and the succeeding one in the closed time histogram. Typically, τ_{crit} was about 1 ms for AChRs activated by 300 μM ACh and 10 ms for AChRs activated by 300 μM ACh and 50 μM pyrantel. To minimize errors in assigning cluster boundaries we analyzed only recordings from patches with low channel activity. In addition, any clusters showing double openings were rejected.

2.3. Ligand binding measurements

Binding of pyrantel and ACh was measured by competition against the initial rate of [^{125}I]α-Bungarotoxin ([^{125}I]α-BTX) binding as described previously (Sine and Taylor, 1979; Sine et al., 1994). Cells were resuspended in high potassium Ringer's solution and divided into aliquots for ligand binding measurements. Cells were first incubated for 30 min with different concentrations of pyrantel or ACh; [^{125}I]α-BTX was subsequently added to a final concentration of 5 nM, and the cells were incubated for an additional 20 min. The total number of binding sites was determined by incubating cells with 5 nM [^{125}I]α-BTX for 2 h. Binding was finished by the addition of potassium Ringer's solution containing 20 mM carbamylcholine. Fractional occupancy by pyrantel or ACh was fitted by the Hill equation :

$$1-\text{fractional occupancy}=[1/(1+([x]/K_d)^{nH}]$$

where x is the concentration of pyrantel or ACh, K_d is the apparent dissociation constant, and n_H is the Hill coefficient.

Data are expressed as mean±SD of 3–8 different experiments for each condition. Statistical comparisons are done using the Student's t-test. A level of $p<0.05$ is considered significant.

3. Results

3.1. Single-channel characterization of mammalian AChRs activated by pyrantel

When activated by the endogenous neurotransmitter ACh, HEK cells transfected with α, β, δ and ε cDNAs

exhibit channel openings typical of adult AChRs (Bouzat et al., 1994 and Fig. 1). Open time histograms show a main open component of 860±80 μs (Fig. 1 and Bouzat et al. 1994, 1998). AChRs activated by pyrantel show openings briefer than those activated by ACh (Fig. 1). Open time distributions of 1 μM pyrantel-activated AChRs can be well fitted by a main component of 400±90 μs (relative area >0.6) and a minor component of 80±20 μs. The decrease in the mean open time is statistically significant ($p<0.005$).

We studied the AChR gating kinetics as a function of pyrantel concentration. As shown in Fig. 1, increasing pyrantel concentration displaces the open time distributions to briefer durations. At 300 μM pyrantel, open time histograms can be well fitted by a single exponential with a mean time of 58±10 μs (n=4). Such concentration-dependent decrease in the mean open time is indicative of an additional open-channel blockade. We therefore used the classical linear kinetic scheme to describe the inhibitory action of the drug acting as an open-channel blocker (Scheme 1):

$$C \underset{\alpha}{\overset{\beta}{\longleftrightarrow}} O \underset{k_{-b}}{\overset{k_{+b}[B]}{\longleftrightarrow}} OB \qquad \text{Scheme (1)}$$

where C is closed, O open and OB blocked states. In agreement with Scheme 1, a linear relationship between the reciprocal of the mean open time and pyrantel concentration ([B]) is observed (Fig. 2). The calculated value for the forward rate constant of the blocking reaction (k_{+b} in Scheme 1), given by the slope of the curve, is 5×10^7 M^{-1} s^{-1}. The apparent closing rate constant of pyrantel-activated AChRs determined by the value at the ordinate intercept is 4050 s^{-1}.

At ACh concentrations higher than 10 μM, wild-type AChRs open in clusters of well-defined activation episodes (Bouzat et al., 2000). Each activation episode begins with the transition of a single receptor from the desensitized to the activatable state and terminates by returning to the desensitized state. In contrast, when

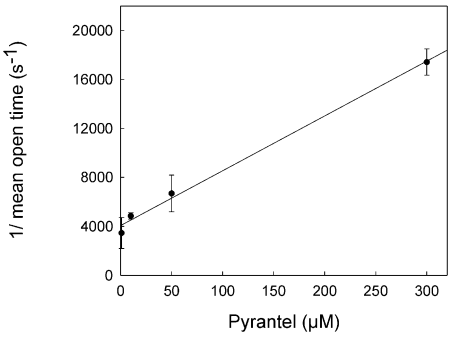

Fig. 2. Relationship between the mean open time and pyrantel concentration. The mean open times were obtained from the corresponding open time histograms. The data are fit by the equation (1/mean open time)=$\alpha+k_{+b}\times$[pyrantel], where k_{+b} is the association rate for pyrantel at the channel block and α is the apparent channel closing rate. Data are shown as mean±SD of 3–5 patches.

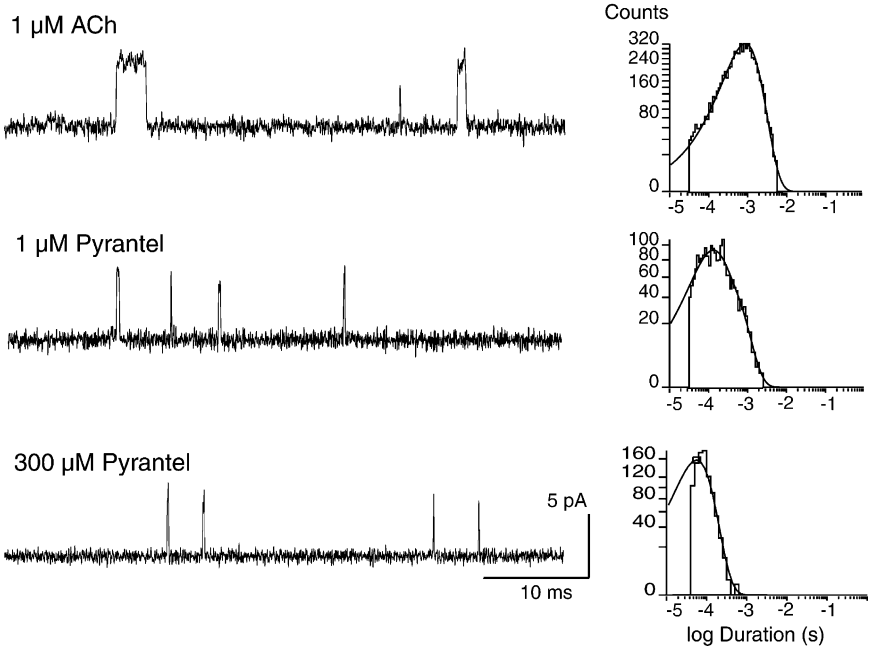

Fig. 1. Adult mammalian muscle AChRs activated by pyrantel. Channels activated by 1 μM ACh, 1 and 300 μM pyrantel were recorded from HEK cells expressing $\alpha_2\beta\varepsilon\delta$ AChRs. Left: Traces of currents are displaced at a bandwidth of 9 kHz with channel openings as upward deflections. Right: Open time histograms corresponding to each condition. Membrane potential: −70 mV.

AChRs are activated by pyrantel, even at concentrations as high as 300 μM, clusters are not observed (Fig. 1). In agreement, closed time histograms do not show a main component clearly sensitive to pyrantel concentration (not shown).

3.2. Effects of pyrantel on ACh-activated AChR channels

We next studied the combined action of pyrantel and ACh at mammalian AChRs. Increasing concentration of pyrantel systematically displaces to briefer duration the open time distributions of AChR channels activated by 1 μM ACh. Mean open time decreases from 860±80 μs to 590±30, 360±20, 210±30, 180±22 and 100±2 μs at 1, 10, 30, 50 and 100 μM pyrantel, respectively. For all conditions such decreases are statistically significant ($p<0.01$). At all pyrantel concentrations, the open time distributions are well described by a single exponential. Thus, the main modification of pyrantel is a dose-dependent reduction in the mean open time, such reduction being about 10-fold when the concentration of pyrantel is 100 μM. Channel activity is observed as isolated events at every pyrantel concentration tested.

Again, we used Scheme 1 to describe the inhibitory action of the drug acting as an open-channel blocker. A linear relationship between the reciprocal of the mean open time of AChRs activated by 1 μM ACh and pyrantel concentration is observed (not shown). The calculated value for the forward rate constant of the blocking reaction (k_{+b} in Scheme 1) is 8×10^7 M^{-1} s^{-1} and the value of the ordinate intercept is 1600 s^{-1}. This value corresponds to the apparent closing rate of ACh-activated channels in the absence of pyrantel.

On the basis of Scheme 1, if pyrantel dissociates quite rapidly, openings would be expected to occur in bursts as the channel blocks and unblocks several times before entering a closed period. However, no flickering is observed probably because blocked periods are relatively long and closings due to channel closing and channel blockade become indistinguishable at low ACh concentrations. In order to identify blocked periods, we studied the action of pyrantel in the presence of a high ACh concentration. At 300 μM ACh, clusters of events corresponding to a single channel can be clearly identified (Fig. 3). A cluster starts when one AChR recovers from desensitization and continues with the receptor undergoing cycles of agonist association/dissociation and channel gating.

At this high concentration sojourns in open and closed states within clusters correspond mostly to the closing and reopening of the biliganded AChR ($A_2R\leftrightarrow A_2R^*$). Pyrantel greatly alters the cluster properties which show both briefer openings and prolonged intracluster closings than control ones (Fig. 3). Under these conditions, open time histograms reveal a homogeneous population of AChRs. Clusters are also homogeneous, thus discarding the presence of kinetically different channel populations. Typically, cluster duration histograms of control recordings obtained at 300 μM ACh show a main component of 33±5 ms whose relative area is bigger than 0.9 (n=4) and the number of openings per cluster is 43±9. In the presence of 50 μM pyrantel the mean cluster duration is 48±3 ms and the openings per cluster, 33±3.

As shown in Fig. 3, recordings of AChRs activated by 50 μM pyrantel alone are greatly different from those obtained in the presence of 300 μM ACh plus 50 μM pyrantel and clusters cannot be clearly identified.

In the absence of pyrantel, closed time histograms corresponding to 300 μM ACh-activated channels can be well fitted by three or four components (Fig. 3 and Bouzat et al., 2000). Typically, there is a main fast component whose duration is briefer than 150 μs, which corresponds to more than 90% of all closings and is sensitive to ACh concentration, and small variable slow components associated with periods between independent activation episodes. The presence of pyrantel significantly changes the closed time distributions of ACh-activated channels and a new closed component is systematically observed (Fig. 3). At a membrane potential of −70 mV and in the presence of 50 μM pyrantel, the mean duration of this component is 1.50±0.12 ms and its relative area, 0.60±0.14 (n=4). This closed component corresponds mostly to the blockade of ACh-activated channels by pyrantel. On the basis of Scheme 1, the blocked time equals $1/k_{-b}$. Thus, the apparent dissociation constant for the blocking process ($K_d=k_{-b}/k_{+b}$) is 8 μM at −70 mV.

To study the voltage dependence of the unblocking process, we measured the mean blocked time of AChRs recorded at different membrane potentials in the presence of 300 μM ACh and pyrantel. As shown in Fig. 4(a), the duration of the blocked periods at 50 μM pyrantel increases with higher negative membrane potentials and the blocked time changes e-fold per 80 mV, indicating that the unblocking process is voltage dependent. The mean blocked time is not affected by pyrantel concentration (Fig. 4(b)). However, the relative area of the closed component associated with blocked periods shows an important increase when pyrantel increases from 10 to 50 μM (Fig. 4(c)). Increasing ACh concentration from 300 μM to 600 μM does not introduce significative changes in pyrantel blockade: In the presence of 50 μM pyrantel, the mean blocked time (1.28±0.18 ms) and the relative area of the corresponding closed component (0.53±0.07) remain constant (non significant changes, Student's t-Test, n=5).

3.3. Measurements of equilibrium binding of pyrantel

We studied the ability of pyrantel to interact with acetylcholine binding site by measuring the inhibition of α-

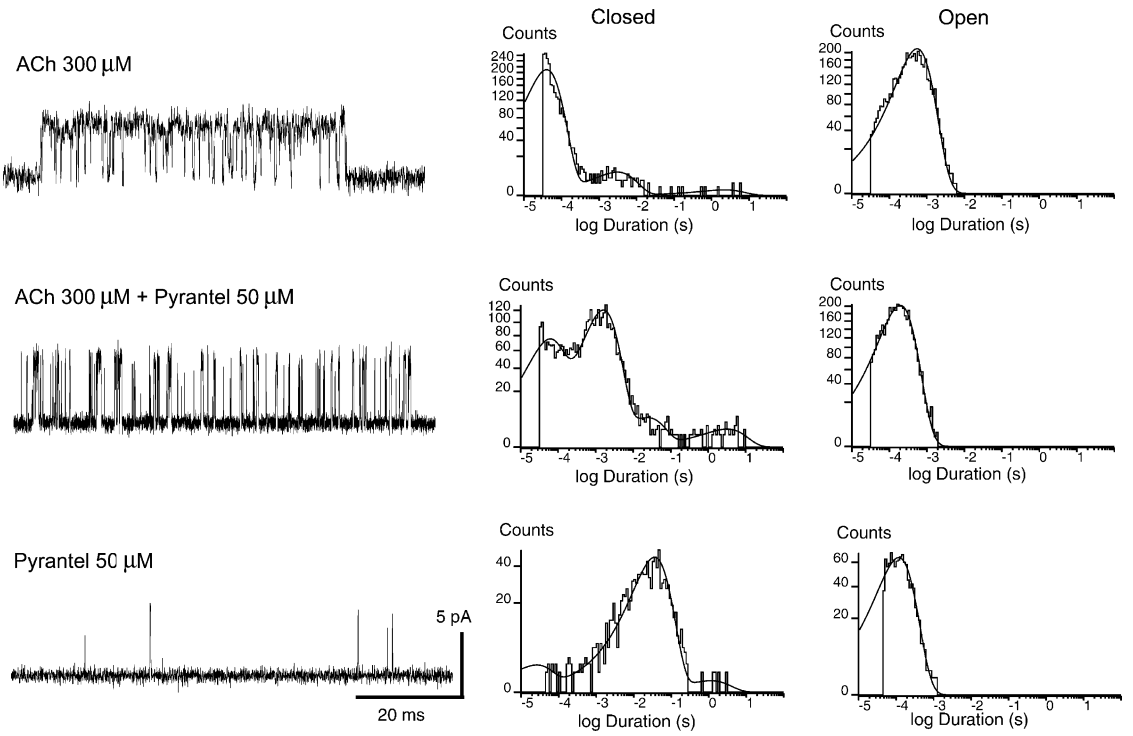

Fig. 3. Combined action of ACh and pyrantel on muscle AChRs. Left: Traces of currents recorded in the presence of 300 μM ACh, 300 μM ACh plus 50 μM pyrantel and 50 μM pyrantel alone. Currents are displaced at a bandwidth of 9 kHz with channel openings as upward deflections. Membrane potential: −70 mV. Right: Closed and open time histograms of the corresponding recordings.

BTX binding. The concentration dependence of equilibrium of pyrantel binding is shifted towards higher concentrations with respect to that of ACh binding (Fig. 5). The apparent affinity constant (K_d) is 10-fold higher for pyrantel than for ACh (1.8 μM and 17 μM for ACh and pyrantel, respectively). Cooperation is maintained in the presence of pyrantel with a Hill coefficient similar to that determined for ACh (n_H=1.2).

4. Discussion

This study uses single-channel recordings to describe at the molecular level the action of the anthelmintic pyrantel on mammalian AChR. The basic observations indicate that pyrantel drives an activation process plus open-channel block at a similar concentration range.

Single-channel openings of AChRs activated by ACh are clustered into activation periods with the receptor undergoing cycles of agonist association/dissociation and channel gating. Experimental evidence showing that pyrantel-activated AChRs do not cluster over a range of concentrations suggests that pyrantel's efficacy as an agonist is very low. In contrast, the efficacy of pyrantel is high at AChRs from *Ascaris suum* muscle (Robertson et al., 1994). In addition, the open durations of mammalian AChRs activated by pyrantel are briefer than those of AChRs activated by ACh. Accordingly, the apparent closing rate of pyrantel-activated channels determined as shown in Fig. 2 is faster than that of ACh-activated channels. In contrast, AChRs from *Ascaris suum* muscle show mean open times that are similar when activated either by ACh or by pyrantel (Robertson et al., 1994).

The low efficacious activation observed in mammalian AChRs is in agreement with measurements of muscle contractility and membrane potential which showed that neuromuscular transmission is much more affected by pyrantel in *Haemonchus contortus* than in the rat (Atchison et al., 1992).

The mean open time of pyrantel-activated channels decreases as a function of agonist concentration, indicating an additional open-channel block. The value for the blocking rate constant calculated on the basis of the linear blocking scheme is similar to those reported for classical open-channel blockers, such as QX-222 and ephedrine (Neher and Steinbach, 1978; Bouzat, 1996). Comparison of the forward blocking rate determined in the absence or presence of ACh indicates that AChRs activated either by pyrantel or by ACh are blocked by pyrantel at a similar rate. Pyrantel also acts as an open-channel blocker of *Ascaris suum* AChRs (Robertson et al., 1994). However, in contrast to what we observe in mammalian AChRs, flickering was evident when helmintic AChRs were exposed to 100 μM pyrantel (Robertson et al., 1994).

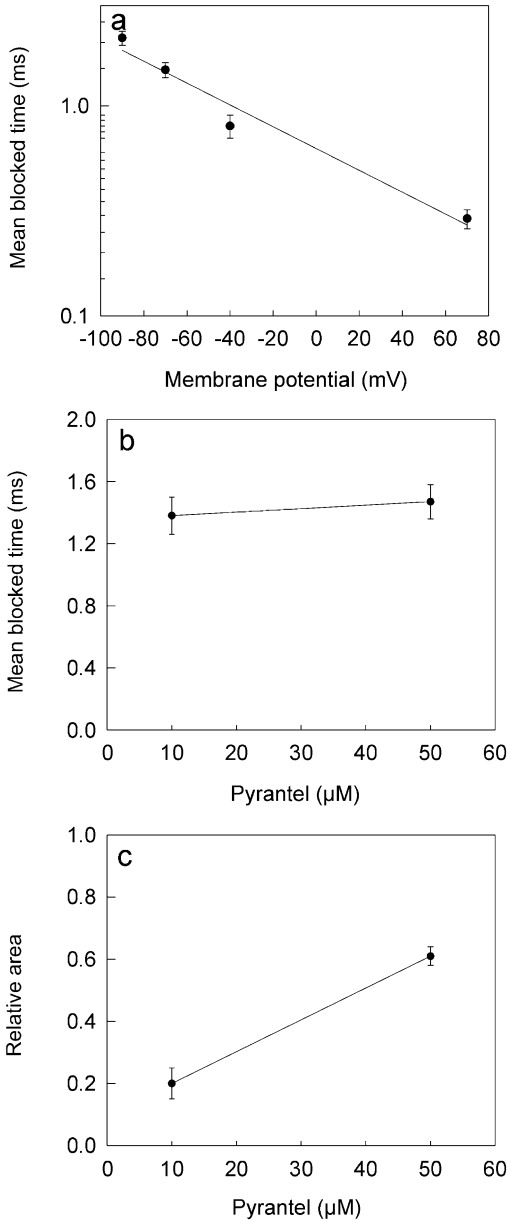

Fig. 4. Characterization of pyrantel block. AChR currents were recorded in the presence of 300 μM ACh plus pyrantel. The mean blocked times were obtained from the corresponding closed time histograms. Data are shown as mean±SD of 3–5 different recordings for each condition. (a) Relationship between mean blocked time and membrane potential. Pyrantel concentration: 50 μM. (b) Relationship between mean blocked time and pyrantel concentration. (c) Relationship between the area of the closed component corresponding to blocking events and pyrantel concentration.

Because flickering was not observed, we were not able to identify closings due to blockade in the absence of ACh or at low ACh concentrations. Therefore, we analyzed its action on AChRs activated by 300 μM ACh. At this high concentration, single-channel activity takes place in easily recognizable clusters which represent activity from a single ion channel. Because ACh blockade is fast (Bouzat et al., 2000), dwellings in the blocked

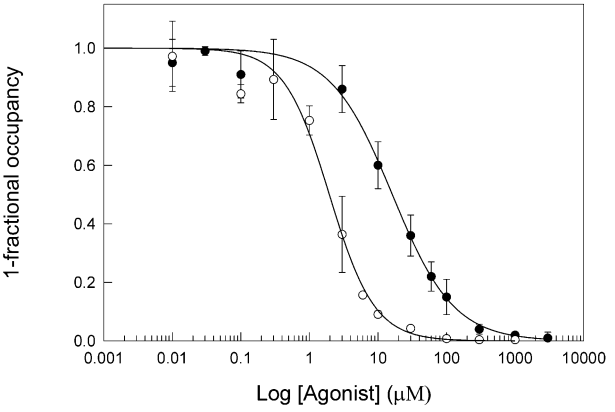

Fig. 5. Equilibrium binding of pyrantel and ACh. Pyrantel and ACh binding was measured by competition against the initial rate of α-bungarotoxin binding. The curves are fits to the Hill equation. Each point represents the mean±SD of 3 different experiments.

state resulting from ACh block should be easily distinguished from those associated with pyrantel block. Given that dwell times within clusters at 300 μM ACh correspond mostly to sojourns in the closed and open states of the biliganded AChR, we hypothesize that the new closed component observed in the presence of pyrantel could correspond to residence in the blocked state. Hence recordings at high ACh concentrations allowed us to measure the mean blocked time and, in turn, the apparent dissociation constant. Thus, this is the first experimental evidence showing that pyrantel is a very potent agonist channel blocker of mammalian AChRs, being the block dissociation constant lower than 10 μM. Block dissociation constants reported for other agonists of mammalian AChRs are in the order of mM for acetylcholine (Sine and Steinbach, 1984; Bouzat et al., 2000), 100 μM for epibatidine (Prince and Sine, 1998) and 9–20 μM for decamethonium (Marshall et al., 1991). Hallmarks of open-channel block are: (i) a concentration dependent decrease in mean open time; (ii) a concentration dependent increase in the fractional area of the block component; and (iii) constant mean duration of the blocked intervals across all blocker concentrations. These statements are confirmed in the presence of pyrantel, indicating thus that pyrantel acts as an open-channel blocker of mammalian AChR.

An alternative explanation for the new intracluster closed component could be that pyrantel is displacing ACh from its binding site thus resulting in a lower effective concentration of ACh. Several lines of evidence allowed us to discard this hypothesis: (i) the voltage-dependence behaviour of the closed component associated with blocking events is compatible with an open-channel block mechanism (see Fig. 4). At positive membrane potentials the duration of this closed component is significatively reduced (~200 μs at +70 mV); and (ii) clusters are kinetically homogeneous and cluster duration histograms show an increase in the area of the

component corresponding to isolated events. The mean duration of this channel population agrees with that of AChR activated by pyrantel alone. Therefore we can infer that under these conditions ACh governs mostly the activation process and pyrantel, the blocking process.

In the absence of ACh, the ~1.5 ms closed component associated with blocked periods is not detected. This could be explained by a low probability of channel opening and a channel block mechanism deviating from Scheme 1. Thus, channels activated by pyrantel alone might close after being blocked by pyrantel. Induction of a closed channel block has been reported for other open-channel blockers such as ephedrine (Milone and Engel, 1996; Bouzat, 1996), MK-801 (Amador and Dani, 1991). Accordingly, the number of openings per cluster of AChRs activated by 300 μM ACh decreases in the presence of pyrantel, suggesting that the anthelmintic is not acting exclusively as an open-channel blocker. An alternative explanation could be that pyrantel blocks closed AChR channels. From our experiments it is not possible to differentiate between both mechanisms.

Pyrantel could open mammalian AChRs either by acting at the agonist binding site or by affecting the equilibrium between the closed and open conformations of unliganded AChRs. Because it is able to displace α-BTX from its binding site, our results show that it acts as an agonist of the mammalian AChR, with an apparent affinity 10-fold lower than that of ACh. The K_d thus determined reflects a weighted average of the affinity of the agonist for the two forms of the receptor, low and high affinity, as well as the fraction of receptor in each state (Sine and Taylor, 1979). Although the K_d for carbamylcholine is about 20 μM (Bouzat et al., 1998), AChRs open in clusters of well-defined activation episodes when activated by carbamylcholine at concentrations higher than 10 μM (Zhang et al., 1995). Given that the K_d calculated for pyrantel is similar to that of carbamylcholine, the lack of clustering could not be attributed only to a decreased affinity. Thus it is possible to infer that pyrantel is much less effective than ACh and carbamylcholine to open the channel. In agreement, comparison of kinetics between ACh- and carbamylcholine-activated channels demonstrated that the opening rates are strongly dependent on the type of agonist (Zhang et al., 1995). As it is not possible to identify clusters when AChRs are activated by pyrantel, kinetic analysis cannot be performed in order to determine accurately the rate constants for pyrantel binding and channel gating steps (Zhang et al., 1995; Bouzat et al., 2000).

The low opening rate together with a faster closing rate and high-affinity blockade lead to the overall consequence of a low probability of opening of mammalian AChR by pyrantel. This finding could explain why mammalian muscles show lower sensitivity to pyrantel. Interestingly, it has been suggested that the agonist activity, and not the blocking activity, is essential for the therapeutic effect of this type of anthelmintics (Dale and Martin, 1995). Pyrantel is employed as the pamoate salt and it is poorly absorbed from the gastrointestinal tract. Considering 10% of absorption of a 100 mg/kg dose, a peak plasma concentration around 200 μM would be achieved. Assuming complete dilution in plasma, this concentration would be high enough to alter the host mammalian AChRs, although loss before delivery to muscle should be taken into account. Accordingly, when given parenterally, pyrantel can produce complete neuromuscular blockade; if given orally, toxic effects are produced only by very large doses (Tracy and Webster, 1996). Under the light of the present results it is possible to infer that the toxic effects are mainly due to pyrantel blocking activity.

In summary, we show that pyrantel acts as a weak agonist of mammalian AChRs because of reduced channel opening coupled with high-affinity open channel block. In contrast to what is observed in the nematode, kinetics of pyrantel-activated mammalian AChRs greatly differ from those of AChRs activated by the natural neurotransmitter ACh.

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