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Paraherquamide and 2-Deoxy-paraherquamide Distinguish Cholinergic Receptor Subtypes in *Ascaris* Muscle

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ABSTRACT

Paraherquamide is a novel natural anthelmintic product with a mode of action that is incompletely characterized. Nicotine and cholinergic-anthelmintic agonists of different chemical classes were used to produce contraction in *Ascaris* muscle strips. Paraherquamide and a semisynthetic derivative, 2-deoxy-paraherquamide, antagonized these responses. Analysis of the actions of the antagonists was made using the simple competitive model and nonlinear regression to estimate the pK_B values of the antagonists. The analysis was tested using Clark plots. The pK_B values for paraherquamide were: nicotine, 5.86 ± 0.14; levamisole, 6.61 ± 0.19; pyrantel, 6.50 ± 0.11; and bephenium, 6.75 ± 0.15. The pK_B of nicotine was significantly different from the pK_B values for levamisole, pyrantel, and bephenium, showing that paraherquamide can distinguish a subtype of cholinergic receptors sensitive to nicotine and a subtype of cholinergic receptors sensitive to levamisole, pyrantel, and bephenium. The pK_B values for 2-deoxy-paraherquamide were: levamisole, 5.31 ± 0.13; pyrantel, 5.63 ± 0.10; and bephenium, 6.07 ± 0.13. The Clark plots of the antagonism illustrated the degree of fit to the competitive model for 2-deoxy-paraherquamide. 2-Deoxy-paraherquamide selectively antagonized the effects of bephenium; the pK_B values of levamisole and pyrantel were significantly different from the pK_B of bephenium. Paraherquamide and 2-deoxy-paraherquamide are selective competitive cholinergic antagonists that distinguish subtypes of cholinergic receptor in *Ascaris* muscle corresponding to nicotine-, levamisole-, and bephenium-sensitive receptors.

Nematode parasite infections of humans and animals cause disease with loss of productivity, debility, and occasionally death. Ascariasis and hookworm infections are carried by 1.6 billion people throughout the world and in 2% of cases cause loss of life. The use of therapeutic compounds forms a major component of control, and the development of novel therapeutic agents is required to deal with the increasing levels of resistance to existing drugs.

Paraherquamide (Fig. 1) is a novel anthelmintic (Yamazaki et al., 1981) that is an alkaloid fermentation product originally isolated from *Penicillium paraherquii*. The anthelmintic property of paraherquamide was first identified using jirds infected with *Trichostrongylus colubriformis* (Ostlind et al., 1990). Paraherquamide produces paralysis of parasitic nematodes in culture, without an effect on ATP, suggesting that it does not act as a metabolic poison (Thompson et al., 1996). Interestingly, one of the toxic effects of paraherquamide in the dog (Shoop et al., 1990) is a prolapsed nictitating membrane, an effect that suggests antagonism of neuronal nicotinic receptors (nAChRs). Recently, it has been reported (E. W. Zinser, M. L. Wolfe, S. J. Bowman, E. M. Thomas, V. E. Groppi, J. P. Davis, D. P. Thompson, and T. G. Geary, manuscript submitted for publication) that paraherquamide and 2-deoxy-paraherquamide act as antagonists of nematode nAChRs and mammalian neuronal nAChRs.

Nematode nAChRs have some pharmacological similarities to vertebrate neuronal receptors, but there are important differences. This is fortunate because it allows drugs like levamisole and pyrantel to be used for therapeutic purposes. Levamisole and pyrantel are potent anthelmintics that act as selective agonists on nematode nAChRs (Martin et al., 1996). Nematode nAChRs, like vertebrate neuronal nAChRs, are taken to be composed of a pentamer of subunits, and a number of different subtypes are present on muscle (Robertson et al., 1999; Richmond and Jorgensen, 1999). The distinctive pharmacology of some nematode nAChRs seems to relate to the distinctive molecular structure of the agonist binding site formed by part of the α-subunit (Fleming et al., 1997).

In this article, we report the quantitative investigation of the cholinergic antagonistic effects of paraherquamide and 2-deoxy-paraherquamide, using the simple competitive an-

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ABBREVIATIONS: nAChRs, neuronal acetylcholine receptors; DMSO, dimethyl sulfoxide.

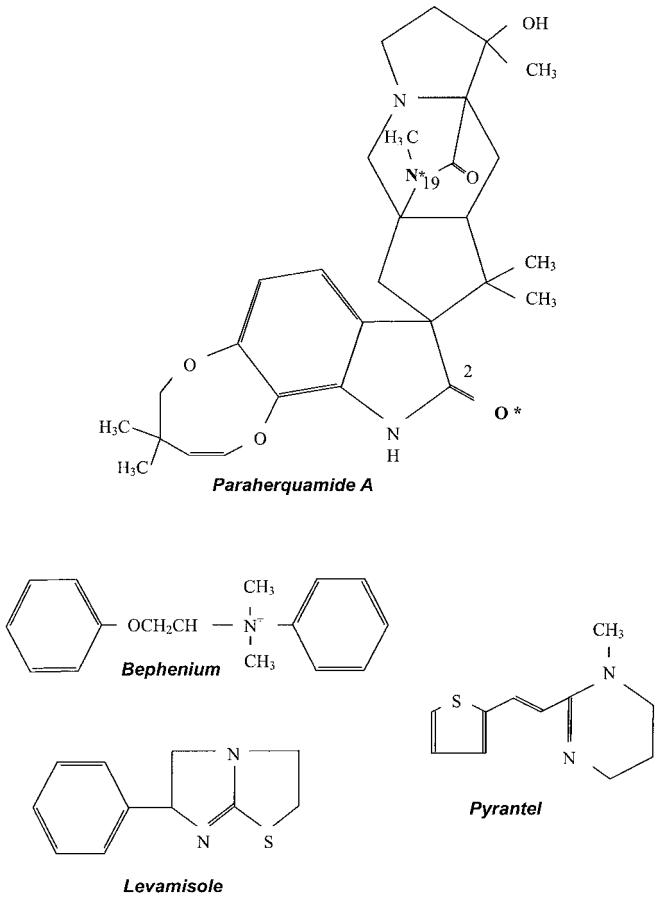

Fig. 1. Structure of anthelmintics paraherquamide, bephenium, levamisole and pyrantel. In paraherquamide, note the presence of the groups connecting N* in position 19 and O* in position 2 with a similarities to acetylcholine. The O* is missing in 2-deoxy-paraherquamide.

tagonist model on nematode nAChRs in *Ascaris* body wall muscle. We report that paraherquamide is a potent antagonist in nematodes that distinguishes levamisole- and nicotine-sensitive nAChRs of nematodes and that 2-deoxy-paraherquamide distinguishes levamisole- and bephenium-sensitive receptors. These findings are important because they demonstrate multiple subtypes of acetylcholine receptor with different anthelmintic sensitivities in a parasitic nematode and they further define the mode of action for these anthelmintics.

Materials and Methods

***Ascaris* Preparation.** Adult *Ascaris suum* were collected weekly from the IPB packing plant (Storm Lake, IA) and returned to the laboratory in Locke's solution at around 35°C in a metal vacuum flask. The *Ascaris* were used for the contraction studies within 72 h because the ability to contract vigorously to cholinergic agonists declined after this period.

Two 2-cm body-flap preparations, one dorsal and one ventral, were made from each *Ascaris* female from the region anterior to the genital pore. The gut was teased away from the muscle strip and the lateral lines were removed from the edge of the flaps to produce two preparations (one dorsal and one ventral). Each flap, composed of the muscle field and the cut nerve cord, was monitored isometrically by attaching a force transducer in an experimental bath, maintained at 37°C, containing 10 ml of *Ascaris* Ringer (23 mM NaCl, 110 mM Na-acetate, 24 mM KCl, 6 mM $CaCl_2$, 5 mM $MgCl_2$, 11 mM glucose, 5 mM HEPES, pH 7.6, with NaOH), and bubbled with nitrogen. Eight baths were used simultaneously. After dissection, the preparations were allowed to equilibrate for 15 min under an initial tension of 2.0 g. The antagonist was then added to the preparation 15 min before the application of the first concentration of the agonist. We ran two controls (no antagonist) and three replicate antagonist concentrations during each experiment (eight preparations). The dorsal and ventral flaps were assigned randomly in the experiments to reduce any potential error associated with differences in receptor populations. The agonists were added cumulatively with 2- to 3-min intervals between applications, and the responses were steady changes in tension. Figure 2B is a representative trace of a control agonist concentration-response plot with bephenium. Results for individual flaps were rejected if the maximum change in tension, at the highest agonist concentration, did not exceed 0.5 g. The mean maximum tension of the preparations, produced at each concentration of antagonist, was not significantly different ($p > 0.05$, F-test). The responses for each concentration were expressed as the percentage of the maximum tension produced by each individual flap preparation. The addition of small volumes of agonist did not significantly reduce the antagonist concentration in the tissue bath.

Drugs. Paraherquamide and 2-deoxy-paraherquamide were obtained from Pharmacia Animal Health (Kalamazoo, MI). Nicotine hemi-sulfate, levamisole hydrochloride, pyrantel citrate, bephenium hydroxynaphthoate, and dihydro-β-erythroidine were purchased from Sigma-Aldrich (St. Louis, MO). Pyrantel tartrate, bephenium hydroxynaphthoate, paraherquamide, and 2-deoxy-paraherquamide were dissolved in DMSO and added to the bath. Concentrations of DMSO did not exceed 0.1% in the bath. Control experiments demonstrated that this concentration of DMSO had no effect on concentration-response relationships (data not shown).

Recording and Analysis. Changes in isometric muscle tension responses were monitored using a PowerLab System (ADInstruments Pty Ltd., Castle Hill, Australia) that consists of the PowerLab hardware unit and Chart for Windows software (Microsoft, Redmond, WA). The system allows for recording, displaying, and analysis of experimental data. Sigmoid concentration-response curves for

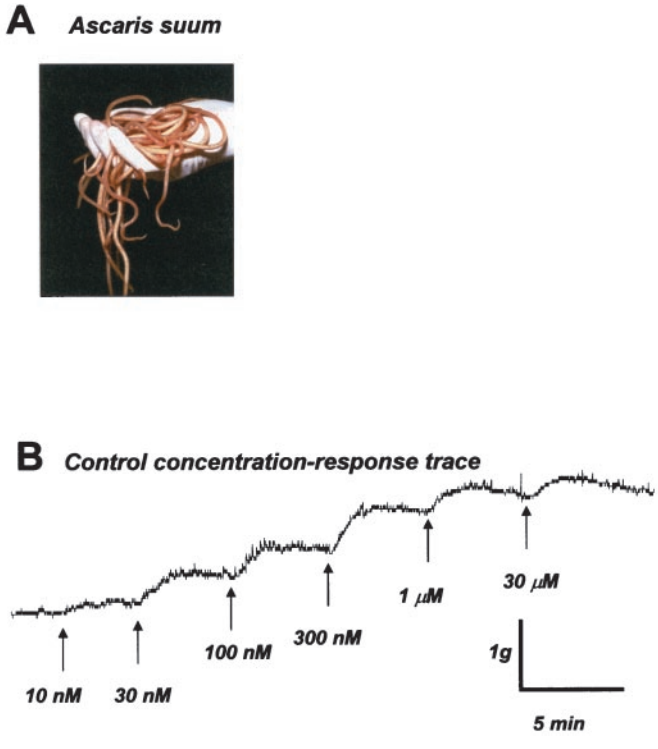

Fig. 2. A, *A. suum* in hand for scale. B, representative *Ascaris* muscle contraction cumulative-concentration-response trace. The trace shows the effect of cumulative addition of bephenium to the muscle strip.

each individual flap preparation at each concentration of antagonist were described by the Hill equation.

$$\% \text{ Response} = 1/(1 + [EC_{50}/X_A]^{n_H}), \quad (1)$$

where EC_{50} is the concentration of agonist (X_A) producing 50% of the maximum response and n_H is the Hill coefficient (slope). On the limited occasions when desensitization was evident (exclusively in control or low antagonist conditions), the maximum response was taken as 100%, and responses at higher agonist concentrations set at 100% to prevent the desensitization phenomenon from biasing the concentration-response plots. Prism 2.01 (GraphPad Software, San Diego, CA) was used to estimate the constants EC_{50} and n_H in eq. 1 by nonlinear regression for each preparation. The effect of antagonist concentration on n_H values was tested using analysis of variance. The pEC_{50} was calculated as the negative logarithm of EC_{50}. To illustrate the agonist concentration-response relationship at each concentration of antagonist, responses are plotted using the mean ± S.E. percent responses (n = 6–10 flap preparations), and the lines of fit are obtained for the figure displays by nonlinear regression without constraining the lines to be parallel. These lines of fit were not used for estimation for the pK_B values. If a compound behaves as a simple competitive antagonist, estimation of pK_B, the negative logarithm of the dissociation constant of the antagonist, is best made by nonlinear regression.

$$pEC_{50} = -\log([X_B] + 10^{-pK_B}) - \log C \quad (2)$$

where pEC_{50} values are as before, X_B is the concentration of the antagonist, and $-\log C$ is a constant and is the difference between the antagonist pK_B and the agonist control curve pEC_{50}. pK_B and $\log C$ were estimated by nonlinear regression as before. The advantage of estimating pK_B with this method is that there is no over-reliance on the control concentration-response relationship for estimating dose ratios for the Schild plot. Also, an error estimate for pK_B can be made from the covariance matrix and is expected to be normally distributed (Lew and Angus, 1995).

The slope factor, equivalent to the slope of the Schild plot, was estimated using nonlinear regression and the following equation.

$$pEC_{50} = -\log([X_B]^N + 10^{-pK_B}) - \log C \quad (3)$$

where N is equivalent to the slope of the Schild plot and the other variables are the same as before.

To display the effect of the antagonists paraherquamide and 2-deoxy-paraherquamide on the agonist EC_{50} values, Clark plots of $\log EC_{50}$ versus $\log([X_B] + pK_B)$ were used. This display allows the distribution of the data to be compared with the ideal for simple competitive block.

Results

Dihydro-β-Erythroidine. Richmond and Jorgensen (1999) have described the presence of nicotine- and levamisole-sensitive cholinergic receptors on muscle of *Caenorhabditis elegans* and have used single concentrations of the antagonist dihydro-β-erythroidine to block electrophysiological responses to nicotine but not levamisole. We tested, in *Ascaris*, the effects of different concentrations of dihydro-β-erythroidine on the contraction-responses to nicotine and levamisole.

Figure 3 shows the effect of different concentrations (10–100 μM) of dihydro-β-erythroidine on the concentration-response relationships of levamisole and nicotine. Dihydro-β-erythroidine had no detectable effect on the levamisole response (N = 7, 5, 5, and 5 for the 0, 10, 30, and 100 μM concentrations of dihydro-β-erythroidine) but produced a modest parallel shift to the right, in the concentration-response curves for nicotine (N = 8 for each concentration of dihydro-β-erythroidine). The dose-ratio produced by 100 μM dihydro-β-erythroidine was 2.16. If the antagonism is taken to be competitive, we can make an estimate for the antagonist pK_B using eq. 4.

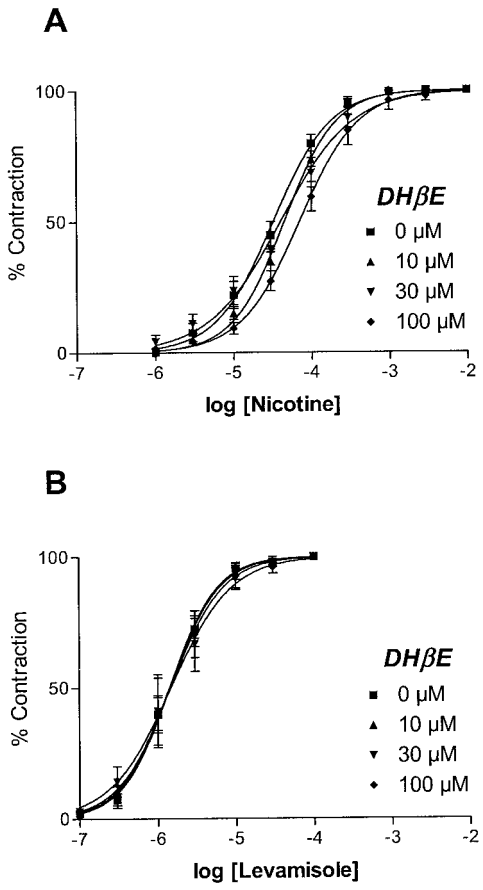

Fig. 3. Dihydro-β-erythroidine (DHβE) as an antagonist of nicotine (A) and levamisole (B). Note that dihydro-β-erythroidine produces some dose-dependent antagonism of the effects of nicotine but has no effect on the levamisole response ($n \geq 5$ for control and each antagonist concentration).

$$pK_B = -\log(X_B/dr - 1) \quad (4)$$

where dr is the dose-ratio and X_B is the concentration of dihydro-β-erythroidine. The pK_B for nicotine was 4.07. The selective effect of dihydro-β-erythroidine suggests the presence of nicotine- and levamisole-sensitive receptors in *Ascaris* and in *C. elegans*. However, the modest pK_B of dihydro-β-erythroidine shows that it is not potent in *Ascaris*.

Paraherquamide. We found that paraherquamide was a more potent antagonist than dihydro-β-erythroidine in *Ascaris*, and we therefore examined the antagonism in greater detail. We selected agonists that were cholinergic anthelmintics belonging to different chemical classes for further examination. We chose (Fig. 1) the tobacco alkaloid nicotine, the imidazothiazole levamisole, the tetrahydropyrimidine pyrantel, and the quaternary nitrogen compound bephenium.

Figure 4 shows the antagonistic effects of paraherquamide (0.03–30 μM) on concentration-response plots for nicotine (N = 8, 5, 5, and 5 for the 0, 0.3, 3, and 30 μM paraherquamide), levamisole (N = 8, 5, 6, and 5 for 0, 0.3, 3, and 30 μM

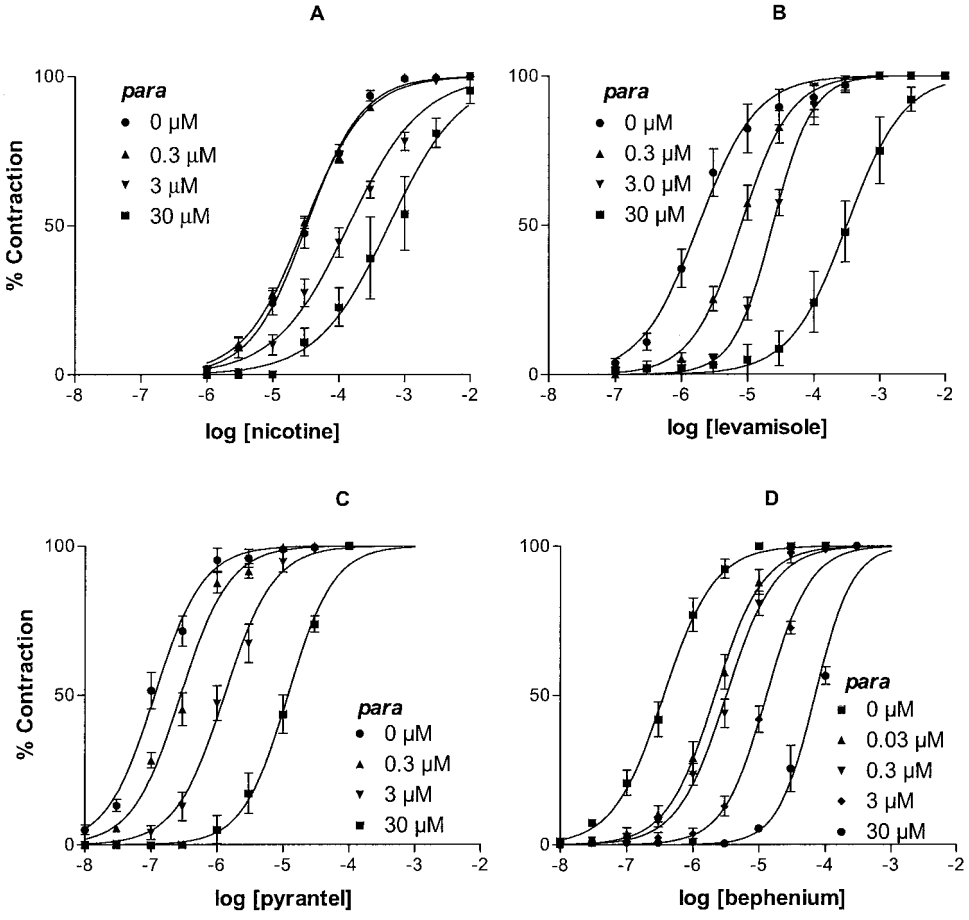

Fig. 4. Concentration-response plots for the agonists nicotine (A), levamisole (B), pyrantel (C), and bephenium (D) in the presence of different concentrations of paraherquamide ($n \geq 5$ for control and each paraherquamide concentration).

paraherquamide), pyrantel (N = 6, 5, 6, and 5 for 0, 0.3, 3, and 30 μM paraherquamide) and bephenium (N = 7, 6, 8, 7, and 3 for 0, 0.3, 3, 10, and 30 μM paraherquamide). The antagonism produced clear shifts to the right in all the concentration-response curves. As a test for the parallel nature of the shifts, we fitted all the concentration-response plots of single flap preparations (n = 5–8 at each antagonist concentration) with eq. 1, to estimate n_H and EC_{50}. We then tested the effect of antagonist concentration on n_H and found no significant effect ($p > 0.05$, F-tests) for nicotine, levamisole, pyrantel, or bephenium and concluded that the shifts could be taken as parallel.

Since the concentration-response curves were shifted to the right in a parallel manner, we used the simple competitive antagonist model (eq. 2) to describe the data. We also estimated the parameters of eq. 3 using nonlinear regression to make an estimate of the Schild slope factor, N.

Table 1 shows the values that were estimated for nicotine, levamisole, and pyrantel. The Schild slopes for these agonists were not significantly different from 1. The slope for bephenium was significantly less than 1, suggesting deviation from the simple competitive antagonist model. For reasons we discuss later, we described the antagonism of all the agonists with the simple competitive model using eq. 2.

The mean ± S.E. values for the parameters of eq. 2 are shown in Table 1 along with their confidence limits. Interestingly, the goodness of fit, R^2, showed that the fit for bephenium was comparable to nicotine and levamisole. As a further test of the fit of the simple competitive antagonist model, we plotted log EC_{50} versus log ($[B] + K_B$) as the Clark plot. EC_{50} values were obtained from eq. 1, B is the concentration of paraherquamide, and K_B is the antagonist dissociation constant (the antilog of $-pK_B$). Figure 5 shows these plots for nicotine, levamisole, pyrantel, and bephenium. These plots show the relationship between the experimental data and the line predicted by the simple competitive antagonist model.

The important point that we can make here is that the pK_B

TABLE 1

Paraherquamide parameter and error estimates for the Schild slope factor N in eq. 3, the negative log of the antagonist dissociation constant pK_B, in eq. 2, with degrees of freedom, and the goodness of fit R^2

Agonist	N (95% Confidence Limits)	pK_B ± S.E.	95% Confidence Limits for pK_B	Degrees of Freedom	R^2
Nicotine	0.85 (0.51–1.20)	5.86 ± 0.14*	5.58–6.15*	21	0.81
Levamisole	0.81 (0.51–1.11)	6.61 ± 0.19	6.21–7.01	22	0.81
Pyrantel	0.93 (0.73–1.13)	6.50 ± 0.11	6.27–6.73	20	0.93
Bephenium	0.55 (0.44–0.66†)	6.75 ± 0.15	6.45–7.06	29	0.80

† Significantly different to 1.
* Significantly different to pK_B values of other agonists in the table.

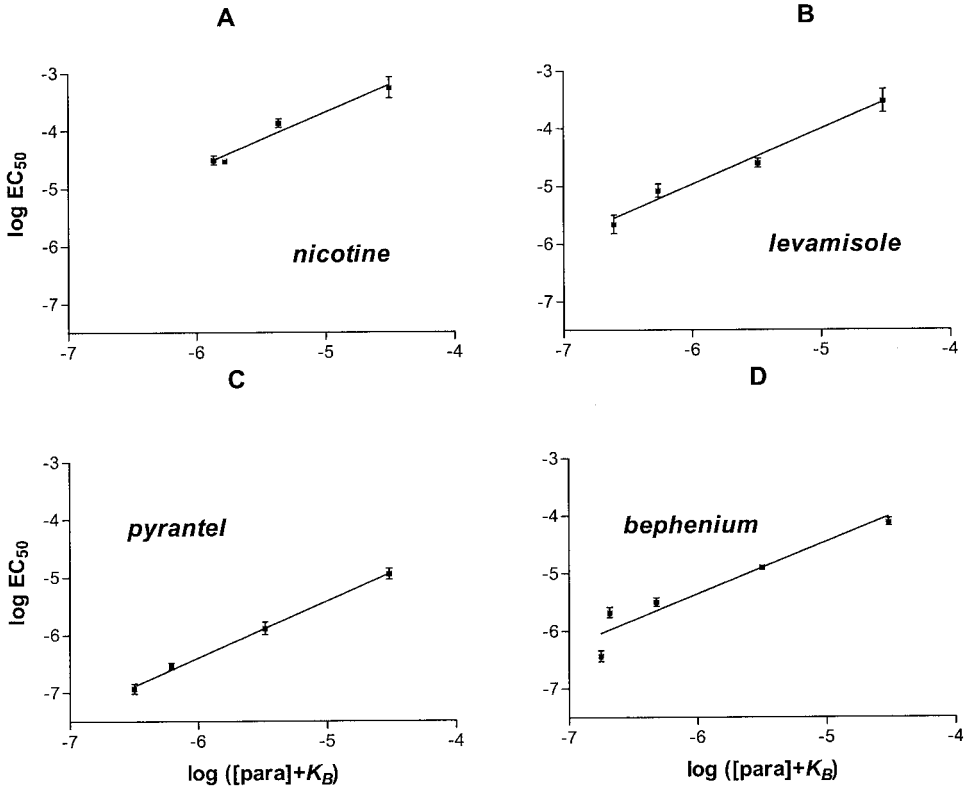

Fig. 5. Clark plots for the agonists nicotine (A), levamisole (B), pyrantel (C), and bephenium (D). Ordinate: mean ± S.E. log EC_{50}. Abscissa: log (paraherquamide concentration + agonist dissociation constant). Note the degree of fit between the observed values and the line predicted by the simple competitive block model.

of paraherquamide for nicotine is 5.86 ± 0.14, and this is significantly less ($p < 0.001$, t test, degrees of freedom 43–52) than for levamisole (6.61 ± 0.19), pyrantel (6.50 ± 0.11), or bephenium (6.75 ± 0.15). The observations demonstrate that the receptors activated by nicotine are not the same as those activated by levamisole, pyrantel, and bephenium.

2-Deoxy-Paraherquamide. We also tested the paraherquamide analog 2-deoxy-paraherquamide (see Fig. 1). We found that like paraherquamide it was an antagonist of nicotine, levamisole, pyrantel, and bephenium. Figure 5 shows the effect of 2-deoxy-paraherquamide on the log-concentration response plots. Concentration-response plots for levamisole (N = 6, 6, 6, and 6 for 0, 0.3, 3, and 30 μM antagonist), pyrantel (N = 6, 8, 7, and 8 for 0, 0.3, 3, and 30 μM antagonist), and bephenium (N = 7, 6, 6, 6, and 6 for 0, 0.03, 0.3, 3, and 30 μM antagonist), but not nicotine (N = 9, 10, 9, and 30 for 0, 0.3, 3, and 30 μM antagonist) were shifted to the right in a parallel manner by 2-deoxy-paraherquamide. Again, as a test for the parallel nature of the shift, we fitted all the concentration-response plots of single preparations with eq. 2. We then tested the effect of 2-deoxy-paraherquamide concentrations on n_H; we found no significant effect ($p > 0.05$, F-tests) for levamisole, pyrantel, or bephenium. The effect of 2-deoxy-paraherquamide on n_H of the nicotine slopes was significant ($p < 0.0004$, $F = 10.2$, degrees of freedom 3,18), an observation inconsistent with a simple competitive antagonism model.

We estimated N using eq. 3. Table 2 shows the mean values and 95% confidence limits for N. The slope, N, for nicotine was clearly less than 1, with a mean value of 0.39 (95% confidence limits 0.10–0.69), again emphasizing that a simple competitive action was not adhered to. As with paraherquamide, we again fitted the simple competitive model of antagonism to describe the action of the antagonist and to estimate the pK_B values with eq. 2. Table 2 shows the mean values and error estimates for the pK_B values for levamisole, pyrantel, and bephenium. We do not include the error estimates for nicotine because the simple competitive antagonism model did not describe the experimental data well, and R^2 was 0.50. To illustrate the fit of the experimental data to the simple competitive antagonist model and the estimated dissociation constant, we again used the Clark plots (Fig. 6). The plots suggest that the model is suitable and describes the data for levamisole, pyrantel, and bephenium.

The pK_B values estimated for 2-deoxy-paraherquamide are less than the pK_B values of paraherquamide for the same agonist showing that paraherquamide is more potent than

TABLE 2

2-Deoxy-paraherquamide parameter and error estimates for the Schild slope factor N in eq. 3, the negative log of the antagonist dissociation constant pK_B, eq. 2, with degrees of freedom, and the goodness of fit R^2

Errors for nicotine are omitted because of the poor fit of the antagonism to the simple competitive model.

Agonist	N (95% Confidence Limits)	pK_B ± S.E.	95% Confidence Limits for pK_B	Degrees of Freedom	R^2
Nicotine	0.39 (0.10–0.69†)	5.30		36	0.50
Levamisole	0.70 (0.33–1.07)	5.31 ± 0.13	5.03–5.58	22	0.71
Pyrantel	0.83 (0.56–1.10)	5.64 ± 0.10	5.43–5.84	27	0.84
Bephenium	0.75 (0.50–1.00†)	6.07 ± 0.13*	5.81–6.34	29	0.80

† Significantly different to 1.
* Significantly different to pK_B values of other agonists in the table.

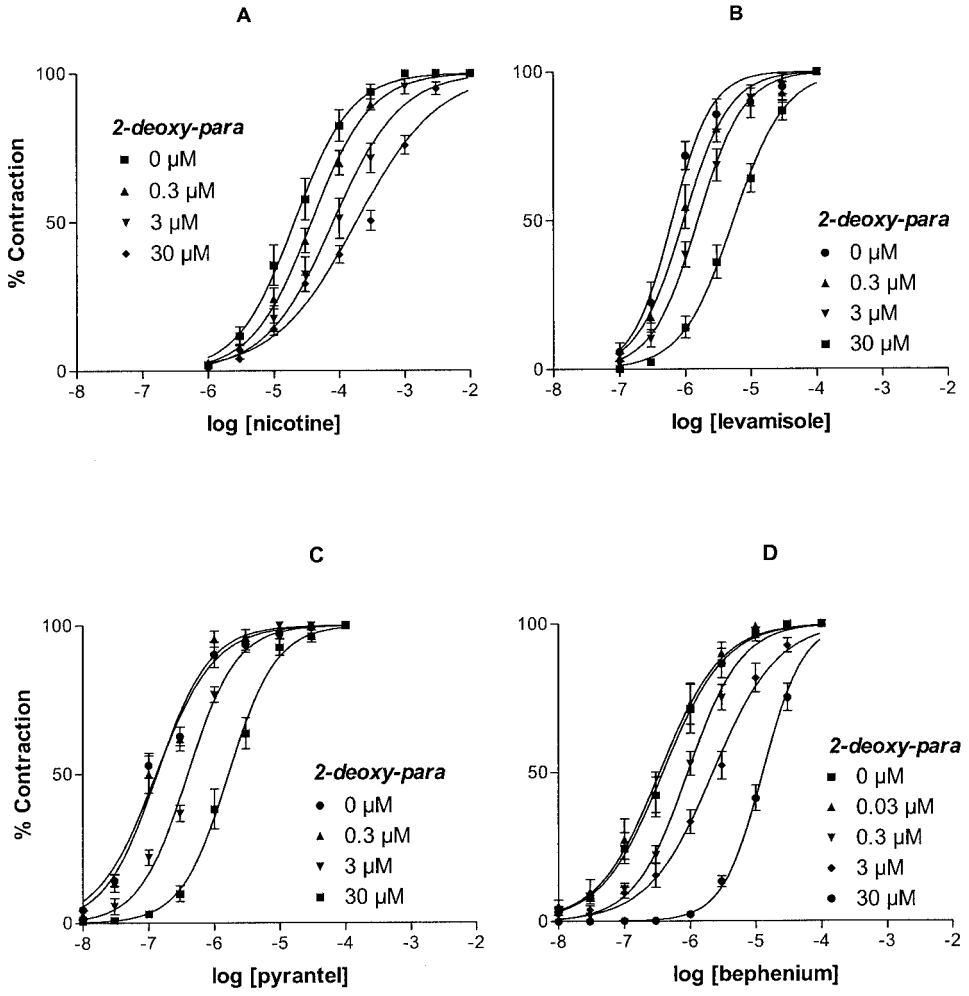

Fig. 6. Concentration-response plots for the agonists nicotine (A), levamisole (B), pyrantel (C), and bephenium (D) in the presence of different concentrations of 2-deoxy-paraherquamide [0 (control), 0.3, 3, and 30 μM] ($n \geq 6$ for each agonist/antagonist combination).

2-deoxy-paraherquamide as an antagonist in *A. suum*. Also, the pK_B of 2-deoxy-paraherquamide was significantly greater with bephenium as agonist than with levamisole and pyrantel as agonist. This implies that bephenium does not act on the same population of nAChRs as levamisole and pyrantel.

Discussion

Use of the Simple Competitive Antagonism Model. We used classical pharmacological techniques to analyze the antagonist action of paraherquamide and 2-deoxy-paraherquamide. The simple model of competitive action is based on the law of mass action, using the reaction scheme below.

$$RB \overset{K_B}{\longleftrightarrow} R \overset{K_A}{\longleftrightarrow} RA*$$

where, R is the receptor not occupied, RA* is the receptor occupied by one molecule of agonist, RB is the receptor occupied by one molecule of antagonist. KB and KA are the dissociation constants of the antagonist and agonist, and * indicates activation of the receptor. In the presence of agonist and antagonist, equation 2 (see *Materials and Methods*) should describe the degree of antagonism.

This simple model may not be universally valid for ion-channels that have two or more binding sites for agonist and antagonist molecules. Under these conditions, the slope of the Schild plot is predicted to be reduced under the law of mass action and under limiting conditions is described by the modified Schild equation (Williams et al., 1988), which has a slope less than 1. For the paraherquamide antagonism of bephenium and the 2-deoxy-paraherquamide antagonism of nicotine, we saw that the Schild slope factor was less than 1. One explanation then for the reduced slope factor may lie along the lines prescribed by Williams et al. (1988).

When a Monod-Wyman-Changeux allosteric model for competitive antagonism is used for the operation of the receptor ion-channel, the dose-ratio turns out to be a linear function of the antagonist concentration, even in the general case (Colquhoun, 1973). So the explanation for when the slope factors are less than one may lie elsewhere and relate to the presence of more than one nAChR receptor subtype. If there are multiple subtypes of nAChR present in the preparation and the agonist and antagonists are not sufficiently selective, it is possible, at low agonist concentrations, that the K_B value of the antagonist for receptors with high agonist affinity dominates the shift in the concentration-response curve. At high concentrations of antagonist, the K_B of the antagonist for low-agonist-affinity receptors may dominate the shift in the concentration-response curve. This could reduce the Schild slope factor and/or give rise to nonparallel shifts in concentration-response plots, as we saw with the antagonism of nicotine by 2-deoxy-paraherquamide.

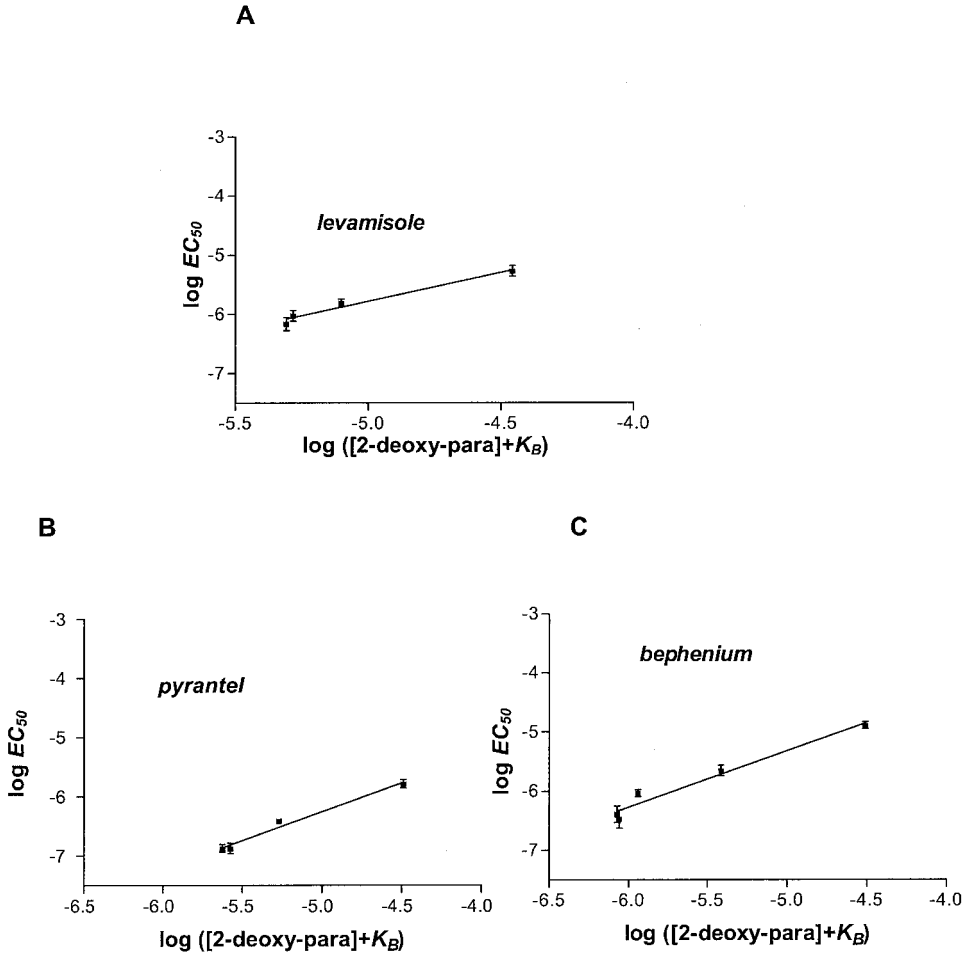

Fig. 7. Clark plots for the agonists levamisole (A), pyrantel (B), and bephenium (C). Ordinate: mean ± S.E. log EC_{50}. Abscissa: log (2-deoxy-paraherquamide concentration + agonist dissociation constant). Note the relationship between the observed values and the line predicted by the simple competitive model of competitive antagonism.

By using eq. 2, we have imposed a slope factor of 1 for all the agonists to estimate the pK_B for reasons of parsimony and because the approach also reduces over estimates of pK_B (Black and Shankley, 1985). Thus, despite some known uncertainties, we feel justified in using the competitive model. Waud and Parker (1971) have commented that "I do not blush when caught using a competitive kinetic analysis heuristically when the underlying model may in fact be more along the allosteric lines. . . if you tell me the K_B of a new antagonist, I know a lot about the drug." The conclusions we draw in this article are based on the assumption that agonists acting on identical receptors will be affected to the same degree by the antagonist. Classically, agonists that act on the same receptors are expected to produce the same pK_B (Arunlakshana and Schild, 1959).

Competitive Action of Paraherquamide and 2-Deoxy-Paraherquamide and nAChR Subtypes. In this study, we have been able to show that paraherquamide and 2-deoxy-paraherquamide can behave as competitive antagonists at nematode nAChRs. We know that paraherquamide is a potent anthelmintic (Shoop et al., 1990). The potency of the competitive antagonist action seen here, with dissociation constants in the micromolar range, suggests that competitive nAChR antagonism contributes to the anthelmintic properties of this compound.

2-Deoxy-paraherquamide lacks a carbonyl group at the 2-position (Fig. 1) and was around 10× less potent as an antagonist in our assays. In whole-worm preparations with a cuticle barrier to penetrate this order of potency is reversed (Thompson et al., 1996), perhaps for reasons of access. Our observations here suggest that the presence of a carbonyl group, which presumably acts in hydrogen bonding to the receptor, is important for binding. The classic model of the cholinergic receptor consists of a site which binds the quaternary nitrogen, N^+, and an osteophilic site that binds to =O of acetylcholine; the separation between the N^+ and =O is a chain length of four atoms. We can see a similar structure in paraherquamide, starting with the carbonyl group in position 2 and ending in the methylated nitrogen in position 19 (Fig. 1). This portion of paraherquamide might bind to the nAChR. We point out that the amino acid structure of the ligand-binding has now been resolved at the atomic level, and the quaternary nitrogen of ligands is thought to stack onto tryptophan (position 143) making cation-π interactions (Brejc et al., 2001).

Interestingly, our analysis revealed that paraherquamide and 2-deoxy-paraherquamide showed pK_B values that varied significantly when different agonists were used. We saw that paraherquamide discriminated between receptors sensitive to nicotine and receptors sensitive to levamisole, pyrantel, and bephenium. This observation is consistent with the observations of Richmond and Jorgensen (1999), who described the presence of nicotine- and levamisole-sensitive receptors on *C. elegans* muscle. We found that 2 deoxy-paraherquamide had a pK_B for bephenium that was significantly different to that of levamisole and pyrantel. Again the evidence suggests that the receptors acted on by levamisole and pyrantel are not the same

as those of bephenium. It is interesting to point out that a comparison of levamisole-sensitive and levamisole-resistant isolates of *Haemonchus contortus* showed that muscle contraction became less sensitive to levamisole with resistance but did not change its sensitivity to bephenium (Sangster et al., 1991). Both these lines of evidence support the view that bephenium-sensitive receptors are different to levamisole-sensitive receptors.

Multiple Subtypes of nAChRs in Mammals and Nematodes and Selective Ligands. Ionotropic acetylcholine receptors are taken to be pentameric structures (Corringer et al., 2000; Sharples and Wonnacott, 2001). In vertebrate muscle, the subunits α, β, δ, and ϵ form the ion-channels with a stoichiometry of α_2, β, δ, ϵ at the endplate. In vertebrate neuronal receptors, the channels are usually formed by a heteropentameric combination of α and β subunits, α2 to 7, α9, α10, and β2 to 4 in mammals, with an additional subunit, α8, in avian species. Homomeric combinations of some α subunits may occur, e.g., by α7, α8, or α9, but most channels are composed of α and β subunits. The rules governing the ability to combine to form functional ion-channels vary with the structure of the subunit.

The ligand binding sites (Corringer et al., 2000: Brejc et al., 2001) of individual nAChR receptors are composed of six amino acid loops, three (loops A, B, and C) from the α subunit and three (D, E, and F) from the adjacent β subunit. Since nAChRs have two or more α subunits, there are two or more ligand binding sites. Different nAChRs formed by different α and β subunit combinations have different agonist binding sites because the loops, ABC and DEF, will vary with the particular α and β subunits that form the ion-channel receptor. It is also a consequence that nAChRs composed of two identical α subunits and three dissimilar β subunits will have two nonequivalent binding sites. Consequently, selective agonists and antagonists are expected to distinguish between receptor subtypes and between nonequivalent sites on an individual nAChR.

A distinct but related family of nAChR subunits has been found in *C. elegans* in which there are 42 candidate nAChR subunits (Fleming et al., 1996., 1997; Baylis et al., 1997; Bargmann, 1998). The α subunits include: UNC-39, UNC-63, ACR-16 or Ce21, and ACR-3. The β subunits include: ACR-1, ACR-2, LEV-1, and UNC-29. An nAChR, sensitive to levamisole, may be composed of UNC-38 as an α-subunit along with UNC-29 and LEV-1 on muscle. Expression of ACR-2 + UNC-38, UNC-38 + ACR-3, and UNC-38 + UNC-29 + LEV-1 in *Xenopus* oocytes produces small currents in response to levamisole. In contrast, expression of ACR-16, which is 47% identical to chicken α8, in *Xenopus* oocytes, produces channels sensitive to acetylcholine but insensitive to levamisole or pyrantel (Ballivet et al., 1996). In the intact nematode, levamisole pyrantel, morantel, and oxantel activate nAChRs on *Ascaris* muscle (Martin et al., 1996), and single channel recordings of nAChRs in *Oesophogostomum dentatum* reveal the presence of four subtypes on somatic muscle (Robertson et al., 1999). There is therefore evidence for the presence of multiple subtypes of nAChR in nematodes with ligands that can be selective for different subtypes of receptor.

Significance of nAChR Receptor Subtypes for Anthelmintic Resistance. There is strong evidence for nAChR receptor subtypes in vertebrates and, now, nematodes and that the different subtypes have different sensitivities to ligands. Also, we have presented evidence that nicotine, levamisole, and bephenium are selective ligands for different subtypes. Sangster et al. (1991) have described isolates of levamisole resistant *H. contortus* that have a reduced sensitivity to levamisole but not to bephenium. We suggest that one form of levamisole resistance is associated with a switch from levamisole-sensitive nAChRs to other nAChR subtypes. This mode of anthelmintic resistance would not be revealed as a change in the amino acid sequence of particular nAChR subunits (Hoekstra et al., 1997) but as a change in proportion of expressed nAChR subtypes.

The effect of paraherquamide and 2-deoxy-paraherquamide on whole nematodes seems to be that of paralysis by competitive antagonist action at different nAChR subtypes. If the potency of these antagonists is sufficient against all of the nAChR subtypes present in nematode parasites, these compounds could be useful for the control of types of levamisole resistant nematodes associated with changes in proportion of nAChR subtypes.

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