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The anthelmintic levamisole is an allosteric modulator of human neuronal nicotinic acetylcholine receptors

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Abstract

L-[−]-2,3,5,6-Tetrahydro-6-phenylimidazo[2,1*b*]-thiazole hydrochloride (levamisole) is an anthelmintic that targets the nicotinic acetylcholine receptors of parasitic nematodes. We report here the effects of levamisole on human neuronal α3β2 and α3β4 nicotinic receptors, heterologously expressed in *Xenopus* oocytes and studied with the voltage clamp method. Applied alone, levamisole was a very weak partial agonist for the two subunit combinations. When co-applied with acetylcholine, micromolar concentrations of levamisole potentiated responses, while millimolar concentrations inhibited them; these effects were complex functions of both acetylcholine and levamisole concentrations. The differences in the levamisole effects on the two receptor combinations suggest that the effects are mediated by the β subunit. Several combinations of agonist and anthelmintic gave the dual potentiation/inhibition behavior, suggesting that the modulatory effects are general. Levamisole inhibition showed macroscopic characteristics of open channel block. Several results led us to conclude that levamisole potentiation occurs through noncompetitive binding to the receptor. We propose pseudo-site binding for noncompetitive potentiation by levamisole.

Keywords: Nicotinic receptor; Levamisole; Potentiation; Inhibition; Allosteric modulator; Oocyte

1. Introduction

The nicotinic acetylcholine receptors are a large family of ligand-gated ion channels with diverse subunit composition, pharmacology and biophysical properties (Sargent, 1993; Papke, 1993). These diverse properties underlie an apparent diversity of functions, which range from mediation of fast synaptic transmission in sympathetic peripheral systems to modulation of synapse function in the central nervous system (McGehee and Role, 1995). There is strong interest in the roles of nicotinic receptors in neuronal tissues due to their possible involvement in a host of disorders ranging from Alzheimer's and Parkinson's diseases to schizophrenia and epilepsy (for a review, see Lindstrom, 1997). In addition, neuronal nicotinic receptors are the central focus of molecular studies of nicotine abuse (Dani and Heinemann, 1996).

From the molecular perspective, considerable effort continues to be directed at determining the composition of neuronal nicotinic receptors in situ and characterizing their properties in order to find specific differences among subtypes that can be exploited therapeutically. For example, the novel ligands (*S*)-3-methyl-5-(1-methyl-2-pyrrolidinyl) isoxazole (ABT-418; Arneric et al., 1994), (±)-2-(6-chloro-3-pyridinyl)-7-azabicyclo[2.2.1]-heptane (epibatidine; Gerzanich et al., 1995) and (*E*)-*N*-methyl-4-(3-pyridinyl)-3-buten-1-amine (RJR-2403; Bencherif et al., 1996) display high selectivity for defined subtypes of the nicotinic receptor family. Maelicke and Albuquerque have suggested that *modulators* of nicotinic receptor activity, in contrast to strict agonists or antagonists, hold promise as agents capable of tuning malfunctioning cholinergic systems (Maelicke et al., 1995; Maelicke and Albuquerque, 2000). Such compounds, in spite of low potency or efficacy alone, might potentiate the effects of endogenous acetylcholine, resulting in effective up-regulation of a given system. Several compounds have been shown to have potentiation effects on nicotinic receptor activity, including curare (Cachelin and Rust, 1994), acetylcholi-

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nesterase antagonists (Pereira et al., 1994; Schrattenholz et al., 1996; Zwart et al., 2000), fenamates (Zwart et al., 1995), atropine (Zwart and Vijverberg, 1997) and choline (Zwart and Vijverberg, 2000).

The anthelmintic compound L-[−]-2,3,5,6-tetrahydro-6-phenylimidazo[2,1*b*]-thiazole hydrochloride (levamisole) acts on the muscle nicotinic acetylcholine receptors of nematodes to cause spastic (excitatory) paralysis of the worm (Martin et al., 1997). Fleming et al. (1997) reported that levamisole was a weak agonist of a heterologously expressed *Caenorhabditis elegans* nicotinic receptor, and Robertson and Martin (1993) demonstrated that levamisole activates muscle-type receptors of *Ascaris suum*. Levamisole and the related compound pyrantel are also open channel blockers of the nematode muscle nicotinic receptor (Robertson and Martin, 1993; Robertson et al., 1994; Ballivet et al., 1996). In addition to its traditional use as an anthelmintic in livestock, levamisole is widely used in humans as an adjuvant in certain cancer therapies (e.g., MacDonald, 1999), as well as treatment for a host of other disorders (e.g., Sanchez, 2000), presumably for its immunomodulatory activity. Levamisole administered in humans in such cases might also act at nicotinic receptors.

We investigated the effects of levamisole on human neuronal nicotinic receptors, because the compound is a known nicotinic receptor ligand in nematodes. We report here the first comparison of levamisole pharmacology on the subunit combinations α3β2 and α3β4. Levamisole, co-applied with acetylcholine, can either potentiate responses or inhibit them, depending on the concentrations of the two compounds. We show that these effects are general in that they occur with several combinations of agonist and anthelmintic; in other words, this modulation appears to be a fundamental aspect of nicotinic receptor behavior. Although several compounds have been reported to potentiate nicotinic responses, the phenomenon is not well understood. We provide evidence that levamisole inhibition occurs through open channel block and that potentiation is through a noncompetitive mechanism. We discuss our results in the context of a novel pseudo-site model for the interaction of ligands with neuronal nicotinic receptors.

2. Materials and methods

2.1. Chemicals and human nicotinic receptor subunit constructs

Unless otherwise noted, all chemicals were reagent grade from Sigma (St. Louis, MO). Vectors bearing the cDNA genes for the human α3, β2 and β4 subunits were the generous gift of Jon Lindstrom (University of Pennsylvania). Plasmids bearing cDNAs of the human α3, β2 and β4 subunits were prepared using standard procedures (cf. Levandoski et al., 1999). cRNA transcripts were generated using the SP6 or T7 MessageMachine kit from Ambion (Austin, TX).

2.2. Oocyte preparation and injections

Oocytes were collected from mature *Xenopus laevis* frogs by survival surgery and were prepared for injection essentially as described by Bertrand et al. (1991) and Levandoski et al. (1999). Procedures for the care and treatment of *Xenopus* were approved by the Grinnell College Institutional Review Board and are in accord with National Institutes of Health (USA) guidelines. Following brief collagenase treatment to remove the follicular cell layer, healthy stage V–VI oocytes were manually selected and incubated for 1 day in Barth medium (in mM: 88 NaCl, 1.0 KCl, 2.5 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES; pH 7.6) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 50 μg/ml Gentamicin (Gibco-BRL; Gaithersburg, MD). Oocytes were then injected with cRNAs and maintained in the Barth–antibiotics medium for 2–3 days at 16 °C before recording nicotinic responses. A Drummond Nanoject was used to inject ~23 ng total cRNAs (in 46 nl), which for the two subunit combinations were combined in equimolar ratios.

2.3. Electrophysiological recordings and data analysis

Acetylcholine-evoked currents were measured from injected oocytes using the two-electrode voltage clamp method with a Warner (Hamden, CT) 752C amplifier. The membrane potential was −60 mV, unless otherwise noted. Cells were perfused with oocyte Ringer's medium (OR2; in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES; pH 7.3). Concentrated stocks of the various ligands (in water) were diluted directly in OR2. Recordings were performed in a Warner RC-3Z chamber (with an incubation volume of ~300 μl) with gravity perfusion flow (~5 ml/min). The flow of various drug solutions into the chamber was regulated using solenoid valves driven by Warner VC-6 valve controllers. Electrodes of resistance 0.5–4.0 MΩ were filled with 3 M KCl. Data were acquired on a personal computer using Chart software through a PowerLab analog/digital converter (ADInstruments, Castel Hill, NSW, Australia).

Measurements of current response for drug applications were performed one to three times on each oocyte; after each drug application (typically 5 s), the oocyte was subjected to washout by continuous perfusion with OR2 for 1.5–2.5 min. Control concentrations of acetylcholine were administered periodically in the course of test recordings on a given oocyte to ensure full recovery prior to the next drug application. No appreciable rundown of the response was observed.

Peak current values obtained from traces for replicate measurements on each oocyte were averaged independently, and then values obtained for replicate oocytes were

averaged. Data are reported as means ± the standard error of the mean (S.E.M.), and measurements from replicate oocytes are noted in each case. Individual experiments were performed on one to four batches of oocytes. Apparent affinities of acetylcholine for α3β2 and α3β4 receptors were determined by nonlinear fits of the concentration–response data to the Hill equation function: fractional response $= 1/\{1 + (EC_{50}/[ACh])^{n_H}\}$, where EC_{50} is the concentration of agonist-eliciting half-maximal response and n_H is the Hill coefficient. Voltage dependence of inhibition data (Fig. 3B) were fit nonlinearly to a single-site model (Zarei and Dani, 1995; Buisson and Bertrand, 1998) as $I_{+\text{ levamisole}}/I_{-\text{ levamisole}} = 1/\{1+[\text{levamisole}]/K_d\exp(\delta zFV/RT)\}$, where z, F, R and T have their usual meanings, K_d is the apparent dissociation constant of the inhibitor at 0 mV, and δ is the fraction of the electric field sensed by the inhibitor. Fitting was done using the Origin 5.0 software (Microcal, Northampton, MA). Statistical comparisons of data were made using Student's t test and are reported significant as $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

3. Results

Levamisole potentiated the acetylcholine-evoked responses of both α3β2 and α3β4 human neuronal nicotinic receptors. The two sets of three traces shown in Fig. 1A demonstrate the potentiation effect of levamisole for the α3β4 combination, using an acetylcholine concentration near EC_{50}. The first three traces (left) are controls showing that acetylcholine-evoked currents are blocked by co-application of 100 μM hexane-1,6-bis[trimethylammonium] (hexamethonium) and recover completely following sufficient washout and agonist challenge. Levamisole, co-applied with acetylcholine to the same oocyte, increased the evoked current by ~30% under these conditions (compare traces 4 and 6 to traces 1 and 3). Like the control case of acetylcholine alone, the currents evoked by co-application of acetylcholine and levamisole were blocked reversibly by hexamethonium (traces 5 and 6). Fig. 1B shows that levamisole potentiates acetylcholine-evoked responses of α3β2 receptors as well; under these conditions, potentiation was nearly fourfold.

Fig. 1C shows representative traces for control experiments testing whether levamisole acts as an agonist. For both α3β2 and α3β4 receptors, 3 mM levamisole applied alone evoked a nearly negligible current compared to that evoked by control concentrations of 160 μM acetylcholine; this concentration is near EC_{50} for the α3β4, and near EC_{75} for α3β2. Peak currents evoked by 3 mM levamisole were 5.1 ± 2.7% that evoked by 160 μM acetylcholine for α3β2 oocytes ($n = 3$), and 7.6 ± 3.8% for α3β4 oocytes ($n = 6$). In addition, we tested 0.3 and 30 μM levamisole alone on these subunit combinations. The magnitudes of these evoked currents, relative to control concentrations of 160 μM acetylcholine alone, were 5.3 ± 2.6% at 0.3 μM and 5.0 ± 3.3% at 30 μM for α3β2, and 2.2 ± 3.0% at 0.3 μM and 1.2 ± 2.1% at 30 μM for α3β4 (three oocytes each set).

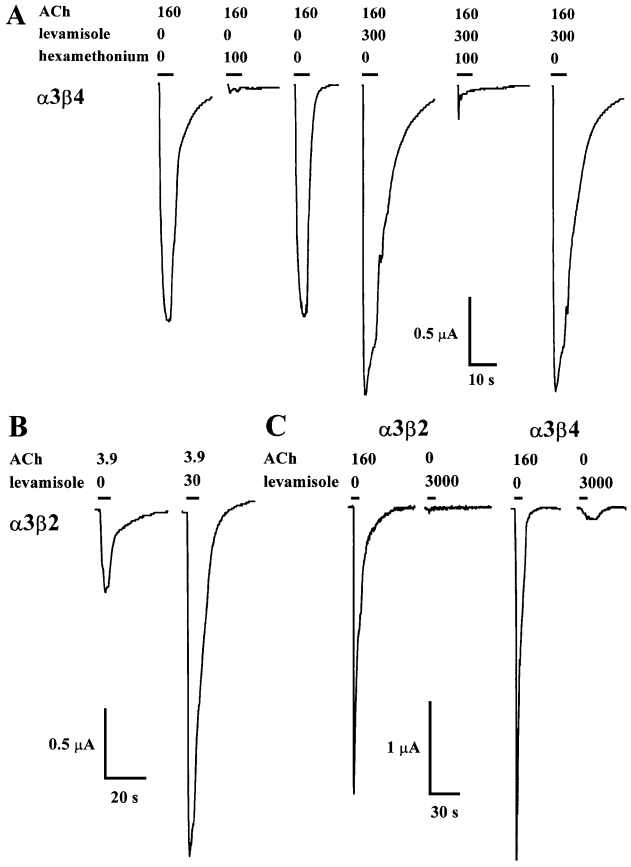

Fig. 1. Levamisole potentiates acetylcholine responses. (A) An oocyte expressing the α3β4 subunit combination was exposed to a series of 5-s applications of drug(s), and the evoked current was measured under two-electrode voltage clamp: Left to right, control application of acetylcholine, block by co-application of acetylcholine and hexamethonium, recovery with acetylcholine alone; co-application of acetylcholine and levamisole, block by co-application of acetylcholine, levamisole and hexamethonium, recovery with co-application of acetylcholine and levamisole. Full recovery after hexamethonium block occurred at 2.8 min for acetylcholine challenge and 8.3 min for acetylcholine/levamisole co-application. (B) Sample experiment for α3β2 receptors. Left trace is acetylcholine alone control and right trace is co-application of acetylcholine and levamisole. (C) Paired experiments for α3β2- and α3β4-expressing oocytes. Left trace of the pair is acetylcholine alone control and right trace of the pair is levamisole alone. Numbers in all panels indicate μM concentrations.

We observed robust expression of human α3β2 and α3β4 receptors in oocytes, as evidenced by currents typically in the range of 1–5 μA evoked by application of micromolar concentrations of acetylcholine. We determined values of EC_{50} for acetylcholine-evoked currents of 80 ± 7 μM for α3β2 receptors and 130 ± 9 μM for α3β4 receptors (data not shown), which are in agreement with previously published values for these human nicotinic receptor combinations (Gerzanich et al., 1995; Chavez-Noriega et al., 1997). The potentiation and inhibition effects observed in this study were very reproducible. In the experiments presented here,

typically five oocytes were tested for any given set of conditions. In addition, for the measurements in Fig. 7A and a subset of those in Figs. 2 and 6, two or three trials of the entire titration series were performed on each oocyte. Measurements were made using several batches of RNAs and oocytes from eight donor frogs. The effects of potentiation and inhibition were also completely reversible.

3.1. Levamisole and acetylcholine concentration dependence of α3β4 receptors

We investigated the potentiation effect of levamisole as a function of both its concentration and the concentration of acetylcholine. Because increasing the concentration of a ligand drives binding to the receptor, it was important to (effectively) titrate receptors with both acetylcholine and levamisole. The data collected for the α3β4 combination are shown in Fig. 2, which plots the ratio of peak current for co-application of acetylcholine and levamisole to that for application of acetylcholine alone ($I_{+\text{ levamisole}}/I_{-\text{ levamisole}}$), as a function of levamisole concentration over the range 1 μM to 3 mM. At very low concentrations (<5 μM), levamisole had very little effect. In the range of ~5–500 μM levamisole, potentiation was observed (i.e., $I_{+\text{ levamisole}}/I_{-\text{ levamisole}}>1$). At concentrations in the millimolar range, inhibition by levamisole was observed ($I_{+\text{ levamisole}}/I_{-\text{ levamisole}}<1$). This resembles the characteristic "bell-shaped" curve of the concentration dependence data previously observed in other nicotinic receptor systems for curare (Cachelin and Rust, 1994), galanthamine (Schrattenholz et al., 1996), atropine and choline (Zwart and Vijverberg, 1997, 2000). In addition, nicotinic receptors can be positively and negatively modulated by a variety of metals such as zinc and mercury (e.g., Mirzoian and Luetje, 2002, and references therein). This general description holds for the levamisole effects measured using 25, 160 and 300 μM acetylcholine as the agonist, which are approximately EC_5, EC_{50} and EC_{75}. Using 1 mM acetylcholine, the effect of levamisole was predominantly inhibitory. In these experiments, intermediate levamisole concentrations did not evoke currents that were significantly greater than the expected maximal current (data not shown). The magnitude of the current ratio generally increased as the acetylcholine concentration was decreased at all constant levamisole concentrations, including 3 mM, where the effect was inhibitory for the four acetylcholine concentrations.

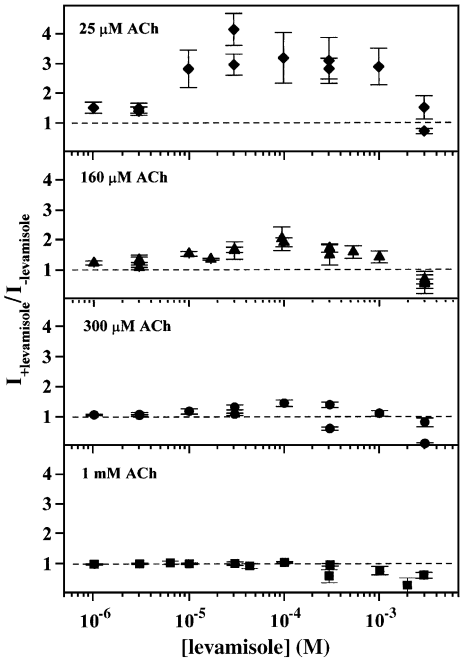

Fig. 2. Concentration dependence of the effects of levamisole on α3β4 receptors. The concentration dependence of the effect of levamisole was investigated as a function of control concentration of acetylcholine. For oocytes expressing the α3β4 combination, measurements of the response to a control concentration of acetylcholine (5-s application) were made, followed by the response to co-applications of that concentration of acetylcholine and the indicated levamisole concentration. Data are plotted as the ratio of the peak inward macroscopic current for co-application of levamisole to that for the control concentration of acetylcholine alone ($I_{+\text{ levamisole}}/I_{-\text{ levamisole}}$). Data collected at different acetylcholine concentrations are shown in separate panels for clarity, with the same ordinate scale to facilitate comparisons. The dashed line at $I_{+\text{ levamisole}}/I_{-\text{ levamisole}}=1$ is shown for comparison of potentiation and inhibition effects. Data are average values of the ratio (±S.E.M.) for measurements on four to six oocytes for each condition.

3.2. Mechanism of inhibition

Under certain conditions, acetylcholine–levamisole co-application resulted in complex current response kinetics for α3β2 receptors (see below); we therefore chose α3β4 receptors to examine the effects of potentiation and inhibition in detail. We first investigated the mechanism of inhibition. Fig. 3A shows that a brief pulse of 3 mM levamisole during a prolonged acetylcholine application induced a rapid and sustained inhibition of the current at two different potentials. As shown in Fig. 3B, the degree of inhibition was dependent on holding potential. Using co-application of 160 μM acetylcholine (near the EC_{50}) and 3 mM levamisole, inhibition decreased from about 90% to 65% as the potential was increased from −120 to −20 mV. The current ratios at the extremes of the range tested were significantly different ($P<0.05$). Fitting these data to a Woodhull-type, single-site model of channel block (Zarei and Dani, 1995; Buisson and Bertrand, 1998) gave the estimates $K_d=2.3\pm0.2$ mM for the apparent dissociation constant at 0 mV and $\delta=0.37\pm0.05$ for the fraction of the field sensed by levamisole. The magnitude of the voltage dependence of levamisole inhibition is similar to that observed for atropine and physostigmine inhibition of rat α4β4 receptors (Zwart and Vijverberg, 1997; Zwart et al., 2000) and for the inhibition by five different open channel blockers on human α4β2 receptors (Buisson and Bertrand, 1998).

The inhibition was also use-dependent (Fig. 3C and D); whereas six repeated applications of acetylcholine

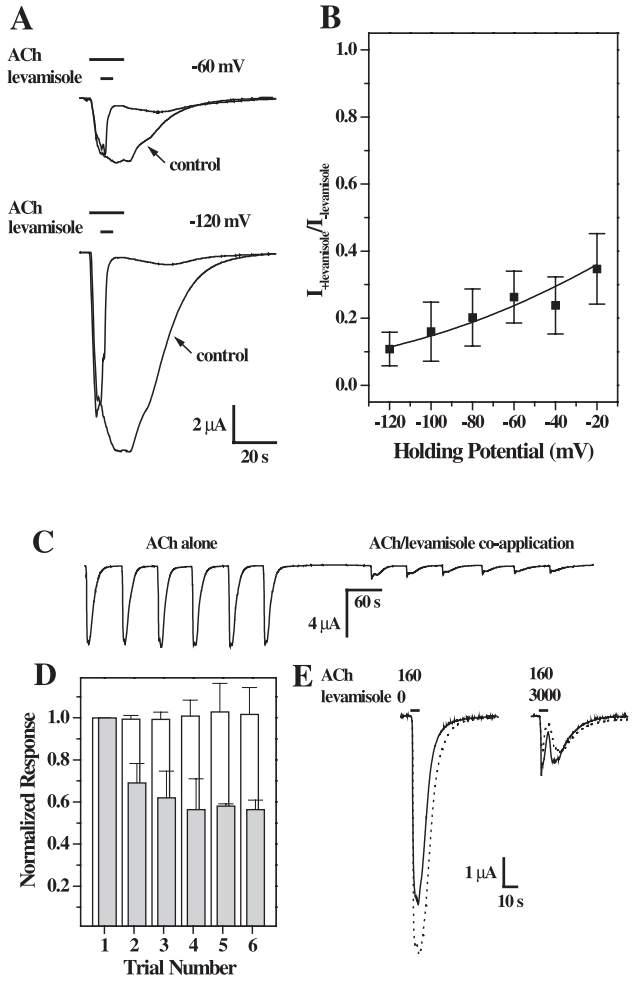

Fig. 3. Levamisole is an open channel blocker of α3β4 receptors. Oocytes expressing α3β4 receptors were tested for macroscopic characteristics of open channel block. (A) A 5-s application of 3 mM levamisole during prolonged 300 μM acetylcholine exposure showed a rapid and persistent inhibition. Pairs of control and inhibition traces are overlaid for the experiments at holding potentials of −60 mV (top) and −120 mV (bottom). (B) The ratio of peak currents with and without levamisole in co-application experiments using 160 μM acetylcholine and 3 mM levamisole is plotted as a function of holding potential. Data points represent averages (± S.E.M.) for 3 oocytes, with two to four trials of the entire series for each. The curve is the best nonlinear fit to a single-site channel block model (see Section 2). (C) A sample experiment testing the use dependence of inhibition by levamisole for α3β4 receptors at −60 mV is shown. Every 60 s, the oocyte was challenged (5 s) with 160 μM acetylcholine (left series) or co-applied 160 μM acetylcholine and 3 mM levamisole (right series). (D) Peak current responses recorded as in panel C were normalized to the first response in the series for acetylcholine control (white bars) or acetylcholine/levamisole co-application (gray bars), and are plotted as sequential trial number. The first co-application response was ~22% of the control under these conditions. Values represent averages (± S.E.M.) for three to four oocytes. (E) Representative current traces demonstrating the "rebound" current are shown at holding potentials of −60 mV (solid traces) and −120 mV (dashed traces). On the left are applications of acetylcholine alone, and on the right are co-applications of acetylcholine and levamisole. Numbers in all panels indicate μM concentrations.

alone evoked identical peak currents, repeated co-application of acetylcholine and levamisole led to further reduction in current amplitude (ranging from $I_{+\text{ levamisole}}/I_{-\text{ levamisole}} \sim 0.22$ to ~ 0.10 under these conditions). A paired comparison t test of peak current responses indicated that while acetylcholine–levamisole co-applications 2–6 were not significantly different from one another, these responses were lower than the first trial ($P < 0.05$). In addition, we observed, under numerous conditions in which both acetylcholine and levamisole concentrations were high enough, that a further inward, "rebound" current occurred immediately upon washout of the co-applied drugs (Bertrand et al., 1992; Maconochie and Knight, 1992; Chavez-Noriega et al., 1997). Fig. 3E shows examples of this behavior for α3β4 receptors at two potentials when challenged with 160 μM acetylcholine and 3 mM levamisole.

3.3. Mechanism of potentiation

In beginning to elucidate the mechanism of levamisole potentiation, we first aimed to distinguish whether levamisole binds at the acetylcholine binding site or elsewhere. We hypothesized that the competitive antagonist dihydro-β-erythroidine (DHβE; e.g., Harvey and Luetje, 1996) should block potentiation if it effectively reduced levamisole bind-

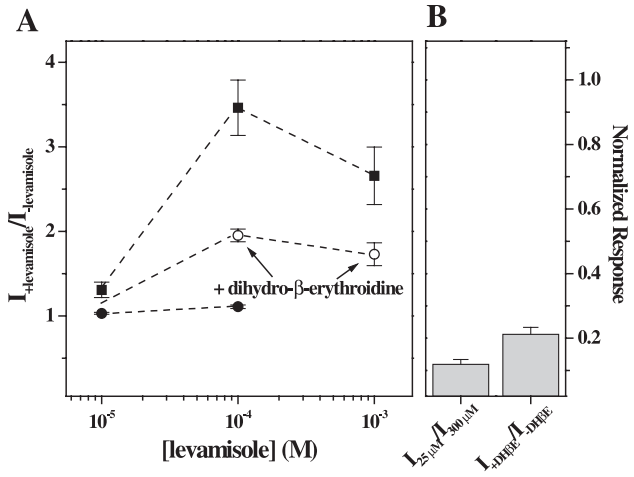

Fig. 4. Effects of dihydro-β-erythroidine block at high levamisole concentrations. Oocytes expressing α3β4 receptors were challenged with various concentrations and combinations of acetylcholine, levamisole and dihydro-β-erythroidine; all data in the figure were collected on the same set of oocytes. (A) The ratio of peak currents in the presence and absence of levamisole ($I_{+\text{ levamisole}}/I_{-\text{ levamisole}}$) is plotted vs. levamisole concentration. Filled circles are data using 300 μM acetylcholine and filled squares are for 25 μM acetylcholine. Open circles are the current ratios for the indicated levamisole concentrations and 300 μM acetylcholine, but in the presence of 100 μM dihydro-β-erythroidine. The dashed lines indicate the behavior at constant acetylcholine concentration (cf. Fig. 2). (B) Two internal control experiments are shown for reference. The ratio of peak currents of responses to 25 and 300 μM acetylcholine was 0.12 ± 0.06. The effect of 100 μM dihydro-β-erythroidine on the response to 300 μM acetylcholine was ~80% inhibition. Data in panels A and B are averages (± S.E.M.) for 18 oocytes from one batch.

ing. Fig. 4 shows the results of an experiment testing this. The filled symbols of Fig. 4A are internal controls for the experiment and indicate the limits of levamisole potentiation under these conditions (cf. Fig. 2). The open circles of Fig. 4A represent the ratio of currents (± levamisole), but in the presence of 100 μM dihydro-β-erythroidine, using 300 μM acetylcholine and the indicated levamisole concentrations. These ratios were greater than those in the absence of DHβE, as expected for dihydro-β-erythroidine reducing the effective acetylcholine concentration. Does dihydro-β-erythroidine also reduce the effective levamisole concentration by blocking binding sites? For concentrations below ~100 μM, increasing the levamisole concentration increases the degree of potentiation monotonically at fixed acetylcholine concentrations, both in general (cf. Fig. 2) and in the presence of DHβE (data not shown). However, note for the +dihydro-β-erythroidine measurements in Fig. 4A, increasing the levamisole concentration tenfold decreased the ratio significantly, from 1.95 ± 0.07 at 100 μM to 1.73 ± 0.14 at 1 mM ($P < 0.001$). Therefore, we conclude that dihydro-β-erythroidine does not reduce the effective levamisole concentration. Two more internal controls for the experiment (Fig. 4B) showed that 100 μM DHβE reduced the current elicited by 300 μM acetylcholine greatly, but not to the level evoked by 25 μM acetylcholine (ratios are different, $P < 0.01$); this is consistent with the 300 μM acetylcholine + 100 μM DHβE data (open circle) values being less than the 25 μM acetylcholine data (filled squares). Additionally, in a separate experiment (not shown), currents potentiated by levamisole in the presence of dihydro-β-erythroidine showed the same acetylcholine concentration dependence as those in the absence of DHβE, suggesting no reduction in effective levamisole concentration. That is, the magnitude of increase in current upon adding levamisole was the same whether using acetylcholine alone at low concentrations or using a higher concentration that was effectively reduced by including dihydro-β-erythroidine. Together, these results suggest that levamisole does not compete directly with dihydro-β-erythroidine, and by extension, that it does not compete directly with acetylcholine.

We also examined the potentiation effect at low concentrations of acetylcholine and levamisole. Fig. 5 shows titration data for the range 4.7–150 μM acetylcholine alone or co-applied with either 10 or 30 μM levamisole; all peak current responses were normalized to the current evoked by 2 mM acetylcholine alone. As expected, with increasing levamisole concentration, the concentration of acetylcholine required to elicit a given response decreased (leftward shift in the concentration–response relationship). The solid curves through the data sets in Fig. 5 represent the best fits to the Hill equation. Importantly, the coefficient n_H is reduced significantly ($P < 0.001$) for co-application with levamisole; the fit values were $n_H = 1.69 \pm 0.09$ (acetylcholine alone), 1.36 ± 0.04 (+10 μM levamisole) and 1.43 ± 0.03 (+30 μM levamisole). The dashed curves in Fig. 5 are plots of the Hill equation with a constant $n_H = 1.69$ and the reported EC_{50} values for the two levamisole data sets, indicating the behavior expected for a competitive mode of potentiation.

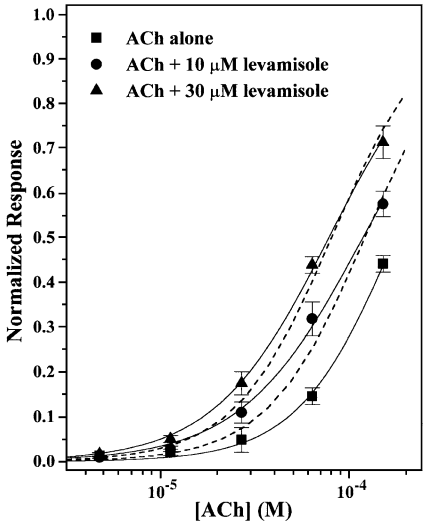

Fig. 5. Levamisole alters the acetylcholine concentration dependence. The acetylcholine concentration dependence of the response for oocytes expressing α3β4 receptors was measured at −60 mV. Peak currents elicited by acetylcholine alone or as co-application of acetylcholine and levamisole were normalized to the response to 2 mM acetylcholine alone. Data are averages of the normalized values (± S.E.M.) for five oocytes from one batch. The solid curves through the data sets represent the best fits to the Hill equation (see Section 2) with the following EC_{50} and n_H values, respectively: 170 ± 5 μM, 1.69 ± 0.09 (acetylcholine alone); 120 ± 3 μM, 1.36 ± 0.04 (+10 μM levamisole); 80 ± 1 μM, 1.43 ± 0.03 (+30 μM levamisole). The dashed curves are plots of the Hill equation with fixed $n_H = 1.69$ and the EC_{50} of 120 μM for the +10 μM levamisole data and 80 μM for the +30 μM levamisole data.

3.4. Levamisole and acetylcholine concentration dependence of α3β2 receptors

For acetylcholine-evoked currents in oocytes expressing α3β2 receptors, levamisole again potentiated responses when applied at low concentrations and inhibited responses at high concentrations, but clearly interacted with these receptors in a different manner than with α3β4 receptors. Fig. 6A shows plots of the ratio of peak currents in the presence and absence of levamisole ($I_{+\text{ levamisole}}/I_{-\text{ levamisole}}$) over the range 1 μM to 3 mM levamisole for four different acetylcholine concentrations. The α3β2 receptors displayed the same general trend observed for α3β4 receptors: At low levamisole concentrations, the magnitude of potentiation increased as the acetylcholine concentration was decreased, and the "bell-shaped" behavior of the levamisole titration was evident at 3.9, 25 and 160 μM acetylcholine. In contrast, at 1 mM acetylcholine, potentiation continued to increase as the levamisole concentration was increased for the entire range. Considering the four data sets together, the levamisole concentration giving the maximum effect (peak $I_{+\text{ levamisole}}/I_{-\text{ levamisole}}$) increased with increasing acetylcho-

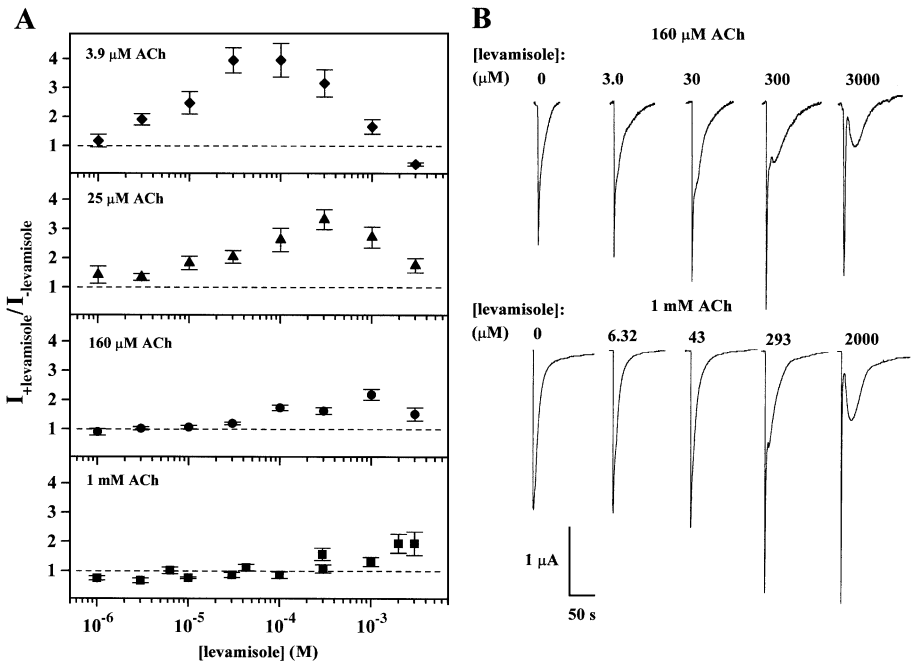

Fig. 6. Concentration dependence of the effects of levamisole on α3β2 receptors. (A) The concentration dependence of the effect of levamisole was investigated as a function of control concentration of acetylcholine. The ratio of the peak currents with and without levamisole ($I_{+\text{ levamisole}}/I_{-\text{ levamisole}}$) is plotted as a function of levamisole concentration for oocytes expressing the α3β2 combination; data were collected and treated as described for Fig. 2. Data collected at different acetylcholine concentrations are shown in individual panels for clarity, with the same ordinate scale to facilitate comparisons. The dashed line at $I_{+\text{ levamisole}}/I_{-\text{ levamisole}} = 1$ is shown for comparison of potentiation and inhibition effects. Data are average values of the ratio (± S.E.M.) for 4–10 oocytes. (B) Representative current traces measured at a holding potential of −60 mV for α3β2 receptors. In each case, the oocyte was challenged with a 5-s application of acetylcholine and the indicated levamisole concentration; data for 160 μM and 1 mM acetylcholine are from two separate oocytes. Scale bars apply to both data sets.

line concentration. In other words, inhibition appeared to be relieved by high acetylcholine concentrations.

As shown in Fig. 6B, the α3β2 responses to co-application of acetylcholine and levamisole were more complicated kinetically than those of α3β4 receptors. As the levamisole concentration was increased, the time required for the current to return to baseline increased. This was presumably due to the increasing contribution of the "rebound" current (e.g., Maconochie and Knight, 1992), which was well-defined at millimolar concentrations. The "rebound" currents were dependent on acetylcholine and levamisole concentrations, and as was the case for α3β4 receptors, the effect was voltage-dependent (not shown). In addition, even at the lower agonist concentrations of 3.9 and 25 μM acetylcholine, high levamisole concentrations gave the "rebound" current upon washout. The effect of levamisole concentration on the current kinetics evident in Fig. 6B, in marked contrast to the case of α3β4 receptors (cf. Fig. 3E), is similar to effects reported for atropine and choline acting on rat α4β4 receptors (Zwart and Vijverberg, 1997, 2000).

Total charge analysis of the current responses (Papke and Porter Papke, 2002) for each of the data sets shown in Fig. 6A revealed that the transient potentiation observed at high levamisole and acetylcholine concentrations (as in Fig. 6B) did not reflect an overall potentiation under these conditions (data not shown). For example, at 1 mM acetylcholine, the ratios of total charge (± levamisole) had the "bell-shaped" behavior across the range, including ratios less than unity at millimolar levamisole concentrations. We interpret these results to mean that the dual potentiation and inhibition effects still occur for α3β2 receptors, but with a time dependence different from α3β4 receptors. Our levamisole titration results for the α3β2 and α3β4 combinations underscore the importance in such studies of measuring modulation effects over wide concentration ranges of modulatory agent *and* agonist.

3.5. Generality of the effects

We hypothesized that the modulatory effects of levamisole were general, that is, that levamisole could potentiate and inhibit the responses evoked by agonists other than acetylcholine. Fig. 7A shows the ratio of peak currents with and without co-application of levamisole using nicotine as the agonist, plotted as a function of the levamisole concentration, for α3β2 and α3β4 receptors. The nicotine concentrations used were approximately their respective EC_{50}'s. As observed when acetylcholine was used as the agonist, levamisole potentiated the nicotine-evoked currents of α3β2 receptors (filled circles), up to $I_{+\text{ levamisole}}/I_{-\text{ levamisole}} \sim 2.6$ at 300 μM levamisole. No direct inhibition of nicotine currents by levamisole was

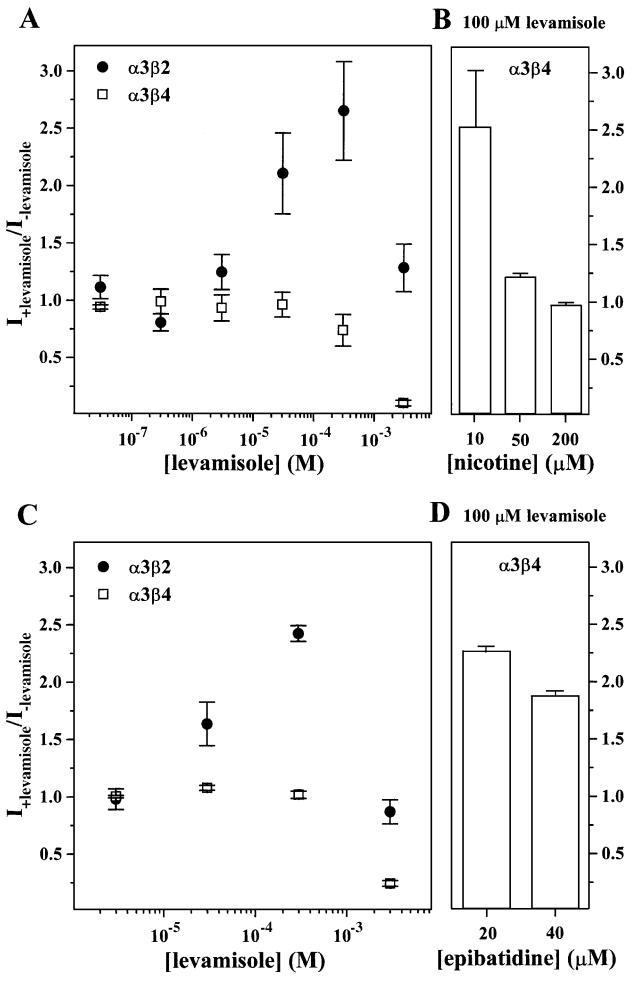

Fig. 7. Levamisole potentiates responses evoked by other nicotinic receptor agonists. (A) The concentration dependence of the effect of levamisole was investigated using nicotine as an agonist at 6.7 μM for oocytes expressing α3β2 receptors (filled circles); for α3β4 receptors (open squares), the nicotine concentration was 110 μM. These nicotine concentrations are the EC_{50}'s reported in Gerzanich et al. (1995). Data are average values of the ratio (± S.E.M.) for six oocytes for each condition. (B) The ratio of currents (± 100 μM levamisole) is shown for α3β4 receptors using the indicated nicotine concentrations ($n = 4$ oocytes). (C) The concentration dependence of the effect of levamisole was investigated using (±)-epibatidine as an agonist at 60 nM for oocytes expressing α3β2 receptors (filled circles) and α3β4 receptors (open squares). The EC_{50}'s with (±)-epibatidine were 160 ± 26 nM for α3β2 and 44 ± 12 nM for α3β4 (data not shown); these are in the ranges of EC_{50}'s determined for the separate enantiomers (Gerzanich et al., 1995). Data are average values of the ratio (± S.E.M.) for 4 α3β2 oocytes and 5 α3β4 oocytes for each condition. (D) The ratio of currents (± 100 μM levamisole) is shown for α3β4 receptors using the indicated (±)-epibatidine concentrations ($n = 4$ oocytes). Data in all panels were collected and analyzed as described in Fig. 2.

observed, but such inhibition is expected at higher levamisole concentrations due to the "bell-shaped" behavior of the data over the range 30 nM to 3 mM. When 50 μM nicotine was used as the control concentration in combination with 30 μM levamisole (cf. $I_{+\,levamisole}/I_{-\,levamisole} = 2.6$ with 6.7 μM nicotine), the ratio of peak currents was 1.2 ± 0.1 ($n = 3$). This shows that the magnitude of levamisole potentiation increases for decreasing nicotine concentrations, as was observed using acetylcholine as the agonist. In contrast, very little, if any, potentiation of nicotine-evoked currents was observed in oocytes expressing α3β4 receptors (open squares) using 110 μM nicotine (EC_{50}) over the same range of levamisole concentrations. However, the nicotine response was inhibited at concentrations ≥ 100 μM levamisole. Nonetheless, 100 μM levamisole did potentiate the responses for α3β4 receptors when lower nicotine concentrations were used (Fig. 7B).

Similarly, as shown in Fig. 7C, levamisole potentiated the responses of α3β2 receptors evoked by 60 nM exo-(±)-2-(6-chloro-3-pyridinyl)-7-azabicyclo[2.2.1]-heptane (epibatidine), but showed only inhibition of α3β4 receptors using the same concentration of agonist. However, as shown in Fig. 7D, α3β4 responses were potentiated using lower concentrations of (±)-epibatidine.

4. Discussion

Considering the possible roles of neuronal nicotinic acetylcholine receptors in numerous neurological disorders (e.g., Lindstrom, 1997), development of nicotinic receptor ligands as therapeutic agents with high specificity remains a significant challenge to the field. An exciting possibility in this regard is a ligand that can modulate nicotinic receptor activity without acting as an agonist itself (Maelicke and Albuquerque, 2000). In this study, we have shown that levamisole has these properties acting on human α3β2 and α3β4 nicotinic receptors expressed in oocytes: Levamisole potentiation is blocked by the classical neuronal nicotinic antagonist hexamethonium (e.g., Nelson and Lindstrom, 1999; Fig. 1A), indicating that the effect of levamisole is mediated by the receptors on the oocyte surface. Levamisole itself is a weak partial agonist (Fig. 1C) but shows potentiation of acetylcholine-evoked currents at micromolar concentrations (Fig. 1A and B). These effects appear to be general for this class of anthelmintics, insofar as they occur whether acetylcholine, nicotine or epibatidine is the agonist, and whether levamisole or the related anthelmintic pyrantel (data not shown) is the modulatory agent (Figs. 2, 6 and 7). That multiple drug pairs give the same behavior suggests that such modulation is an important, fundamental property of nicotinic receptors.

4.1. The β subunit mediates levamisole effects

The effects of levamisole on human α3β2 and α3β4 nicotinic receptors are qualitatively and quantitatively distinct. While the peak potentiation occurs at 100 μM levamisole for α3β4 receptors using an acetylcholine concentration near EC_{50} compared to 300 μM levamisole for the α3β2 combination, the magnitude of the effect is smaller in the former case ($I_{+\,levamisole}/I_{-\,levamisole} = 1.9$ vs. 3.3; Figs. 2 and 6). 1-Methyl-2-[2-(2-thienyl)ethenyl]-1,4,5,6-tetrahydropyrimidine (pyrantel) potentiated the ace-

tylcholine-evoked responses of α3β4 receptors better than for α3β2: $I_{+\,pyr}/I_{-\,pyr} = 1.6$ at 3 μM for α3β4 vs. 1.1 at 30 μM for α3β2 (data not shown). In contrast, levamisole potentiated nicotine-evoked responses of the α3β2 combination better than those of α3β4 using equipotent nicotine concentrations (Fig. 7A); this behavior was repeated using ±-epibatidine as agonist (Fig. 7C). Furthermore, levamisole appears to have an important kinetic effect on α3β2 receptors at high concentrations, allowing rapid potentiation followed by open channel block (see below and Fig. 6B). These results emphasize the importance of β subunits in determining the pharmacology of neuronal nicotinic receptors for such ligands as acetylcholine, nicotine, epibatidine and dihydro-β-erythroidine (Luetje and Patrick, 1991; Gerzanich et al., 1995; Chavez-Noriega et al., 1997), as well as modulatory agents such as curare, fenamates and choline (Cachelin and Rust, 1994; Zwart et al., 1995; Zwart and Vijverberg, 2000). One interpretation of the diversity of the effects of levamisole is that the β subunit contributes directly to the binding site for levamisole (see discussion below), but we cannot rule out indirect effects. We have yet to determine if the α subunit mediates levamisole effects.

4.2. Inhibition is by open channel block

Several compounds that display both potentiation and inhibition effects act as open channel blockers, including atropine (Zwart and Vijverberg, 1997) and physostigmine (Pereira et al., 1993; Zwart et al., 2000). While we cannot rule out contributions by other mechanisms (cf. Cachelin and Rust, 1994), our data strongly suggest that the inhibition of human α3β2 and α3β4 receptors by levamisole occurs via an open channel block mechanism. As shown in Fig. 3 for α3β4 receptors, inhibition at high concentrations of acetylcholine and levamisole has the macroscopic characteristics of open channel block. The change in current ratio of ~ 0.2 over a 100-mV range is comparable to the effects shown by five open channel blockers used at high concentrations (Buisson and Bertrand, 1998) and to other modulatory agents (Zwart and Vijverberg, 1997; Zwart et al., 2000) on nicotinic receptors. A fit to a single-site model of channel block (Fig. 3B; Zarei and Dani, 1995) gave reasonable values for K_d and δ. We used a protocol of repeated co-application, following Buisson and Bertrand (1998), to demonstrate use dependence of inhibition. The effect (Fig. 3C and D) was modest, most likely due to the rather long (5 s) applications, wherein significant block occurred already with the first exposure. Finally, the currents inhibited by levamisole showed dramatic inward "rebound" currents (Fig. 3E), which have been observed in a variety of nicotinic receptor subtypes and experimental systems (Bertrand et al., 1992; Maconochie and Knight, 1992; Zwart et al., 1995; Chavez-Noriega et al., 1997; Van den Beukel et al., 1998). This phenomenon, although often observed, has not been well-characterized; one explanation is that the open channel blocker dissociates upon drug removal, leaving a transiently open, current-passing channel (Bertrand et al., 1992). In general, we interpret the decrease in the ratio $I_{+\,levamisole}/I_{-\,levamisole}$ (beyond the peak effect) for increasing levamisole concentrations as the contribution of some channel block in a fraction of the receptor population. Furthermore, macroscopic and single channel measurements have shown levamisole and pyrantel to be open channel blockers of nematode muscle-type receptors (Robertson and Martin, 1993; Robertson et al., 1994; Ballivet et al., 1996).

4.3. Potentiation is noncompetitive

Does levamisole bind the acetylcholine sites on human nicotinic receptors or elsewhere? Because we observe levamisole effects indirectly through the *functional* measurement of agonist-evoked currents, we cannot at present definitively answer this question. While there is precedent for levamisole acting as agonist on nematode muscle-type receptors (Robertson and Martin, 1993; Fleming et al., 1997) and while we cannot rule out some contribution of competitive potentiation at present, our data suggest that levamisole binds noncompetitively to human neuronal nicotinic acetylcholine receptors. We discuss below the observations supporting this conclusion.

Dihydro-β-erythroidine is a competitive antagonist of neuronal nicotinic receptors (e.g., Pereira et al., 1994; Harvey and Luetje, 1996). Because the currents elicited by levamisole alone are effectively negligible (Fig. 1C), we examined whether dihydro-β-erythroidine blocked the potentiation by levamisole. As shown in Fig. 4A, the current ratios at constant acetylcholine and levamisole concentrations increased in the presence of DHβE, consistent with the effective reduction of acetylcholine binding. The current ratio at 1 mM levamisole was smaller (1.73) than that at 100 μM (1.95), following the typical behavior for high levamisole concentrations. If levamisole binding were blocked by dihydro-β-erythroidine, increasing the levamisole concentration tenfold should outcompete the DHβE, leading to an increase in the ratio, not a decrease. Furthermore, levamisole potentiation was the same using low absolute acetylcholine concentrations or equivalent effective acetylcholine concentrations achieved by DHβE addition.

If levamisole bound nicotinic receptors competitively, potentiation should occur when the (presumed) two sites of the receptor are occupied by one molecule each of acetylcholine and levamisole (cf. Cachelin and Rust, 1994; Zwart and Vijverberg, 1997). In this case, one expects that for a constant levamisole concentration, different agonists used at the same *effective* concentration would result in the same degree of potentiation. We did not observe this, as evidenced by the negligible potentiation of α3β4 receptors when using nicotine and epibatidine at their EC_{50}'s compared to the peak $I_{+\,levamisole}/$

$I_{-\text{levamisole}} = 1.9$ at 100 μM levamisole using 160 μM acetylcholine (compare Figs. 2 and 7). For both α3β2 and α3β4 receptors, side-by-side comparisons of currents in the same oocyte showed that levamisole gave different degrees of potentiation with acetylcholine and nicotine at their respective EC_{50}'s (data not shown). An alternative interpretation is that levamisole binds competitively but has an efficacy that depends on the agonist used; however, because levamisole alone has nearly no efficacy, this seems unlikely. Regardless of the implications about mechanism, the variable potentiation of levamisole with the same effective concentration of various agonists is a direct demonstration of the allosteric interaction of levamisole with neuronal nicotinic receptors (Colquhoun, 1998; Wyman and Gill, 1990).

Fig. 5 shows that low concentrations of levamisole potentiate acetylcholine-evoked currents such that the Hill coefficient is significantly reduced relative to the titration of acetylcholine alone. Curves drawn with the relevant lower values of EC_{50} but using the $n_H = 1.69$ determined for acetylcholine alone—the predicted behavior if binding of levamisole were competitive—systematically underestimate the response for the 10- and 30-μM levamisole data sets. Preliminary results using multiple-state allosteric models to fit such data (Wyman and Gill, 1990) indicate that noncompetitive binding of levamisole lowers n_H.

Again, levamisole elicited very little current when applied alone to α3β2 and α3β4 receptors (Fig. 1C). Therefore, if it bound competitively to nicotinic receptors, levamisole should inhibit currents at high concentrations. In fact, inhibition occurs in the millimolar concentration range, but with characteristics of open channel block. Furthermore, close examination of the data for α3β4 reveals that as the acetylcholine concentration is increased, the levamisole concentration needed to achieve the same degree of inhibition *decreases*. Estimates of IC_{50} from the data in Fig. 2 are 12, 3.8, 3.4 and 2.3 mM for the acetylcholine concentrations 25, 160, 300 and 1000 μM, respectively. This is completely in contrast to the expectation for a competitive mechanism, wherein more levamisole would be needed to displace acetylcholine at higher concentrations.

We focused on elucidating the mechanisms of potentiation and inhibition by levamisole for the α3β4 subtype because the two effects appeared to be clearly defined and separable. Nonetheless, several experiments of the type described using dihydro-β-erythroidine gave similar results for the α3β2 combination (not shown). In addition, although few sets of conditions led to absolute inhibition for α3β2 receptors, at high levamisole concentrations the "rebound" current was marked, and total charge analysis of the data revealed inhibition trends similar to those of α3β4 receptors. While the kinetic effects of high levamisole concentrations on α3β2 receptors may indicate more complex interactions, the observation of "rebound" currents suggests channel block. Therefore, while further experiments are needed to understand the importance of the current kinetics for levamisole effects, we consider the actions of the compound on α3β2 receptors to be fundamentally the same as those on the α3β4 subtype.

4.4. Pseudo-sites for nicotinic ligands

The large body of work indicating that binding sites for nicotinic receptor agonists and competitive antagonists are formed by neighboring α and non-α subunits (for reviews, see Karlin and Akabas, 1995; Galzi and Changeux, 1994) was recently substantiated by the crystal structure of the molluscan acetylcholine binding protein (Brejc et al., 2001). This structure also demonstrated that the subunits, each with a radial asymmetry or "polarity," were arrayed in a head-to-tail fashion. The overall homology of all nicotinic acetylcholine receptor subunits suggests the possibility that, in addition to the two sites for acetylcholine binding on a neuronal receptor of stoichiometry $(\alpha x)_2(\beta x)_3$ (Anand et al., 1991; Cooper et al., 1991), three pseudo-sites for nicotinic ligands are present. Support for this idea comes from studies of Maelicke et al. who found that physostigmine, which allosterically modulates the activity of nicotinic receptors (Pereira et al., 1993, 1994), can be crosslinked to lysine 125 of α1 subunits (Schrattenholz et al., 1993) and that this site does not overlap with the acetylcholine binding site (Pereira et al., 1993). This residue is highly conserved across nicotinic subunits, and occurs in one of the consensus, non-α, ligand-binding subsite regions (Tsigelny et al., 1997; Karlin and Akabas, 1995; Galzi and Changeux, 1994). That levamisole and other nicotinic allosteric modulatory agents (Maelicke and Albuquerque, 2000) potentiate through such pseudo-sites is an attractive hypothesis because the behavior of these ligands is reminiscent of the action of benzodiazepines on $GABA_A$ receptors, to which nicotinic receptors are closely related (Macdonald and Olsen, 1994; Karlin and Akabas, 1995). Our continuing work is aimed at developing rigorous models of the potentiation effects of levamisole and identifying determinants of its binding sites.

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