Investigating the Effects of Anthelmintic Compounds at the Site of Zinc Potentiation on Alpha4Beta4 Neuronal Nicotinic Acetylcholine Receptors

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INVESTIGATING THE EFFECTS OF ANTHELMINTIC COMPOUNDS AT THE SITE OF ZINC POTENTIATION ON α4β4 NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

By

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INVESTIGATING THE EFFECTS OF ANTHEMINTIC COMPOUNDS AT THE SITE OF ZINC POTENTIATION ON α4β4 NEURONAL NICOTINIC ACETYLCOLINE RECEPTORS

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Investigating the Effects of Anthelmintic Compounds at the Site of Zinc Potentiation on α4β4 Neuronal Nicotinic Acetylcholine Receptors

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Neuronal nicotinic acetylcholine receptors can have their function modulated by zinc. Depending on concentration and subunit composition, zinc either inhibits or potentiates receptor function. The zinc ion potentiates the α4β4 receptor at non-agonist binding interfaces or “pseudo sites.” Zinc potentiation is reduced if certain residues are mutated or spatially interfered with. The residue contributing most to this potentiation reduction effect is histidine 162 on the α4 subunit. The anthelmintic compound levamisole potentiates acetylcholine response of certain neuronal nicotinic receptors. Levamisole and its functional analogues morantel, oxantel, and pyrantel all were found to potentiate α4β4 receptors at low (µM) concentrations and inhibit them at high (mM) concentrations. Oxantel showed the greatest degree of potentiation, about a third of the maximal zinc potentiation measured. Oxantel was screened using the substituted cysteine accessibility method (SCAM) against the residue histidine 162 as well as nearby α4 residues histidine 61 and glutamate 59 and the β4 residue aspartate 195. Screening was carried out by mutating said residues into cysteine, followed by covalent linkage with a disulfide bridge of that residue with a methanethiosulfonate compound. SCAM experiments allowed testing of the effects of a single residue and the area
immediately adjacent to it. Receptors that lost zinc potentiation capacity from site-directed mutagenesis at the his 162 residue and subsequent methanethiosulfonate reaction still showed regular potentiation following oxantel treatment. Although these compounds exhibit similar biphasic potentiation dose-response curves as zinc, their mechanism for potentiation is not through the same mechanism.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2 METHODS</td>
<td>6</td>
</tr>
<tr>
<td>3 RESULTS</td>
<td>8</td>
</tr>
<tr>
<td>4 DISCUSSION</td>
<td>13</td>
</tr>
<tr>
<td>References</td>
<td>14</td>
</tr>
</tbody>
</table>
Chapter 1 - Background

The nicotinic acetylcholine receptors (nAChRs) are a large subfamily of neurotransmitter receptors that function as ligand-gated ion channels. All nAChRs belong to a larger superfamily of ligand-gated ion channel receptors including GABA-, glycine-, and serotonin-gated ion channels (Cockcroft et al., 1992). All nAChRs exist as pentamers of individually translated polypeptide chains. Many different permutations of subunit compositions exist leading to diverse pharmacology and a wide range of biophysical properties of nAChRs. These receptors’ functions range from modulatory roles at central nervous system synapses to direct mediation of fast synaptic transmission in the sympathetic peripheral nervous system (McGehee et al., 1995). A general division of the nAChR family is between those receptors found at the neuromuscular junction or neuronal nAChRs. Each class exhibits distinct subunit stoichiometry (Corringer et al., 2000). Neuronal nAChRs are implicated in numerous disorders, from the degenerative diseases Alzheimer’s and Parkinson’s, to conditions like schizophrenia and epilepsy (Lindstrom, 1997). Nicotinic receptors are also prominent in the studies of nicotine abuse (Dani et al., 1996).

Discerning what each specific subunit contributes to overall receptor function is a formidable task. Different methods helped establish the current understanding of which subunits are implicated in which functions. Photoaffinity labeling studies performed on muscle nAChRs with a variety of compounds
indicate binding sites for nicotinic agonists and competitive antagonists are located at interfaces between the subunits, with one subunit exhibiting much higher affinity. Experiments using the competitive antagonists p-(dimethylamino)benzenediazonium fluoroborate (DDF) (Dennis et al., 1988), d-tubocurarine (dTC) (Pedersen et al., 1990), and α-bungarotoxin (Oswald et al., 1982) and the agonist nicotine (Middleton et al., 1991) all show probes mainly labeling the α subunit. The δ and γ subunits are labeled as well, but only at about 10%-25% of α subunit labeling. This data lead to the categorization of the α subunit as “principal components” and the δ/γ subunits as “complementary components” (Corringer et al., 1995). Both the principal and complementary components are comprised of three amino acid loops, A, B, and C for the principal and D, E, and F for the complementary. Upon comparison of amino acid sequence between the muscle nAChRs and neuronal nAChRs, the A, B, C, and D loops are conserved across all subunits, the F loop is conserved across some subunits, and the E loop is highly variable (Corringer et al., 2000). Site-directed mutagenesis studies indicated which specific residues within these loops contribute to the ACh binding site in neuronal α7 nicotinic receptors (Galzi et al., 1991). NAcHR subunit genes can be split into the classes of α subunits and non-α subunits. The α subunits posses two adjacent cysteine residues that are required for ACh binding. The non-α subunits represent all other subclasses of subunit, β, γ, δ, and ε (Sargent, 1993).

The acetylcholine binding protein (AChBP) is a homopentameric soluble protein released from glial cells of the snail *Lymnaea stagnalis* to modulate
synaptic transmission (Smit et al., 2001). Each AChBP monomer is closely related to α subunits of nAChRs. All residues important to ligand binding in nAChRs are conserved in the AChBP. The extracellular domain of nAChRs can be compared directly to the soluble AChBP because the transmembrane and intracellular domains do not contribute to ligand binding. This is shown in chimera experiments joining the N-terminal domain of the α7 nAChR to the transmembrane and cytoplasmic regions of the 5HT3 receptor leading to channel activation by ACh (Eisele et al., 1993). The resolved crystal structure of the AChBP (Brejc et al., 2001) confirms the idea of agonist binding sites at the subunit interfaces. The AChBP structure indicates a rotational symmetry allowing five agonist binding sites, with each subunit contributing a principal and complementary component on opposite sides. This confirms the predicted agonist binding of the homomeric α7 neuronal nAChR (Corringer et al., 1995).

Most neuronal nAChRs are heteromeric though, and based upon the stoichiometric contributions of each subunit type, only two agonist binding sites are formed. These sites are at the interfaces of appropriate sides of dissimilar subunits, such as the side of the α4 subunit containing the A, B, and C loops interfacing with the side of the β4 subunit containing the D, E, and F loops. This means that in the heteromeric receptors, there are three interfaces not involved in binding agonist. Studies have shown ionic zinc (Hsiao et al., 2006) potentiates receptor activity at this site. The potentiation shown from the zinc ion acting at subunit interfaces indicates these interfaces act as “pseudo sites”, similar to the
site of action of benzodiazepines on the closely related GABA<sub>A</sub> receptors (Karlin et al., 1995).

The site of potentiation of Zn<sup>2+</sup> is of great interest due to the strong potentiation shown on certain neuronal nAChRs, depending on the subunit composition. Zn<sup>2+</sup> causes inhibition of the ACh response of some neuronal nAChRs. Other neuronal nAChRs have their ACh response potentiated by Zn<sup>2+</sup> at low concentrations and inhibited at higher concentrations (Hsiao et al., 2001). Previous work has mapped the Zn<sup>2+</sup> site by protein modeling combined with site-directed mutagenesis and the substituted cysteine accessibility method (SCAM). Analysis of different subunit combinations narrowed the potentiation effects of Zn<sup>2+</sup> to the α4, β4, and β2 subunits (Hsiao et al., 2006). The α4β4 nAChRs show the greatest magnitude of zinc potentiation and least desensitization, so they were chosen for this study. Zn<sup>2+</sup> is not viable for use as a therapeutic, so other compounds acting at the same site to cause potentiation are of great interest.

Levamisole is an anthelmintic compound that acts to paralyze nematodes through action on the muscle nAChRs in these species (Martin et al., 1997). Studies on neuronal nAChRs indicate levamisole can both inhibit and potentiate receptors, depending on concentration and the subunit composition of the receptor. Experiments detailing levamisole pharmacology on α3β2 and α3β4 receptors show both have ACh responses that are potentiated but the magnitude is greater for the α3β2 receptors (Levandoski et al., 2003). The actual mechanism of this action is unknown. This makes levamisole and similar compounds intriguing to investigate at the zinc site.
We used site-directed mutagenesis combined with the SCAM to screen levamisole and its functional analogues morantel, pyrantel, and oxantel on α4β4 neuronal nAChRs to determine if they act at the zinc site. The specific residues used were the ones showing greatest loss of potentiation upon mutation and covalent linkage to a methanethiosulfonate (MTS) reagent (Hsiao et al., 2006). The residue with the greatest degree of zinc potentiation loss is α4H162. The others exhibiting significant loss of zinc potentiation are α4H61 and α4E59. We also screened the residue β4D195 due to its proximity to the zinc site and the indication that the β subunit might be important in levamisole potentiation (Levandoski et al., 2003). Results obtained indicate that these compounds show similar biphasic dose-response curves of ACh potentiation, with oxantel potentiating to the greatest degree. Oxantel does not show the loss of potentiation that zinc does after mutagenesis at the H162 residue and SCAM treatment.
Chapter 2 – Methods

**Molecular Biology.** Rat neuronal nAChR subunit cDNAs were cloned and ligated into plasmids. Plasmids were linearized and capped cRNA transcripts were generated using mMessage mMACHINE kits (Ambion, Austin, TX). All mutations were previously generated (Hsiao et al., 2006) using the GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI) and confirmed by sequencing.

**Oocyte Preparation and Injection.** Mature *Xenopus laevis* frogs (Nasco, Fort Atkinson, WI) were anesthetized and oocytes were surgically removed. Follicle cells were removed by 2 hour treatment at room temperature by with Collagenase B (Roche Diagnostics, Indianapolis, IN). Stage V oocytes were injected with 5-15ng of each cRNA in 20-50nL of water and incubated at 18°C in Barth’s saline solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM CaNO₃, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 100 µg/ml gentamicin, 15 mM HEPES, pH 7.6) for 3 to 7 days.

**Electrophysiology and Data Analysis.** All experiments were conducted using an OpusXpress 6000A Parallel Oocyte Voltage Clamp system running OpusXpress 1.1 and Clampfit 9.1 software (Molecular Devices). Micropipettes for OpusXpress microelectrodes were filled with 3M KCl and had resistances between 0.3 and 1.8 MΩ. Holding potential for the voltage clamp was -70mV in all experiments. Current responses were filtered (4-pole, Bessel, low pass) at 20 Hz (-3 db) and sampled at 100 Hz. Oocytes were perfused with ND-96 (96mM
NaCl, 2mM KCl, 1mM CaCl₂, 1mM MgCl₂, 5mM HEPES, pH 7.5) at room temperature (20-25°C). All Zn²⁺-containing solutions were prepared from a 1M stock of Zn(CH₃COO)₂. Concentrations of solutions used were 1µM ACh, 100µM Zn²⁺, and 1µM oxantel. Optimal ACh and Zn²⁺ concentrations were determined previously (Hsiao et al., 2006) and oxantel was used at the concentration showing the greatest potentiation of the ACh response (fig. 1). All anthelmintic compounds were purchased from Sigma-Aldrich (St. Louis, MO). In the SCAM experiments, we first measured zinc potentiation, followed by measurement of oxantel potentiation. Then 1mL of 1mM MTSET (Toronto Research Chemicals, Inc., North York, ON, Canada) was applied for 2 minutes, followed by a 5 minute wash with perfusion solution before re-measuring potentiation.

All data analysis was performed with Prism 4 software (GraphPad, San Diego, CA). Statistical significance was assessed using one-way analysis of variance followed by the Dunnett’s post-test.
Chapter 3 – Results

I first determined the effects of the compounds levamisole, morantel, pyrantel, and oxantel on ACh response of α4β4 receptors. Each compound exhibited a biphasic curve from potentiating ACh response to inhibiting, with peaks at different levels (Fig. 1). ACh concentration was 1µM in all experiments, and the compounds were analyzed from 100nM to 300µM. Oxantel showed the greatest degree of potentiation, reaching about three times the original ACh response at a concentration between 1 and 3µM. We decided to use oxantel for the SCAM experiments due to this pronounced superiority in magnitude of potentiation achieved.

I screened oxantel at the zinc site of the mutant receptors α4-2H162Cβ4C75S (α4H162), α4-2H61Cβ4C75S (α4H61), α4-2β4C75SD195C (β4D195) and α4-2E59Cβ4C75S (α4E59) as well as wild type α4β4 and pseudo-wild type (α4-2β4C75S). The pseudo-wild type showed the same ACh response and zinc potentiation of the wild-type. It was necessary to create the pseudo-wild type receptor because the free cysteine residues present on the wild-type need to be removed for the SCAM experiments due to potentially confounding effects of the MTS reagent. The mutants used were chosen based upon previous work indicating they were most involved in coordinating Zn$^{2+}$ ions (Hsiao et al., 2006). Inward current response to ACh treatment was lower after MTSET treatment, indicating a lower number of total receptors responding. The degree of
potentiation induced by both compounds after MTSET treatment was the same for pseudo-wild type (Fig. 2A). The mutant receptors showed a lower degree of zinc potentiation even before MTSET treatment, but the zinc potentiation was significantly decreased after the treatment for the α4H162 mutant, while the oxantel potentiation was unchanged (Fig.2B). The α4H162 mutant was the only one to show statistically significant decrease in zinc potentiation after treatment with MTSET (Fig. 3A). Oxantel potentiation was not significantly altered in any of the mutants before or after treatment with MTSET (Fig. 3B). All potentiation figures shown are normalized to the percent of the pre-MTSET treatment potentiation of ACh response. Although slight variability is observed, the only statistically significant difference is the zinc potentiation reduction at the residue α4H162.
Fig 1. Dose response curves for anthelmintic compounds used. All values normalized to percent of current response shown from ACh treatment alone. Concentrations ranging from 100nM to 300µM
Fig. 2. Representative traces of zinc and oxantel potentiation of ACh response. In A and B, zinc potentiation measured followed by oxantel potentiation. After MTSET treatment and wash, measurement of zinc followed by oxantel potentiation again. Thin line, ACh application. Thick line, zinc application. Dotted line, oxantel application. Arrow, MTSET application.
Fig. 3. Effect of MTSET reagent on potentiation. All ACh concentrations 1µM. A, zinc potentiation after MTSET treatment as percentage of pre-MTSET potentiation (mean ± S.E.M., n = 3-8). All zinc concentrations 100µM. B, oxantel potentiation after MTSET treatment as percentage of pre-MTSET potentiation. All oxantel concentrations 1µM. Red line indicates control (pseudo-wild type) response. (⁎, p<0.05)
Chapter 4 – Discussion

Previous work has identified residues on the α4β4 receptor at or near the site of Zn^{2+} action of potentiation (Hsiao et al., 2006). The specific residue α4His162 is involved in coordinating the zinc ion directly. This allowed us to use this as a control for investigating the capability of other compounds to potentiate α4β4 receptors at this site. Anthelmintic compounds have been shown to potentiate and inhibit nAChRs (Levandoski et al., 2003). We have shown the anthelmintic compound levamisole and its functional analogues morantel, pyrantel, and oxantel all exhibit biphasic potentiation-inhibition of ACh response reminiscent of Zn^{2+} effects (Fig. 1). SCAM experiments screening oxantel against α4His162 and the residues in closest proximity to it show no change in potentiation from oxantel (Fig. 3). We can conclude that despite the interesting effects of these compounds on α4β4 neuronal nAChRs they do not act through the zinc site. Since the zinc site is a “pseudo” ligand-binding site and holds the most promise for a target for potential therapeutics, this removes these anthelmintic compounds from being considered for this purpose.
References


