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ACTIONS OF VESAMICOL ON AN α-BUNGAROTOXIN-SENSITIVE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR

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Summary

Electrophysiology and binding studies were used to determine the actions of vesamicol [2-(4-phenylpiperidino)cyclohexanol, (AH5183)] on an α-bungarotoxin-sensitive, neuronal nicotinic acetylcholine receptor in the nervous system of the cockroach, *Periplaneta americana*. Electrophysiological studies on an identified motor neurone revealed a reversible blocking action of (±)-vesamicol on the response to ionophoretically applied acetylcholine with an IC_{50} value of 8.0×10^{-6} mol l^{-1}. The block was weakly voltage-dependent over the membrane potential range of −50mV to −90mV, and appeared to be non-competitive. No difference in potency was observed between the resolved stereoisomers. (±)-Vesamicol was found to suppress specific binding of ^{125}I-labelled α-bungarotoxin to cockroach nervous tissue with an IC_{50} value of 5.1×10^{-3} mol l^{-1} and an estimated Hill coefficient of 0.73. Differences in the Hill coefficients were found when the resolved stereoisomers were tested separately. These data provide the first demonstration of a blocking action by vesamicol of a neuronal nicotinic acetylcholine receptor.

Introduction

Vesamicol, 2-(4-phenylpiperidino)cyclohexanol, also known as AH5183, exists in two stereoisomeric forms (Fig. 1). Studies on the action of vesamicol at vertebrate peripheral and central cholinergic synapses have revealed two sites of action. The first of these, observed by Marshall (1970), is presynaptic. Vesamicol inhibits vesicular acetylcholine storage, acting in a stereoselective, non-competitive manner at a site distinct from the transport ATPase and the acetylcholine transporter of synaptic vesicle membranes (Marshall and Parsons, 1987). Similar findings have been reported for cholinergic nerve terminals of rat cerebral cortex (Jope and Johnson, 1986). Enomoto (1988) has detected a postsynaptic blocking action of vesamicol on the decay of the end-plate current recorded

Key words: vesamicol (AH5183), nicotinic acetylcholine receptor, identified neurone, insect, *Periplaneta americana.*

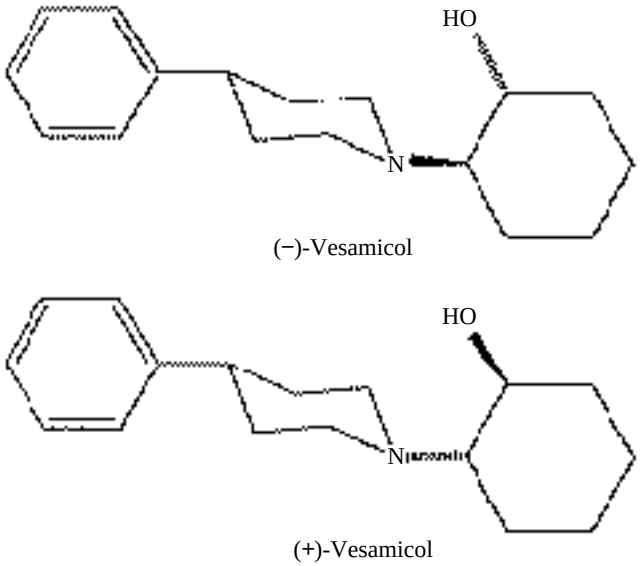

Fig. 1. Chemical structure of vesamicol, also known as AH5183, showing (+) and (−) stereoisomers.

from a vertebrate neuromuscular synapse. This suggests a second, direct, postsynaptic receptor or ion channel site of action.

To explore this possible receptor action further we have examined for the first time the actions of vesamicol on a neuronal nicotinic acetylcholine receptor, using a tissue source that is amenable to both electrophysiological and ligand-binding experiments – the insect central nervous system. This is one of the richest sources of neuronal, α-bungarotoxin-sensitive, nicotinic acetylcholine receptors (Sattelle, 1980; Breer and Sattelle, 1987). Furthermore, identified cell bodies in the nervous system of the cockroach *Periplaneta americana* provide a convenient electrophysiological preparation for this study. These cell bodies receive no direct presynaptic innervation, yet contain well-characterized, extrasynaptic nicotinic acetylcholine receptors that are sensitive to α-bungarotoxin (Sattelle *et al.* 1980; David and Sattelle, 1984) and κ-bungarotoxin (Pinnock *et al.* 1988). Thus, the preparation allows a detailed study of vesamicol–receptor interactions in the nervous system without interference from possible presynaptic actions.

In addition to providing a source of identifiable neurones for electrophysiological studies, cockroach nervous tissue is also suitable for radioligand binding studies on nicotinic acetylcholine receptors (Lummis and Sattelle, 1985). This study applies electrophysiology and radioligand binding to the same tissue to examine the actions of (±)-vesamicol and its resolved stereoisomers on nervous system nicotinic acetylcholine receptors.

Materials and methods

All experiments were performed on adult male cockroaches (*Periplaneta americana*) reared at 27˚C, with free access to food and water.

Electrophysiology

The cell body of the fast coxal depressor motor neurone (D_f) was located visually in isolated, desheathed, metathoracic ganglia and, following desheathing, was mounted under saline in a Perspex experimental chamber (total volume 0.5ml). The cell body of motor neurone D_f was impaled by two microelectrodes filled with 2.0mol l^{-1} potassium acetate, of resistance (*R*) 10–15 MΩ. Changes in membrane potential and input resistance were recorded as described elsewhere (David and Sattelle, 1984). Current passing through the experimental chamber was monitored using a virtual-earth circuit. Vesamicol was applied into the chamber and acetylcholine was applied ionophoretically to the surface of motor neurone D_f from micropipettes filled with a 1.0mol l^{-1} aqueous solution of acetylcholine chloride (*R*=5–15 MΩ). In all cases, a retaining current of 10–50nA was used to prevent leakage of the drug from the ionophoretic pipette. Cells were bathed in normal saline for 20min prior to any drug application. In voltage-clamp experiments, the clamp current was recorded directly from a Dagan 350 voltage-clamp amplifier. The gain of the voltage-clamp was set between 4×10^3 and 6×10^3. The normal saline used throughout these experiments had the following composition: NaCl, 214.0mmol l^{-1}; $CaCl_2$, 9.0mmol l^{-1}; KCl, 3.1mmol l^{-1}; Tes, 10.0mmol l^{-1} (pH7.2 adjusted with 1.0mol l^{-1} NaOH). During an experiment, the preparation was continuously superfused with normal saline to which vesamicol or other ligands were subsequently added.

Preparation of nervous system extracts

Nerve cords, containing thoracic and abdominal ganglia together with interganglionic connectives, were removed from the cockroach and stored on ice. Nervous tissue from 10–15 animals was homogenized in a glass, hand-held homogenizer in cockroach saline (see above) buffered with 10mmol l^{-1} phosphate buffer (pH7.2) instead of Tes. Samples yielding a final concentration of 0.2–0.3mg ml^{-1} protein were used in the binding assay.

Ligands for pharmacological studies

Ligands were dissolved in saline immediately prior to use. (±)-Vesamicol, and its purified stereoisomers (−)-vesamicol and (+)-vesamicol, were obtained from Research Biochemicals Inc. Unlabelled α-bungarotoxin was obtained from the Miami Serpentarium Laboratories, USA. Acetylcholine chloride, carbamylcholine chloride and nicotine were obtained from Sigma Chemical Co. Ltd, UK.

Filter binding assay

Samples of homogenate (20–30 μg) were incubated at 21°C in normal saline containing 2.0nmol l^{-1} ^{125}I-labelled α-bungarotoxin (135Ci mmol^{-1}, New England Nuclear) in a total volume of 100 μl. After 60min of incubation, the assay mixture was filtered under reduced pressure through wet glassfibre (GF/B) discs (Whatman), which were then washed once with 2.0ml of ice-cold phosphate-buffered saline. Determinations of binding were performed in triplicate, and triplicate samples containing unlabelled α-bungarotoxin (1.0×10^{-6} mol l^{-1}) or nicotine (1.0×10^{-4} mol l^{-1}) were used to determine non-specific binding. Radioactivity was measured in a Beckman Gamma 5500 counter.

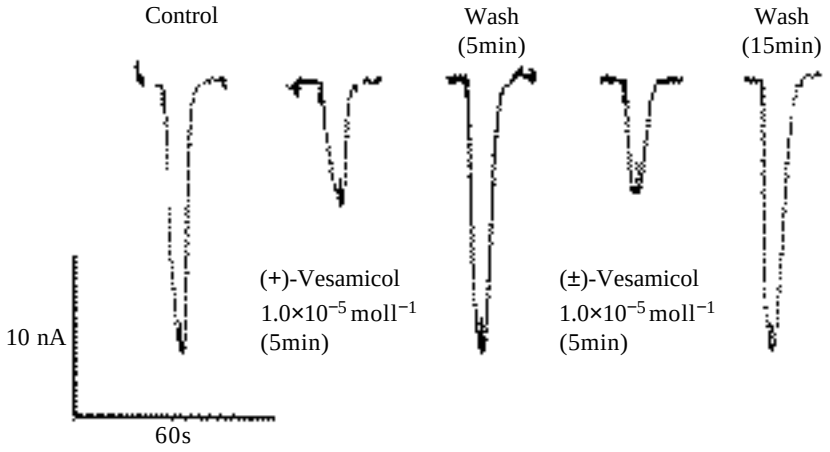

Fig. 2. Bath-applied (+)-vesamicol and (±)-vesamicol at concentrations of 1.0×10^{-5} mol l^{-1} are equally effective in reversibly reducing the inward current recorded under voltage-clamp conditions from motor neurone D_f in response to ionophoretically applied acetylcholine.

Protein assay

Total protein was assayed by the method of Bradford (1976), using bovine serum albumin (Sigma Chemical Co., UK) as a standard.

Results

Actions of vesamicol on the α-bungarotoxin-sensitive, neuronal nicotinic acetylcholine receptor of an identified insect motor neurone

Cholinergic chemosensitivity of the cell body of motor neurone D_f was assayed by ionophoretic application of acetylcholine in the presence or absence of bath-applied (±)-vesamicol. Voltage-clamp experiments showed that concentrations up to 1.0×10^{-6} mol l^{-1} (±)-vesamicol were ineffective, but at higher concentrations the acetylcholine-induced inward current was suppressed (Fig. 2). A similarly effective block was observed when the (+) stereoisomer was tested. Following a 5min application of 1.0×10^{-3} mol l^{-1} (±)-vesamicol, a complete block of the acetylcholine-induced inward current was observed. At 1.0×10^{-5} mol l^{-1} (±)-vesamicol, the partial block of the acetylcholine-induced response was fully reversible on rebathing the preparation in normal saline (Fig. 2).

In a separate series of experiments, membrane potential changes were recorded in response to ionophoretic applications of increasing doses of acetylcholine in the presence and absence of bath-applied (+)-vesamicol. Reduction of the peak amplitude of the response was accompanied by only a slight shift to the right of the dose–response curve (Fig. 3). Similar data were obtained for (±)-vesamicol and (−)-vesamicol (Fig. 4). Thus, vesamicol and its resolved stereoisomers show reversible, non-competitive block of the α−bungarotoxin-sensitive nicotinic receptors of motor neurone D_f. From a dose–response

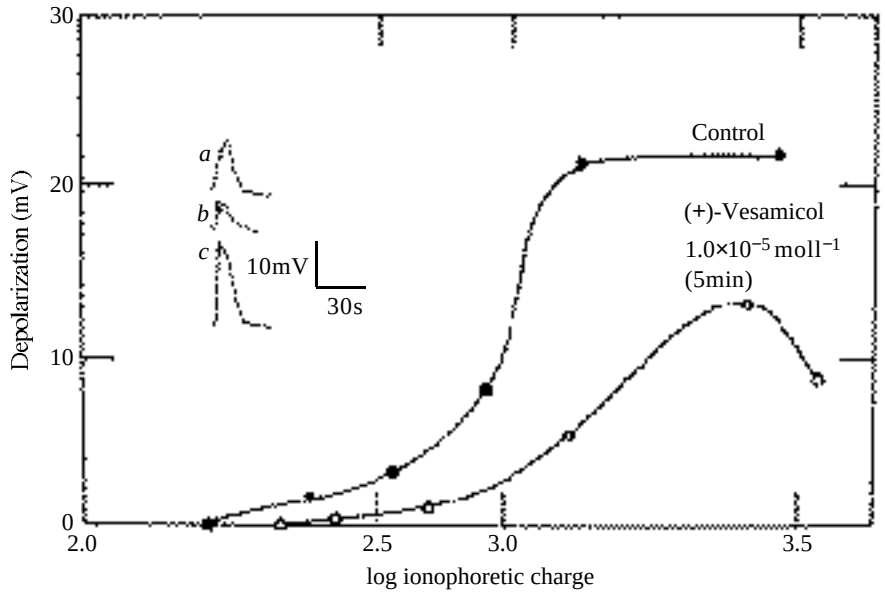

Fig. 3. Bath application of (+)-vesamicol at a concentration of 1.0×10^{-5} moll^{-1} reduces the amplitude of responses to ionophoretically applied acetylcholine. Data shown are from a single cell and are typical of three separate experiments. The decline in the response at the maximal dose observed in the presence of (+)-vesamicol is not a consistent feature of all preparations. Similar results were obtained for the (−) stereoisomer (*N*=2). (*a*) Depolarization induced by a 1000nA pulse of ionophoretically applied acetylcholine in normal physiological saline. (*b*) Depolarizing response to a 1000nA dose of acetylcholine after a 20min exposure to 1.0×10^{-5} moll^{-1} (+)-vesamicol. (*c*) Response to a 1000nA dose of acetylcholine after a 20min wash in normal saline. The ionophoretic charge was measured in nanocoulombs.

curve of inhibition of the functional nicotinic receptor by (±)-vesamicol and its stereoisomers, an IC_{50} of 8.0×10^{-6} moll^{-1} was estimated (Fig. 4).

Partial block of the response to acetylcholine was detected at 1.0×10^{-5} moll^{-1} (±)-vesamicol (Fig. 5A) and this concentration was used to examine the voltage-dependence of the blocking action of (±)-vesamicol. Blocking by (±)-vesamicol was measured under voltage-clamp conditions (*N*=3) and was found to be only weakly voltage-dependent over the range of membrane potential −50mV to −90mV (Fig. 5B). Similar findings were obtained for (+)-vesamicol (*N*=3) and (−)-vesamicol (*N*=3).

Actions of vesamicol on ^{125}I-labelled α-bungarotoxin binding sites in the insect nervous system

(±)-Vesamicol suppressed the specific binding of ^{125}I-labelled α-bungarotoxin to cockroach nervous system membranes at concentrations above 1.0×10^{-5} moll^{-1}, and at 1.0×10^{-2} moll^{-1} inhibited most of the specific ^{125}I-labelled α-bungarotoxin binding. An IC_{50} of 5.1×10^{-3} moll^{-1} and a Hill coefficient of 0.73 were estimated from the binding curve. When the purified (+) and (−) stereoisomers were tested separately, both were found to be effective as inhibitors of ^{125}I-labelled α-bungarotoxin binding (Fig. 6). An

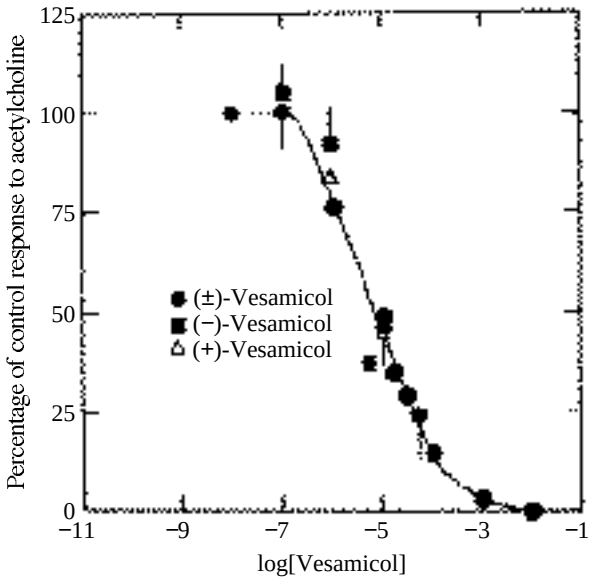

Fig. 4. Reduction of the response to ionophoretically applied acetylcholine is dependent upon the concentration (measured in $mol\,l^{-1}$) of the bath-applied vesamicol. Data for the pure stereoisomers overlie data for the racemic mixture. Points are taken from 26 separate experiments. Individual data points are shown, except in cases where three preparations were tested with the same concentration of ligand, when the mean and one standard error are shown.

IC_{50} value of $7.6\times10^{-3}\,mol\,l^{-1}$ and a Hill coefficient of 1.53 were determined for the (−) isomer and corresponding values for IC_{50} of $2.2\times10^{-3}\,mol\,l^{-1}$ and a Hill coefficient of 0.55 were obtained when (+)-vesamicol was investigated.

Thus, inhibition of ^{125}I-labelled α-bungarotoxin binding to central nervous system membranes was observed at concentrations of vesamicol considerably higher than those required to block functional α-bungarotoxin-sensitive neuronal nicotinic receptors.

Discussion

This study, using electrophysiological and ligand-binding techniques on insect nervous tissue, has shown that (±)-vesamicol and its resolved stereoisomers are all reversible, non-competitive blockers of a central nervous system, α-bungarotoxin-sensitive, nicotinic acetylcholine receptor. Vesamicol has long been known to act on a presynaptic site at vertebrate neuromuscular (Gandiha and Marshall, 1973; Marshall and Parsons, 1987; Estrella *et al.* 1988) and central synapses (Carroll, 1985; Collier *et al.* 1986; Melega and Howard, 1984; Jope and Johnson, 1986; Ricny and Collier, 1986; Suszkiw and Toth, 1986) and in *Torpedo* electric organ (Bahr and Parsons, 1986; Anderson *et al.* 1983), where it acts in a stereospecific, non-competitive manner to inhibit the transport of newly synthesized acetylcholine into synaptic vesicles. Recently, a postsynaptic blocking action of vesamicol has been observed at the frog neuromuscular junction (Enomoto, 1988).

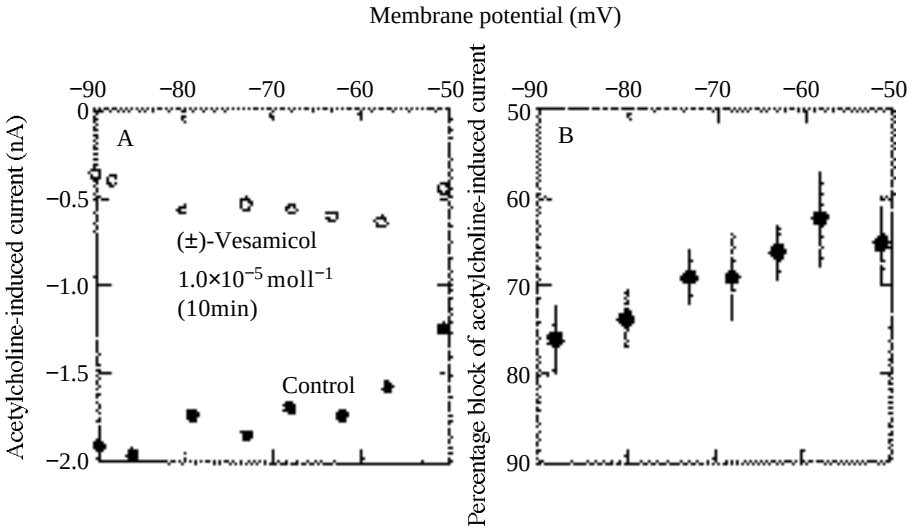

Fig. 5. Voltage-dependence of the block of acetylcholine-induced current by 1.0×10^{-5} mol l^{-1} (±)-vesamicol. (A) Acetylcholine-induced currents at different membrane potentials are recorded in the voltage-clamped cell before (●) and after (○) exposure to 1.0×10^{-5} mol l^{-1} (±)-vesamicol for 10min. These acetylcholine-induced currents were obtained by subtracting the control membrane currents from the membrane currents observed during ionophoretic application of acetylcholine. (B) Percentage block of acetylcholine-induced currents in the voltage-clamped cell at different membrane potentials. Data plotted are mean values ± standard error (*N*=3).

This report demonstrates a central action of vesamicol on a neuronal membrane that has no direct presynaptic inputs. The nicotinic-receptor-blocking actions of vesamicol in the cockroach nervous system are similar to those described for the frog muscle. Enomoto (1988) reported an approximately 66% reduction of the acetylcholine-induced end-plate current (EPC) at the frog neuromuscular junction produced by a 3.0×10^{-5} mol l^{-1} infusion of (±)-vesamicol. This compares with a value of 30% obtained for motor neurone D_f exposed to 5.0×10^{-5} mol l^{-1}, although in the case of the insect receptor, a complete block at higher concentrations was also demonstrated. No directly comparable dose–response data were provided for the frog preparation. Interestingly, Enomoto (1988) reported that (±)-vesamicol was more effective as an inhibitor of the response to ionophoretically applied acetylcholine than it was in suppressing the evoked end-plate current and the miniature end-plate potentials at low (0.5Hz) stimulation frequencies.

The receptor-blocking action of (±)-vesamicol on the cockroach D_f motor neurone is non-competitive and exhibits weak voltage-dependence. The low potency of (±)-vesamicol at inhibiting ^{125}I-labelled α-bungarotoxin binding supports this hypothesis. However, suppression of ^{125}I-labelled α-bungarotoxin binding by (±)-vesamicol at high concentrations indicates that there may also be a weak effect at, or close to, the recognition site. The low Hill coefficients obtained for (±)-vesamicol and one purified stereoisomer provide some support for the view that (±)-vesamicol may act at more than one site.

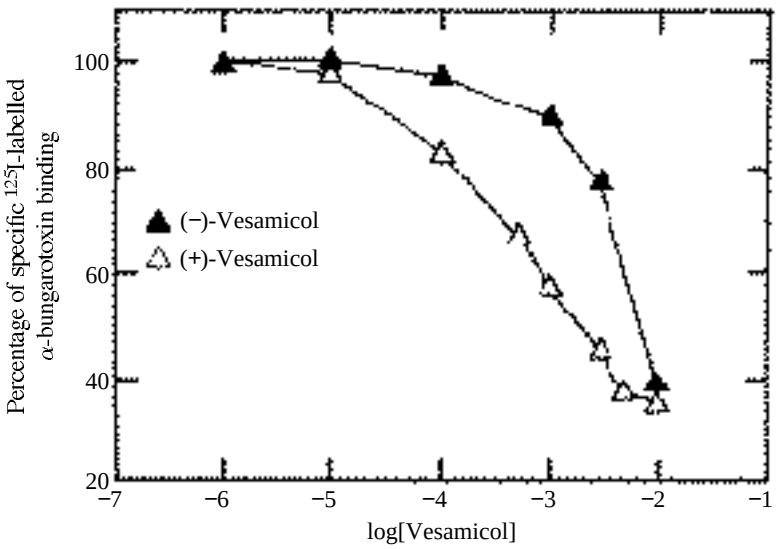

Fig. 6. Both (−)-vesamicol (▲) and (+)-vesamicol (△) inhibit specific binding of ^{125}I-labelled α-bungarotoxin to extracts of cockroach nerve cord. Non-specific binding is determined in the presence of 1.0×10^{-6} mol l^{-1} α-bungarotoxin and is subtracted from the control binding to yield percentage specific binding. 100% binding is determined in the absence of vesamicol. The result is typical of three experiments, each performed in triplicate. Vesamicol concentration is measured in mol l^{-1}.

Radiolabelled α-bungarotoxin binds with high affinity to sites in the cockroach nervous system (Gepner *et al.* 1978; Lummis and Sattelle, 1985). As shown in the present study, inhibition of an ^{125}I-labelled α-bungarotoxin-sensitive neuronal nicotinic receptor by vesamicol is detected at higher concentrations than in physiological studies. Although α-bungarotoxin is inactive on most neuronal nicotinic acetylcholine receptors of vertebrates, in insects it has been shown to block acetylcholine-induced responses at nanomolar concentrations (Sattelle, 1980). The toxin binding site is a constituent of functional neuronal nicotinic acetylcholine receptors at postsynaptic (Sattelle *et al.* 1983) and cell body (David and Sattelle, 1984) locations.

The blocking action of vesamicol on motor neurone D_f lacks any stereospecificity, although the (+)-isomer is more potent in the binding assay. This is in marked contrast to the transport-blocking activity of vesamicol, which is without exception restricted to the (−) isomer. It is of interest that, in the ^{125}I-labelled α-bungarotoxin binding experiments reported here, the (+) isomer reveals a Hill coefficient of 0.55 whereas the (−) isomer yields a Hill coefficient closer to 1.5. This suggests a different action of the two isomers at the receptor site, although there are no apparent differences in their actions on the nicotinic-receptor-operated ion channel. It will be of interest to examine vesamicol actions on vertebrate neuronal nicotinic receptors, most of which are pharmacologically distinguishable from the invertebrate receptor described here by their insensitivity to α-bungarotoxin (see Lindstrom *et al.* 1987). The block by vesamicol observed in the present

study, using both biochemical and physiological methods, is the first demonstration of a blocking action of this ligand at a neuronal nicotinic acetylcholine receptor.

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