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VOLTAGE-INDEPENDENT BLOCK OF A NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR BY *N*-METHYL LYCACONITINE

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Summary

N-Methyl lycaconitine is the most effective low molecular weight antagonist reported for an insect neuronal nicotinic receptor. In the present study, the citrate salt of this neurotoxin from the plant *Delphinium brownii* was found to inhibit [^{3}H]-α-bungarotoxin binding to nerve cord extracts of the cockroach *Periplaneta americana* with a K_i of $1{\cdot}4\times10^{-9}$ mol l^{-1}. At a concentration of $1{\cdot}0\times10^{-7}$ mol l^{-1}, *N*-methyl lycaconitine completely blocked the response to ionophoretically applied acetylcholine recorded from the cell body membrane of the fast coxal depressor motor neurone (D_f) in the desheathed metathoracic ganglion of the cockroach. The block was voltage-independent over the range of membrane potential -100 to -30 mV. The effectiveness of *N*-methyl lycaconitine on the nicotinic receptor, which is present in very high concentrations in the insect nervous system, suggests that this alkaloid is a natural plant protection agent.

Introduction

The principal toxin of the plant *Delphinium brownii* is the alkaloid *N*-methyl lycaconitine (Fig. 1) and its blocking actions on vertebrate cholinergic pathways have been described (Nambi Aiyar *et al.* 1979). By means of an external-electrode recording method based on the sucrose-gap technique of Callec & Sattelle (1973), it has recently been shown that micromolar concentrations of *N*-methyl lycaconitine block synaptic transmission at cholinergic synapses of the cockroach *Periplaneta americana* (Jennings *et al.* 1987). The same authors showed the alkaloid to be an inhibitor of [^{3}H]-α-bungarotoxin binding sites in housefly (*Musca domestica*) head extracts. However, it is not known if synaptic blockade at cockroach nicotinic cholinergic synapses (Sattelle, 1978; Sattelle *et al.* 1983) is due to pre- or postsynaptic actions of the toxin. Though an action on a putative nicotinic cholinergic receptor can be inferred, there is no direct evidence for either

Key words: *N*-Methyl lycaconitine, neuronal nicotinic receptors, acetylcholine receptors, voltage-independent block, identified motor neurone, insect (*Periplaneta americana*).

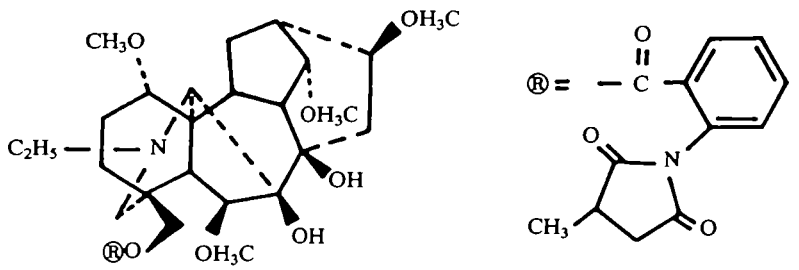

Fig. 1. Chemical structure of *N*-methyl lycaconitine, the principal toxin of *Delphinium brownii*.

an agonist action or an antagonist effect at a functional insect nicotinic acetylcholine receptor.

To test for a receptor action of *N*-methyl lycaconitine, its actions are examined on the nicotinic acetylcholine response (David & Sattelle, 1984) recorded from the cell body membrane of the cockroach fast coxal depressor motor neurone (D_f). A population of nicotinic acetylcholine receptors that are blocked by α-bungarotoxin and κ-bungarotoxin account for the cholinergic chemosensitivity of the cell body membrane of this motor neurone (David & Sattelle, 1984; Pinnock *et al.* 1988). The cell body receives no direct synaptic input, thereby facilitating interpretation of pharmacological data. Voltage-clamp techniques can be applied, and this method, in combination with radioligand binding experiments on the same insect tissue, permits a detailed analysis of neuronal nicotinic acetylcholine receptor actions of *N*-methyl lycaconitine.

Materials and methods

Experimental animals

Adult male cockroaches (*Periplaneta americana*) were used throughout the investigation. They were obtained from cultures maintained at 27°C with access to unlimited food and water.

Radioligand binding

Full details of the methods for examining the binding of [^{3}H]-α-bungarotoxin to homogenized cockroach nerve cords (abdominal and thoracic ganglia) have been described elsewhere (Lummis & Sattelle, 1985). *N*-[Propionyl-^{3}H]propionylated α-bungarotoxin was used for determinations of binding, and samples containing unlabelled α-bungarotoxin ($1{\cdot}0\times10^{-6}$ mol l^{-1}) or nicotine ($1{\cdot}0\times10^{-4}$ mol l^{-1}) were used to determine non-specific binding. Inhibition studies with *N*-methyl lycaconitine and other cholinergic ligands were performed by adding a constant amount of nerve cord homogenate to assay tubes already containing 2·0 nmol l^{-1} [^{3}H]-α-bungarotoxin and the test ligand at various concentrations. Such mixtures were incubated at 22°C for 60 min prior to filtering. The concentration of ligand

required for 50% inhibition of specific [^{3}H]-α-bungarotoxin binding (IC_{50}) was determined graphically, and then K_i was calculated for each ligand using the equation:

$$K_i = IC_{50}/(1 + [L]/K_d),$$

where $[L] = 2{\cdot}0\,\text{nmol}\,l^{-1}$ and K_d (the dissociation constant) for [^{3}H]-α-bungarotoxin $= 4{\cdot}3\,\text{nmol}\,l^{-1}$ (Lummis & Sattelle, 1985).

Electrophysiology

The isolated, desheathed metathoracic ganglion of the cockroach *Periplaneta americana* was mounted under saline (composition: $214\,\text{mmol}\,l^{-1}$ NaCl; $3\,\text{mmol}\,l^{-1}$ KCl; $5\,\text{mmol}\,l^{-1}$ $CaCl_2$; $4\,\text{mmol}\,l^{-1}$ $MgCl_2$; $50\,\text{mmol}\,l^{-1}$ sucrose; $10\,\text{mmol}\,l^{-1}$ TES; pH 7·4) in a Perspex experimental chamber (total volume 0·3 ml). The cell body of the fast coxal depressor motor neurone D_f (Pearson & Iles, 1970, 1971) was located visually and then impaled with two microelectrodes (filled with $0{\cdot}5\,\text{mol}\,l^{-1}$ K_2SO_4, $5{\cdot}0\,\text{mmol}\,l^{-1}$ KCl) each of resistance 20–25 MΩ. Changes in membrane potential, current and resistance were monitored as described in detail elsewhere (David & Sattelle, 1984). Acetylcholine was applied ionophoretically from a micropipette (resistance 5–15 MΩ) filled with $1{\cdot}0\,\text{mol}\,l^{-1}$ acetylcholine chloride (pH 4·0). A retaining current of 50 nA was used to prevent leakage of the drug from the ionophoretic pipette.

Chemicals

N-Methyl lycaconitine citrate was the generous gift of Dr M. H. Benn of the Chemistry Department, University of Calgary, Alberta, Canada.

Results

Radioligand binding

N-methyl lycaconitine citrate caused a dose-dependent inhibition of specific [^{3}H]-α-bungarotoxin binding to cockroach ventral nerve cord homogenates (Fig. 2). The IC_{50} value of $2{\cdot}0\times10^{-9}\,\text{mol}\,l^{-1}$, determined graphically, yields a K_i value of $1{\cdot}4\times10^{-9}\,\text{mol}\,l^{-1}$. Inhibition curves for cholinergic ligands (α-bungarotoxin and *d*-tubocurarine) known to block functional cockroach nicotinic acetylcholine receptors (Sattelle, 1986) are also illustrated. For comparison, the actions of a muscarinic antagonist (atropine) and a nicotinic agonist (nicotine) are also included (Fig. 2).

α-Bungarotoxin, the most effective of all ligands tested at insect neuronal nicotinic receptors (Sattelle *et al.* 1980, 1983; David & Sattelle, 1984), is the only ligand of comparable effectiveness to *N*-methyl lycaconitine. Both ligands are

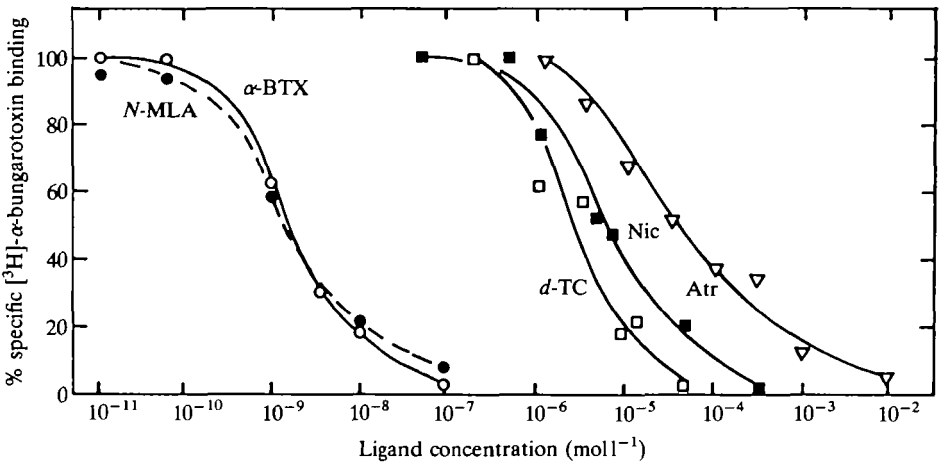

Fig. 2. Inhibition by *N*-methyl lycaconitine and other cholinergic ligands of the specific binding of [^{3}H]-α-bungarotoxin to membranes from cockroach nerve cords. 100% binding is the total binding of $2{\cdot}0\times10^{-9}$ mol l^{-1} [^{3}H]-α-bungarotoxin minus background determined in the presence of $1{\cdot}0\times10^{-6}$ mol l^{-1} unlabelled α-bungarotoxin, or $1{\cdot}0\times10^{-4}$ mol l^{-1} nicotine. *N*-Methyl lycaconitine (●) is by far the best of the low molecular weight inhibitors of toxin binding, being of similar effectiveness to α-bungarotoxin (○), and considerably more effective than *d*-tubocurarine (□), nicotine (■) and atropine (▽). Abbreviations: *N*-MLA, *N*-methyl lycaconitine; α-BTX, α-bungarotoxin; *d*-TC, *d*-tubocurarine; Nic, nicotine; Atr, atropine.

much more effective than *d*-tubocurarine in inhibiting the binding of [^{3}H]-α-bungarotoxin to cockroach nerve cord extracts.

Current-clamp electrophysiology

At resting potential, ionophoretic doses of acetylcholine resulted in depolarization of the cell body membrane of motor neurone D_f, accompanied by a drop in input resistance. Repeated applications (400 nA) at intervals of 2 min or more yielded responses of constant amplitude over a period of several hours in control conditions when the cells were continuously perfused ($2{\cdot}0$ ml min^{-1}) with normal saline. Bath-application of $1{\cdot}0\times10^{-6}$ mol l^{-1} *N*-methyl lycaconitine resulted in a progressive block of the depolarization induced by ionophoretic application of acetylcholine (Fig. 3). After 15 min, the acetylcholine-induced response was completely blocked. Increasing the ionophoretic dose to 600 nA and 1000 nA, or prolonged washing of the preparation (60 min) in normal saline, did not restore the response to acetylcholine. Using a submaximal response to acetylcholine (30–60% of the maximum depolarization), the percentage reduction in amplitude of the original response was determined after 20 min of bath-application of various concentrations of *N*-methyl lycaconitine. In this way a dose-dependent block of

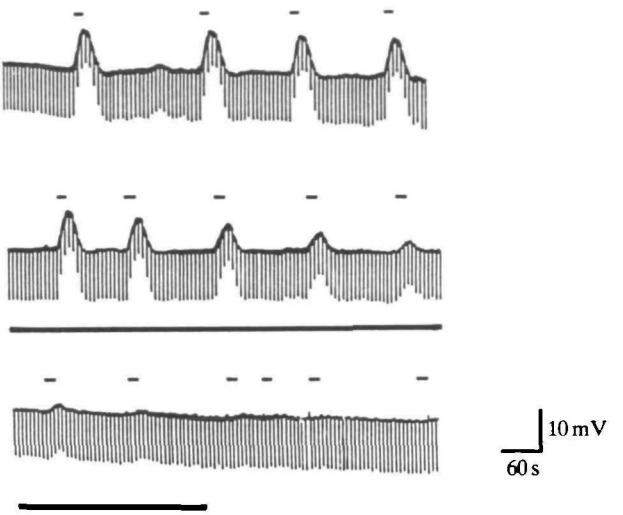

Fig. 3. Depolarization and a decrease in input resistance accompany ionophoretic applications of acetylcholine to the cell body membrane of motor neurone D_f. The short bars above the continuous voltage trace indicate the period of application of a 400 nA dose of acetylcholine. The solid bar below the voltage trace denotes the period of application of $1{\cdot}0\times10^{-6}$ mol l^{-1} *N*-methyl lycaconitine. The brief downward deflections are changes in membrane potential that result from hyperpolarizing constant-current pulses (2 nA, 400 ms) delivered through one of the two intracellular microelectrodes. Progressive block of the acetylcholine-induced response is observed during the application of *N*-methyl lycaconitine. Resting potential of the neurone at the beginning of the trace is −65 mV.

the insect neuronal nicotinic receptor was demonstrated in the range $1{\cdot}0\times10^{-8}$ to $1{\cdot}0\times10^{-6}$ mol l^{-1} (Fig. 4).

Voltage-clamp electrophysiology

To characterize the interaction between *N*-methyl lycaconitine and the nicotinic receptor, acetylcholine-induced currents were recorded in the presence and absence of the alkaloid (Fig. 5). Non-competitive antagonists often cause a voltage-dependent block of insect neuronal nicotinic receptors, whereas the blockade produced by competitive nicotinic antagonists is voltage-independent (Sattelle & David, 1983; David & Sattelle, 1984). To examine whether the blockade produced by *N*-methyl lycaconitine was dependent on membrane potential, the motor neurone cell body was voltage-clamped at −60 mV and a series of square-wave voltage-excursions was imposed over the range −30 to −100 mV in the presence or absence of *N*-methyl lycaconitine. The voltage steps were imposed before and during the acetylcholine-induced responses.

Examples of the current traces obtained in this way are illustrated in Fig. 5 which shows records from a single neurone. Fig. 6A shows, for a different neurone, the acetylcholine-induced current as a function of membrane potential before and after application of $1{\cdot}0\times10^{-7}$ mol l^{-1} *N*-methyl lycaconitine. The

percentage block of acetylcholine-induced currents resulting from this exposure to *N*-methyl lycaconitine was determined for each command potential for three separate cells and the data combined (Fig. 6B). *N*-Methyl lycaconitine block of the

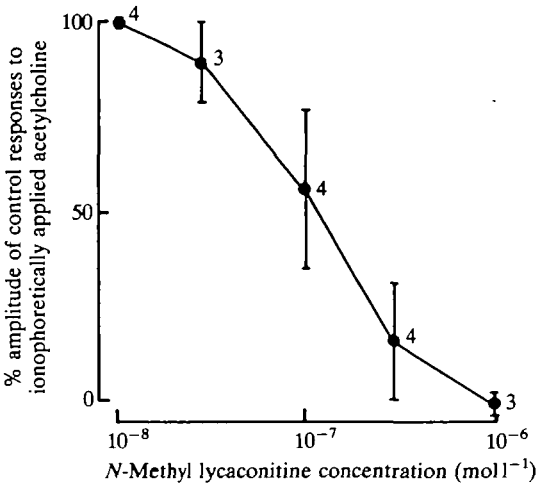

Fig. 4. Dose–response curve for inhibition of acetylcholine-induced depolarization by *N*-methyl lycaconitine. A submaximal acetylcholine response (30–60 % of the maximal dose) is used as a control, and the response amplitude (expressed as a percentage of the control response) is recorded after 20 min of exposure to *N*-methyl lycaconitine. Vertical bars represent the standard deviation of the mean and the numbers beside each mean value denote the number of preparations employed.

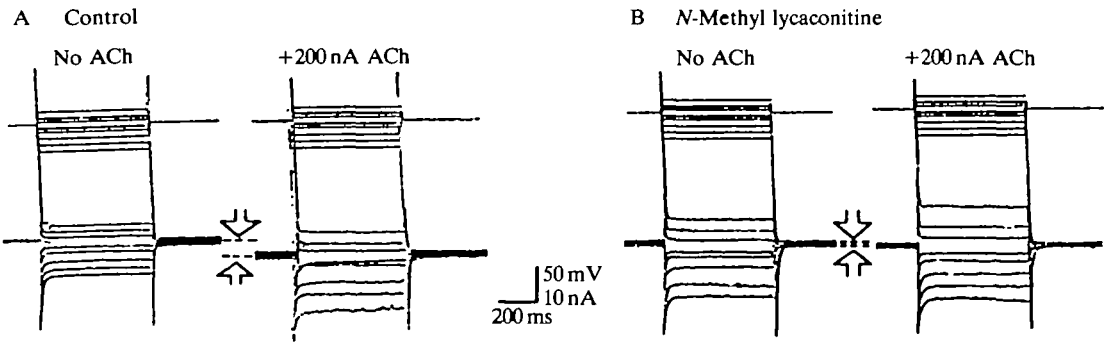

Fig. 5. Control and acetylcholine-induced currents recorded at different membrane potentials before and after exposure to $1{\cdot}0\times10^{-7}$ mol l^{-1} *N*-methyl lycaconitine. (A) Currents (lower sweeps) required to displace the membrane to the same voltage (upper sweeps) in the presence and absence of acetylcholine are shown. The top arrow indicates a current of 0 nA, so the distance between the two arrows indicates the magnitude of the current flowing at resting potential in the presence of acetylcholine. (B) Conditions are identical to A except that the motor neurone is exposed to $1{\cdot}0\times10^{-7}$ mol l^{-1} *N*-methyl lycaconitine for 20 min. A decreased acetylcholine-induced current (arrows) is evident following exposure to *N*-methyl lycaconitine. The holding potential for this cell is −60 mV.

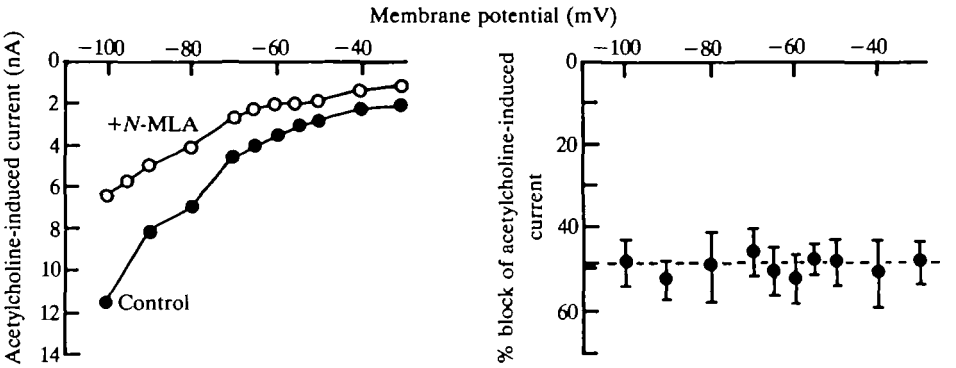

Fig. 6. Voltage-independent block of acetylcholine induced current by $1{\cdot}0\times10^{-7}$ mol l^{-1} *N*-methyl lycaconitine (*N*-MLA). (A) Acetylcholine-induced currents are recorded at different membrane potentials before and after exposure to *N*-methyl lycaconitine. These acetylcholine-induced currents are obtained by subtracting the control membrane currents from the membrane currents observed during ionophoretic application of acetylcholine. Data are from a single neurone, and are representative of three similar experiments. (B) Percentage block of acetylcholine-induced current as a function of membrane potential following bath-application of *N*-methyl lycaconitine for 30 min. Points plotted are mean values ± S.E.M. Data from three neurones are combined. In each case intracellular microelectrodes are filled with potassium sulphate and the holding potential is −60 mV.

nicotinic receptor of motor neurone D_f was independent of membrane potential over the range examined (−100 to −30 mV).

Discussion

Though the insecticidal properties of *Delphinium* seed extract were noted in AD 77 by Pliny the Elder (Book XXIII, Chapter XII), who recorded that 'pounded, they rid the head of lice' (see Jones, 1961), it is only in the present century that this has been confirmed (Nambi Aiyar *et al.* 1979; Grina *et al.* 1986). Recently, Jennings *et al.* (1986, 1987) have demonstrated a potent synaptic blocking action of *N*-methyl lycaconitine, a major active fraction of the seed extract. Here, we provide direct evidence that *N*-methyl lycaconitine is active at an insect neuronal nicotinic acetylcholine receptor. A K_i value of $4{\cdot}0\times10^{-9}$ mol l^{-1} for inhibition of [^{3}H]-α-bungarotoxin binding to cockroach (*Periplaneta americana*) nerve cord extracts is higher than the value ($2{\cdot}5\times10^{-10}$ mol l^{-1}) obtained in binding studies on housefly (*Musca domestica*) head extracts, a preparation which contains a wide variety of tissues in addition to nervous tissue. Differences of this order have been seen for some other ligands when comparing putative receptors from different insect tissues (see Sattelle, 1986). One possibility is that there may be some pharmacological differences between fly and cockroach nicotinic acetylcholine receptors. Alternatively, differences in preparative procedures may account for these discrepancies. Nevertheless, it is clear that *N*-methyl lycaconi-

tine is the most potent low molecular weight blocking agent so far tested on the insect nervous system [^{3}H]-α-bungarotoxin binding site.

Interpretation of receptor–ligand interactions is facilitated when radioligand binding and electrophysiological data are available for the same tissue. The finding that the alkaloid blocks acetylcholine-induced currents in the cell body membrane of an identified cockroach neurone located in the same tissue on which the binding studies have been performed is the first direct evidence for an antagonist action of *N*-methyl lycaconitine. The fast coxal depressor motor neurone (D_f) nicotinic receptors are blocked by α-bungarotoxin (Sattelle *et al.* 1980; David & Sattelle, 1984), κ-bungarotoxin (Pinnock *et al.* 1988) and a wide range of nicotinic ligands (David & Sattelle, 1984). Hitherto, the most effective low molecular weight blocking agent was dihydro-β-erythroidine. *N*-Methyl lycaconitine reduces the acetylcholine-induced current at concentrations above $1{\cdot}0\times10^{-8}$ mol l^{-1}, completely blocking the response following brief exposures to a $1{\cdot}0\times10^{-6}$ mol l^{-1} concentration. Although the IC_{50} value determined from binding studies ($2{\cdot}0\times10^{-9}$ mol l^{-1}) is much lower than that estimated from physiological experiments ($\approx1{\cdot}0\times10^{-7}$ mol l^{-1}), factors such as accessibility to receptors of the *in vivo* preparation may account for these differences. A blocking action at an insect neuronal nicotinic acetylcholine receptor is the simplest explanation of these findings.

The blocking action of *N*-methyl lycaconitine is voltage-independent over the membrane potential range -100 to -30 mV. Other ligands which block the insect nicotinic receptor in a voltage-independent manner include α-bungarotoxin (David & Sattelle, 1984), κ-bungarotoxin (Pinnock *et al.* 1988), dihydro-β-erythroidine and benzoquinonium (David & Sattelle, 1984). By contrast, *d*-tubocurarine, atropine (David & Sattelle, 1984), histrionicotoxin (Sattelle & David, 1983) and amantadine (Artola *et al.* 1984) are all strongly voltage-dependent in their blocking actions over a similar range of membrane potentials. The precise site of action of *N*-methyl lycaconitine remains to be determined. An irreversible blocking action at the acetylcholine recognition site remains a possibility. A noncompetitive, voltage-independent action cannot be ruled out, though the radioligand binding data presented here suggest a competitive action. With the recent detection of acetylcholine-induced single channels in dissociated cockroach neurones (Sattelle *et al.* 1986), it will be of interest to examine the actions of *N*-methyl lycaconitine on the unitary conductance and lifetime properties of this insect nicotinic acetylcholine receptor.

When physiological data from the present study are compared with data obtained on the rat phrenic diaphragm preparation (Nambi Aiyar *et al.* 1979), it appears that *N*-methyl lycaconitine is more effective on the insect neuronal nicotinic receptor than on the vertebrate peripheral nicotinic receptor. When the high affinity of *N*-methyl lycaconitine for the insect nicotinic receptor is considered together with the finding that the insect nervous system is one of the richest sources of neuronal nicotinic receptors in the animal kingdom (see Sattelle, 1986; Breer & Sattelle, 1987), it is possible that the role of the alkaloid in plants is related

to protection from insect damage. All insects so far tested, including such diverse forms as cockroaches (Lummis & Sattelle, 1985), moths, locusts and flies, are rich in neuronal nicotinic receptors (Breer & Sattelle, 1987) which may account for the observation that *N*-methyl lycaconitine exhibits insecticidal activity against a range of insect species.

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