ELECTROPHYSIOLOGICAL PHARMACOLOGY OF THE NICOTINIC AND MUSCARINIC CHOLINERGIC RESPONSES OF ISOLATED NEURONAL SOMATA FROM LOCUST THORACIC GANGLIA

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Summary
1. Mechanically isolated neuronal somata from the thoracic ganglia of the locust Locusta migratoria remain electrophysiologically viable under current- or voltage-clamp in vitro for many hours. Nicotine and muscarine evoke different responses when pressure-microapplied to these somata. The response to acetylcholine is mainly nicotinic but contains a small muscarinic component.

2. The nicotinic (ACh1) response is a rapid depolarisation accompanied by a decrease in membrane resistance. In voltage-clamped somata, the current mediating the ACh1 response is inward over the membrane potential range —30 to —110 mV, decreasing with depolarisation and with a projected reversal potential of about +20 mV.

3. The muscarinic (ACh2) response is a slow depolarisation accompanied by a decrease in membrane resistance. In voltage-clamped somata, the current mediating the ACh2 response is inward, decreasing to zero at potentials of —80 to —90 mV.

4. The ACh1 response is evoked by nicotine, anabasine, tetramethylammonium, DMPP and relatively high concentrations of the nitromethylene heterocycle insecticide, PMNI. Suberyldicholine or decamethonium evoke the response only when acetylcholine is present in the bathing saline. Nicotinic antagonists of the ACh1 response, in descending order of potency, are PMNI > α-bungarotoxin > lobeline > mecamylamine > trimethaphan > chlorisondamine > d-tubocurarine > hexamethonium > gallamine triethiodide > tetraethylammonium. This response is also potently blocked by strychnine and more weakly blocked by δ-philanthotoxin, bicculline and picrotoxin.

5. The ACh2 response is evoked by muscarine, oxotremorine, arecoline, pilocarpine and, very weakly, by the M1-selective agonist McN-A-343. Muscarinic antagonists of the ACh2 response, in descending order of potency, are QNB > scopolamine > atropine > 4-DAMP (M3) > benactyzine > HHSiD (M1/M3) >pirenzepine (M1). QNX (M1), AF-DX 116 (M2), gallamine triethiodide (M2) and methoctramine (M2) are almost or completely inactive.

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Key words: acetylcholine, insect, receptor, nicotinic, muscarinic, Locusta migratoria.
6. With the exception of pirenzepine and QNX, all of the muscarinic antagonists used in this study also block the nicotinic ACh1 response with EC50 values in the range 5 to 50 μmol L⁻¹, similar to those for δ-philanthotoxin, bicuculline and picrotoxin. Pirenzepine is inactive (10 μmol L⁻¹), but QNX is potently active, with an EC50 value of approximately 20 nmol L⁻¹, similar to that of α-bungarotoxin.

7. The extrasynaptic nicotinic and muscarinic receptors of *Locusta migratoria* neurones are pharmacologically distinct from the corresponding mammalian receptors studied so far.

**Introduction**

Early experiments by Roeder and Roeder (1939) showed that the cockroach nervous system responds not only to acetylcholine but also to nicotine and the muscarinic agonist, pilocarpine. The sensitivity of insect neuronal somata *in situ* to acetylcholine was first reported by Callec and Boistel (1967), and Kerkut *et al.* (1969a,b) used ionophoretic microapplication to show that the depolarization of the soma in response to acetylcholine depended on the dose applied. Usherwood and his colleagues showed that neuronal somata mechanically isolated from the thoracic ganglia of the locust *Schistocerca gregaria* remain electrophysiologically viable *in vitro* for many hours and respond with changes in membrane potential and membrane resistance to ionophoretic microapplication of several neurotransmitters, including acetylcholine (Holden *et al.* 1978; Usherwood *et al.* 1980; Suter and Usherwood, 1985). Using the same method of mechanical dissociation to isolate neurones from the thoracic ganglia of the locust *Locusta migratoria*, we have shown that these somata can be readily and stably voltage-clamped for long periods (Lees *et al.* 1985, 1987). The clamped neurones respond characteristically not only to acetylcholine and nicotine but also to muscarine (Benson and Neumann, 1987) and other putative neurotransmitters and neuropeptides (Neumann *et al.* 1987; reviewed by Benson, 1992b). This report defines the pharmacology of these extrasynaptic nicotinic (ACh1) and muscarinic (ACh2) acetylcholine receptors and the voltage-dependence of the currents they activate. Preliminary reports on parts of this work have appeared elsewhere (Benson and Neumann, 1987; Benson, 1988a,b, 1989a).

**Materials and methods**

The methods of cell preparation and drug application used for these experiments have been described in detail by Lees *et al.* (1987) and are based on the method of Usherwood *et al.* (1980). They can be summarized as follows. The three thoracic ganglia were dissected free from adult locusts (*Locusta migratoria* R. & F.), desheathed and aspirated three or four times through a Pasteur pipette tip. The resulting suspension of dissociated neuronal somata was then left in a few drops of physiological saline in a Petri dish for 2–4 h. This allowed the dendritic stumps to seal off and the cell bodies to settle to the bottom of the Petri dish.

The somata used in these experiments ranged in diameter from 50 to 100 μm.
Smaller neurones were less easy to record from for long periods and larger neurones were apparently destroyed during preparation. The experimental somata were thus not selected randomly from the total neuronal population of the ganglia but represent a subpopulation based on size. Every soma tested responded to acetylcholine (>300) and muscarine (>150), and the respective responses were identical in their essential characteristics for all cells. The subtype of receptor present for other putative neurotransmitters can depend on slight changes in the soma isolation method (e.g. for serotonin; Bermudez et al. 1990, 1992). Nevertheless, cross-checks of pharmacology between individual isolated somata supported the assumption made in this study that pharmacologically uniform cholinergic receptor subtypes, nicotinic ACh1 and muscarinic ACh2, were present.

The cells were impaled and voltage-clamped using a conventional single-electrode switching clamp (Axoclamp). The agonists were dissolved in physiological saline and pressure-applied using a Picospritzer (General Valve Corp.) in combination with patch electrodes pulled on a Mecanex electrode puller. The antagonists were applied by continuous perfusion of the bath. The composition of the physiological saline (in mmol l\(^{-1}\)) was: NaCl, 180; MgCl\(_2\), 15; CaCl\(_2\), 10; KCl, 10; Hepes, 10; pH 6.8. All experiments were carried out at room temperature (24–25°C).

The test compounds were obtained as follows: \(\text{d}-\text{tubocurarine}\) was purchased from Calbiochem; tetraethylammonium, DMPP and oxotremorine from Aldrich; nicotine and tetramethylammonium from Fluka; QNB, QNX, 4-DAMP, methoctramine and McN-A-343 from Research Biochemicals Inc.; scopolamine, arco- line and pilocarpine from Merck. HHSiD was a kind gift from Professor G. Lambrecht, J. W. Goethe-Universität, Frankfurt, and Dr R. Tacke, University of Karlsruhe, and AF-DX116 from Dr Karl Thomae, GmbH. PMNI, trimethaphan camyslate, chlorisondamine, Pyrantel, Levamisol and \(\delta\)-philanthotoxin-433 were synthesized at CIBA-GEIGY Ltd, Basel. All other compounds were purchased from Sigma. For an explanation of the abbreviations, please refer to Tables 1–5.

For the pharmacological experiments, the somata were voltage-clamped at \(-50\) mV for test pulses of acetylcholine or nicotine, and at \(-40\) mV for test pulses of muscarine. The resting potential of the isolated neurones was approximately \(-50\) mV. All compounds were assayed for both agonism and antagonism. A compound was classified as an agonist if, when bath-applied, it induced an appropriate shift in holding current with a concentration-dependence similar to its action in reducing the evoked test response. The latter reduction in such cases can be assumed to be due to a combination of desensitisation and competition at the ligand binding site, but it is used here simply as a method to quantify agonistic effects for the purposes of comparison. In addition, an ‘agonist’ was required to evoke a response when pulse-microapplied to the soma at a dose of 1 mmol l\(^{-1}\) for 2 s or less, while ‘weak agonists’ were those compounds that evoked a current only when bath-applied and at a concentration of 100 \(\mu\)mol l\(^{-1}\) or more. The compounds were tested for antagonistic effects at concentrations up to 100 \(\mu\)mol l\(^{-1}\). A
compound was classified as an antagonist if it reduced the evoked test response without a corresponding shift in the holding current, or at least reduced the response at a concentration much lower than the threshold for a shift in holding current. To obtain dose–response data, the test compounds were bath-applied, beginning at low concentrations and, after a steady-state reduction in the evoked response had been achieved, in increasing doses, without an intervening washout. Both agonist potency and antagonism were quantified in the same way, by obtaining EC50 values from individual dose–response curves and calculating the average EC50 value and the standard deviation (s.d.).

To obtain current–voltage (I–V) curves, the somata were clamped at different voltages via a series of 10 mV steps, held at each potential until the membrane current reached a constant level and then challenged with an agonist pulse. The voltage-dependence of the antagonist action was determined from the percentage blockade at different clamp potentials at approximately 50% blockade in comparison with the control.

**Results**

*Cholinergically evoked changes in membrane potential and resistance*

Pressure-microapplication of a 200 ms pulse of 100 μmol l⁻¹ acetylcholine to an isolated soma current-clamped at −30 mV evoked a rapid and transient depolarization of the membrane potential and a decrease in the membrane resistance (Fig. 1A). To evoke a response of comparable amplitude in the same soma at the same membrane potential using muscarine required a comparatively high dose, 1 mmol l⁻¹ for 5 s. The muscarine-evoked depolarization was accompanied by a smaller decrease in resistance than for acetylcholine, the rate of rise of the response being slower (Fig. 1B). Pulse-microapplication of oxotremorine, a muscarinic agonist, had an effect similar to that of muscarine (Fig. 1C). In a different soma, also current-clamped at −30 mV, nicotine evoked a transient depolarisation and decrease in membrane resistance, slower than those for acetylcholine but faster than those for muscarine (Fig. 1D). As for acetylcholine, the dose required to evoke responses of the same amplitude was less for nicotine than for muscarine. Under voltage-clamp conditions, both acetylcholine and muscarine evoked experimentally useful responses at doses lower than those necessary to allow measurement of changes in membrane resistance. The doses used were usually 100 μmol l⁻¹ for 50 or 100 ms for acetylcholine and 1 mmol l⁻¹ for 500 ms for muscarine.

*Membrane currents evoked by nicotine and muscarine*

Under voltage-clamp, acetylcholine, nicotine and muscarine evoked inward currents at membrane potentials more negative than −30 mV, which was the most positive potential at which the somata could be clamped under the experimental conditions used here. However, the membrane-potential-dependence of the current evoked by muscarine differed from that of the response to nicotine, which
Fig. 1. (A–D) Responses to pressure-microapplication of cholinergic agonists recorded from current-clamped, isolated thoracic neuronal somata. The agonist pulses began at the times indicated by the arrows. The membrane resistance is indicated by the amplitude of the membrane potential change in response to repeated intracellular application of brief constant-current pulses. $E_h = E_m =$ membrane potential. A–C were recorded in the same soma.
was almost indistinguishable from the acetylcholine response. This is illustrated by the current–voltage ($I$–$V$) curves in Fig. 2. The nicotinic and acetylcholinergic responses increased with hyperpolarization over the membrane potential range tested (−30 to −100 mV) and had a projected reversal potential of about +20 mV. The muscarinic response, in contrast, decreased to zero at potentials of −80 to −90 mV.

These results strongly suggest that nicotine and muscarine evoke different currents via different receptors. This further implies that the acetylcholine response consists of two components, one mediated by relatively numerous nicotinic receptors and the other by much sparser muscarinic receptors. Since a much higher dose was required for muscarine to evoke a response of a particular amplitude than for acetylcholine and nicotine, it was hypothesized that the muscarinic component would be much smaller than the nicotinic component in the acetylcholine response. To test this hypothesis, almost equal doses of acetylcholine (1 mmol l$^{-1}$, 1 s) and muscarine (1 mmol l$^{-1}$, 2 s) were pulse-microapplied to a soma voltage-clamped at −40 mV. The amplitude of the acetylcholine response was 40 times greater than for muscarine (Fig. 3A). Mecamylamine, which is a potent and highly selective nicotinic antagonist in vertebrate systems and also in this preparation (details below), was applied to the soma at 30 μmol l$^{-1}$. This had no effect on the amplitude of the muscarine response but reduced the acetylcholine response to approximately 5% of the control value (Fig. 3B). The response to the usual experimental dose of acetylcholine (100 μmol l$^{-1}$, 100 ms) was almost
Locust neuronal soma cholinoreceptors

Effects of compounds selective for vertebrate nicotinic acetylcholine receptors

The nicotinic cholinergic receptors of the vertebrates fall into at least two
subtypes, those occurring on muscle fibres at the neuromuscular synapses, neuromuscular (nm), and those found on neurones in the peripheral ganglia and the brain, ganglionic (ga). There are several antagonists that are selective to varying degrees for one or other of these subtypes, while the agonists are usually active at both.

Antagonists

In Table 1, the nicotinic antagonists are grouped according to their vertebrate receptor subtype specificity and ranked within those groups in order of decreasing potency against the ACh1 response. The overall rank order of potency against the ACh1 response was: PMNI \( \alpha \)-bungarotoxin (nm) \( \geq \) lobeline \( \geq \) mecamylamine (ga) \( > \) trimethaphan camsylate (ga) \( > \) chlorisondamine (ga) \( > \) \( d \)-tubocurarine (nm) \( \geq \) hexamethonium (ga) \( \geq \) gallamine triethiodide (nm) \( \geq \) tetraethylammonium (ga).

PMNI, 1-(pyridin-3-yl-methyl)-2-nitromethylene-imidazoline, a highly active nitromethylene heterocycle insecticide, was the antagonist most potent at the ACh1 receptor. PMNI, with an EC\(_{50}\) value of \( 1.0 \pm 0.8 \times 10^{-10} \) mol\(^{-1}\), was 15–50 times more active as an antagonist than the two compounds hitherto recognised as most potent at this receptor (\( \alpha \)-bungarotoxin and mecamylamine). In addition to its antagonistic effects, when pulse-applied at concentrations high in comparison to its antagonist action threshold, it evoked an ACh1 response. When applied to \textit{Locusta migratoria} neurones, PMNI is, however, primarily an antagonist, since the threshold for its agonistic activity during bath-application was about 30 nmol\(^{-1}\) compared with an antagonism threshold of below 0.01 nmol\(^{-1}\) (Benson, 1989, 1990, 1992a).

\( \alpha \)-Bungarotoxin was the next most potent antagonist, with an EC\(_{50}\) value of 20 nmol\(^{-1}\). This toxin is a competitive antagonist selective for the vertebrate neuromuscular junction cholinoreceptor, with no effect on most vertebrate neuronal acetylcholine receptors. Its blocking action was independent of membrane potential (Benson, 1988a). Lobeline and mecamylamine had EC\(_{50}\) values of the same order of magnitude as that of \( \alpha \)-bungarotoxin (Table 1). Lobeline is usually described as a ganglionic nicotinic stimulant with blocking properties (Bowman and Rand, 1980). At the ACh1 receptor it was only very weakly agonistic but it showed strong antagonism. Mecamylamine is a secondary amine that blocks ganglionic cholinergic responses in a non-competitive manner. It is unusually potent for a channel-blocking compound, with an EC\(_{50}\) value of 63 nmol\(^{-1}\) at the ACh1 receptor.

The other ganglionic antagonists, in order of potency, were trimethaphan camsylate, chlorisondamine (an asymmetric bisquaternary compound) hexamethonium (a typical bisquaternary methonium competitive ganglion blocking agent) and tetraethylammonium (TEA\(^{+}\), a ganglionic antagonist that blocks K\(^{+}\) channels at higher concentrations; Stanfield, 1983). They were all moderately potent to weak (EC\(_{50}\) values in the range 700 nmol\(^{-1}\) to 50 \( \mu \)mol\(^{-1}\)) and not distinguishable in this respect from the neuromuscular nicotinic blockers \( d \)-tubocurarine (EC\(_{50}\)
<table>
<thead>
<tr>
<th>Compound name</th>
<th>Agonism</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Reduction of the nicotinic (ACh&lt;sub&gt;n&lt;/sub&gt;) response</th>
<th>Reduction of the muscarinic (ACh&lt;sub&gt;2&lt;/sub&gt;) response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lobeline</td>
<td>Nicotinic</td>
<td>1.0±0.8×10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>PMNI*</td>
<td>Weak agonism</td>
<td>4.3±1.9×10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>α-Bungarotoxin</td>
<td>Weak agonism</td>
<td>2.0±0.8×10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>d-Tubocurarine</td>
<td>No effect</td>
<td>8.5±5.5×10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Gallamine triethiodide</td>
<td>Weak agonism</td>
<td>2.7±2.0×10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>No effect</td>
<td>6.3±1.7×10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Trirnethaphan</td>
<td>No effect</td>
<td>6.7±0.8×10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Chlorisondamine</td>
<td>No effect</td>
<td>4.2±3.1×10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>No effect</td>
<td>1.9±1.2×10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>No effect</td>
<td>4.6±3.5×10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

PMNI, l-(pyridin-3-yl-methyl)-2-nitromethylene-imidazoline.

*data from Benson (1989).

EC<sub>50</sub> values are given as means±s.d., calculated from the specified number (N) of dose–response curves.

PMNI, selective for vertebrate neuromuscular nicotinic acetylcholine receptors.

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EC<sub>50</sub> values are given as means±s.d., calculated from the specified number (N) of dose–response curves.

PMNI, selective for vertebrate neuromuscular nicotinic acetylcholine receptors.
8 μmol l$^{-1}$) and gallamine triethiodide (EC$_{50}$ 30 μmol l$^{-1}$), both quaternary ammonium compounds, like TEA$^+$. The antagonistic action of TEA$^+$ varied with the membrane potential, being greater at more hyperpolarised potentials (Benson, 1988a).

With the exception of lobeline, none of the nicotinic compounds influenced the muscarinic ACh2 response, up to the concentrations indicated.

**Agonists**

Desensitisation is the effect seen at many receptors, including the ACh1 and ACh2 receptors, where continuous application of an agonist drives the receptor through its activated state to an inactive and inactivatable state. As for antagonism, desensitisation is observed as a decrease in the amplitude of the pulse-activated response; it is distinguished from antagonism by showing, in addition, a concurrent agonistic shift in baseline current in the appropriate direction. Agonism was quantified in terms of an EC$_{50}$ value for the reduction in the amplitude of the pulse-evoked response due to desensitisation.

A series of compounds that act as selective nicotinic agonists in vertebrate preparations was tested for agonistic and antagonistic effects on isolated somata (Table 2). Although nicotinic agonists are poorly selective among nicotinic receptor subtypes in comparison with the antagonists, nicotine and DMPP (1,1-dimethyl-4-phenyl-piperazinium) show some preference for vertebrate ganglionic receptors, while suberyldicholine and decamethonium are more effective at the neuromuscular junction (Bowman and Rand, 1980). When bath-applied at concentrations up to 100 μmol l$^{-1}$ or pulse-applied at 1 mmol l$^{-1}$ for up to 2 s, nicotine itself, as well as anabasine (also an alkaloid), tetramethylammonium and DMPP, were potent activators of the ACh1 current (identified by its kinetics, membrane-potential-dependence and pharmacology). When bath-applied, nicotine had an agonist threshold of between 0.1 and 0.3 μmol l$^{-1}$ (although its threshold for desensitisation is lower), and evoked comparatively large responses when pulse-applied for as little as 20 ms at 100 μmol l$^{-1}$. Surprisingly, suberyldicholine and decamethonium, which are potent agonists at vertebrate neuromuscular cholinoreceptors (Bowman and Rand, 1980), showed no agonistic action over this concentration range when tested in control saline. In contrast, when pulse-applied in the presence of acetylcholine in the bathing saline, both compounds evoked the ACh1 current, and the amplitude of the evoked current increased with the concentration of acetylcholine in the bathing saline (Benson, 1988a). This effect could not be mimicked by addition of the cholinesterase inhibitor eserine to the control saline, although eserine itself sometimes enhanced the amplitude of the response to acetylcholine (data not shown). Suberyldicholine and decamethonium, when bath-applied in control saline, blocked the ACh1 response with EC$_{50}$ values of approximately 1 μmol l$^{-1}$ and approximately 40 μmol l$^{-1}$, respectively (Table 2), but neither compound showed agonistic action when bath-applied at concentrations of up to 10 μmol l$^{-1}$.

The nematicidal compounds Pyrantel and Levamisole were low-potency
Table 2. Effects of selective vertebrate nicotinic acetylcholine receptor agonists

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Agonism</th>
<th>N</th>
<th>EC$_{50}$ (mol$^{-1}$)</th>
<th>N</th>
<th>Reduction of the nicotinic (ACh1) response</th>
<th>Reduction of the muscarinic (ACh2) response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>Nicotinic</td>
<td>17</td>
<td>5.0 ± 1.5 x 10$^{-7}$</td>
<td>3</td>
<td>No effect (10$^{-5}$ mol$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>Anabasine</td>
<td>Nicotinic</td>
<td>17</td>
<td>1.0 ± 0.7 x 10$^{-5}$</td>
<td>6</td>
<td>No effect (10$^{-4}$ mol$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>Tetramethylammonium</td>
<td>Nicotinic</td>
<td>5</td>
<td>6.2 ± 2.6 x 10$^{-5}$</td>
<td>3</td>
<td>No effect (10$^{-5}$ mol$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>DMPP</td>
<td>Nicotinic</td>
<td>5</td>
<td>8.5 ± 1.8 x 10$^{-5}$</td>
<td>3</td>
<td>No effect (10$^{-5}$ mol$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>Suberyldicholine* (nm)</td>
<td>No effect</td>
<td>14</td>
<td>4.6 ± 4.7 x 10$^{-5}$</td>
<td>3</td>
<td>No effect (10$^{-4}$ mol$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>Decamethonium* (nm)</td>
<td>No effect</td>
<td>11</td>
<td>1.2 ± 1.5 x 10$^{-6}$</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrantel</td>
<td>Nicotinic</td>
<td>5</td>
<td>3.6 ± 1.4 x 10$^{-5}$</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>Nicotinic</td>
<td>17</td>
<td>3.8 ± 2.4 x 10$^{-5}$</td>
<td>3</td>
<td>No effect (10$^{-4}$ mol$^{-1}$)</td>
<td></td>
</tr>
</tbody>
</table>

EC$_{50}$ values are given as mean ± s.d. calculated from the specified number (N) of dose–response curves.

DMPP, 1,1-dimethyl-4-phenyl-piperazinium.
Morantel, E-1,4,5,6-tetrahydro-1-methyl-2-[2(3-methyl-2-thienyl)ethenyl]pyrimidine.
Pyrantel, E-1,4,5,6-tetrahydro-1-methyl-2-[2-(2-thienyl)vinyl]pyrimidine.
Levamisole, 2,3,5,6-tetrahydro-6-phenyl-imidazo(2,1-b)thiazole.
nm, selective for vertebrate neuromuscular nicotinic acetylcholine receptors.

ga, selective for vertebrate central (ganglionic) neuronal acetylcholine receptors.

* These compounds showed no agonistic effects when pressure pulse applied to somata in control saline, but in the presence of acetylcholine they did evoke a current which depended in magnitude on the bath acetylcholine concentration (Benson, 1988a).
blockers of the ACh1 response and were ACh1 agonists at concentrations above 30 μmol l⁻¹, when tested on Locusta migratoria neuronal soma (Table 2). Both were less potent than acetylcholine (see Table 5).

**Effects of compounds selective for vertebrate muscarinic acetylcholine receptors**

The vertebrate muscarinic receptors can be classified tentatively into at least three pharmacological subtypes, neuronal-M₁, cardiac-M₂ and glandular-M₃ (reviewed by Mei *et al.* 1989).

**Antagonists**

In Table 3, the muscarinic antagonists are grouped according to receptor subtype specificity and ranked within those groups in order of decreasing potency against the ACh2 response. The overall rank order of potency against the ACh2 receptor was: QNB ≥ scopoline > atropine > 4-DAMP (M₃) ≥ benactyzine ≥ HHSiD (M₁/M₃) ≥ pirenzepine (M₁). QNX (M₁), AF-DX116 (M₂), gallamine triethiodide (M₂) and methoctramine (M₂) are almost or completely inactive.

Of the non-subtype-specific compounds, QNB and scopoline were highly active, with thresholds in the subnanomolar range, scopoline being about 10 times as potent as the classical and structurally very similar muscarinic blocker, atropine. Benactyzine was also quite potent (EC₅₀ 0.36 μmol l⁻¹). In other words, all the non-subtype-specific antagonists showed high or very high activity (EC₅₀ values of 3–300 nmol l⁻¹) at the ACh2 receptor.

Among the subtype-specific compounds, pirenzepine (M₁-selective) and 4-DAMP (M₃-selective) both had EC₅₀ values below 1 μmol l⁻¹, but were noticeably weaker than the non-specific compounds. All the other antagonists tested, including QNX, a structural analogue of the very potent QNB, were inactive at the ACh2 receptor. HHSiD, a silicon-containing compound that shows good activity on vertebrate M₁ and M₃ receptors (Lambrecht *et al.* 1989), was active against the ACh2 response with an EC₅₀ value in the same range as those of pirenzepine and 4-DAMP.

Of the antagonists, atropine showed weak ACh2 agonistic activity. High concentrations of atropine have been reported to act in this way in other systems. Gallamine triethiodide had a weak agonistic effect which it was not possible to characterise. There was no reduction of the ACh2 response when the compound was applied at 10 μmol l⁻¹, which suggests that the agonistic effect was mediated via ACh1 receptors. Gallamine triethiodide is known to have nicotinic effects in vertebrates and it blocked the ACh1 response (Table 1), probably by desensitisation.

**Agonists**

In addition to acetylcholine and muscarine, oxotremorine and arecoline evoked the ACh2 response when pulse-applied to the somata (Table 4). Arecoline also evoked the ACh1 response. Pilocarpine had an effect similar to that of muscarine in evoking the ACh2 response but, unlike muscarine and like arecoline, when
Table 3. Effects of selective vertebrate muscarinic acetylcholine receptor antagonists

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Agonism</th>
<th>$N$</th>
<th>$EC_{50}$ (mol$^{-1}$)</th>
<th>$N$</th>
<th>$EC_{50}$ (mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QNB</td>
<td>No effect</td>
<td>12</td>
<td>$1.9\pm0.5\times10^{-5}$</td>
<td>3</td>
<td>$3.7\pm0.6\times10^{-9}$</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>No effect</td>
<td>11</td>
<td>$4.5\pm3.0\times10^{-5}$</td>
<td>3</td>
<td>$6.7\pm5.2\times10^{-9}$</td>
</tr>
<tr>
<td>Atropine</td>
<td>Weak agonism</td>
<td>16</td>
<td>$2.8\pm0.5\times10^{-5}$</td>
<td>4</td>
<td>$2.9\pm1.3\times10^{-8}$</td>
</tr>
<tr>
<td>Benactyzine</td>
<td>No effect</td>
<td>6</td>
<td>$4.9\pm4.0\times10^{-6}$</td>
<td>4</td>
<td>$3.6\pm4.3\times10^{-7}$</td>
</tr>
<tr>
<td>Pirenzepine (M₁)</td>
<td>No effect</td>
<td>5</td>
<td>No effect ($10^{-5}$)</td>
<td>3</td>
<td>$7.1\pm4.5\times10^{-7}$</td>
</tr>
<tr>
<td>HHSiD (M₁/M₃)</td>
<td>No effect</td>
<td>8</td>
<td>$4.1\pm2.7\times10^{-6}$</td>
<td>3</td>
<td>$5.2\pm2.3\times10^{-7}$</td>
</tr>
<tr>
<td>QNX (M₁)</td>
<td>No effect</td>
<td>10</td>
<td>$1.8\pm2.0\times10^{-8}$</td>
<td>4</td>
<td>approx. $-30%$ ($10^{-5}$)</td>
</tr>
<tr>
<td>AF-DX 116 (M₂)</td>
<td>No effect</td>
<td>11</td>
<td>$1.3\pm0.3\times10^{-5}$</td>
<td>3</td>
<td>approx. $-30%$ ($10^{-4}$)</td>
</tr>
<tr>
<td>Gallamine triethiodide (M₂)</td>
<td>Weak agonism</td>
<td>13</td>
<td>$2.7\pm2.0\times10^{-5}$</td>
<td>3</td>
<td>No effect ($10^{-5}$)</td>
</tr>
<tr>
<td>Methoctramine (M₂)</td>
<td>No effect</td>
<td>9</td>
<td>$1.0\pm0.9\times10^{-6}$</td>
<td>3</td>
<td>No effect ($10^{-4}$)</td>
</tr>
<tr>
<td>4-DAMP (M₃)</td>
<td>No effect</td>
<td>11</td>
<td>$1.2\pm0.2\times10^{-6}$</td>
<td>3</td>
<td>$2.4\pm1.1\times10^{-7}$</td>
</tr>
</tbody>
</table>

$EC_{50}$ values are given as mean±s.d. calculated from the specified number ($N$) of dose-response curves.

HHSiD, hexahydro-sila-difenidol.HCl.
QNB, quinuclidinyl benzilate.
QNX, 3-quinuclidinylxanthene-9-carboxylate hemioxalate hydrate.
4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide.
AF-DX 116, 11-2-(diethylamino) methyl-1-piperidinyl acetyl-5,11-dihydro-6H-pyrido 2,3-b 1,4 benzodiazepin-6-one.
M₁, selective for the vertebrate neuronal-M₁ muscarinic receptor subtype.
M₂, selective for the vertebrate cardiac-M₂ muscarinic receptor subtype.
M₃, selective for the vertebrate glandular-M₃ muscarinic receptor subtype.
Table 4. Effects of selective vertebrate muscarinic acetylcholine receptor agonists

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Agonism</th>
<th>( N )</th>
<th>Reduction of the nicotinic (ACh₁) response</th>
<th>Reduction of the muscarinic (ACh₂) response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EC₅₀ (mol l⁻¹)</td>
<td>EC₅₀ (mol l⁻¹)</td>
</tr>
<tr>
<td>Muscarine</td>
<td>Muscarinic</td>
<td>&gt;100</td>
<td>No effect (10⁻⁴ mol l⁻¹)</td>
<td>9.3±9.2x10⁻⁷ mol l⁻¹ 3</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>Muscarinic</td>
<td>3</td>
<td>No effect (10⁻⁴ mol l⁻¹)</td>
<td>Desensitisation 1</td>
</tr>
<tr>
<td>Arecoline</td>
<td>Nicotinic and muscarinic</td>
<td>7</td>
<td>Desensitisation 4</td>
<td>Desensitisation 3</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Muscarinic</td>
<td>12</td>
<td>Desensitisation 8</td>
<td>Desensitisation 3</td>
</tr>
<tr>
<td>McN-A-343 (M₁)</td>
<td>Weak muscarinic</td>
<td>7</td>
<td>1.1±0.6x10⁻⁵ mol l⁻¹</td>
<td>Weak desensitisation 1</td>
</tr>
</tbody>
</table>

EC₅₀ values are given as mean±s.d. calculated from the specified number (\( N \)) of dose-response curves.


M₁, selective for the vertebrate neuronal-M₁ muscarinic receptor subtype.

Desensitisation indicates that bath application of the test compound reduced the response to pressure pulses of transmitter and at the same time evoked an agonistic shift in the holding current.

Table 5. Effects of non-selective compounds

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Agonism</th>
<th>( N )</th>
<th>Reduction of the nicotinic (ACh₁) response</th>
<th>Reduction of the muscarinic (ACh₂) response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EC₅₀ (mol l⁻¹)</td>
<td>EC₅₀ (mol l⁻¹)</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Nicotinic and muscarinic</td>
<td>&gt;250</td>
<td>1.9±1.3x10⁻⁶</td>
<td>Desensitisation 8</td>
</tr>
<tr>
<td>Strychnine</td>
<td>No effect</td>
<td>13</td>
<td>2.3±0.5x10⁻⁸</td>
<td>No effect (10⁻⁴) 3</td>
</tr>
<tr>
<td>δ-Phlanthotoxin 433</td>
<td>No effect</td>
<td>6</td>
<td>1.4±0.1x10⁻⁵</td>
<td>No effect (10⁻⁴) 1</td>
</tr>
<tr>
<td>Bicuculline*</td>
<td>No effect</td>
<td>17</td>
<td>3.1±1.8x10⁻⁵</td>
<td>No effect (10⁻⁴) 10</td>
</tr>
<tr>
<td>Picrotoxin</td>
<td>No effect</td>
<td>14</td>
<td>2.9±1.3x10⁻⁵</td>
<td>No effect (10⁻⁴) 1</td>
</tr>
</tbody>
</table>

EC₅₀ values are given as mean±s.d. calculated from the specified number (\( N \)) of dose-response curves.

Desensitisation indicates that bath application of the test compound reduced the response to pressure pulses of transmitter and at the same time evoked an agonistic shift in the holding current.

*Includes experiments using bicuculline methiodide and methobromide in addition to the free base (Benson, 1988c).
bath-applied it also weakly desensitized the ACh1 response. These non-subtype-specific agonists were approximately equipotent, all evoking an ACh2-type response of about the same amplitude when pulse applied (500 ms, 1 mmol l⁻¹) and desensitising the ACh2 response. As expected from the pulse application data (Table 4, Agonism), when bath-applied, the four active agonists desensitised the ACh2 response while McN-A-343 was only weakly active when bath-applied.

McN-A-343, which has some structural features in common with oxotremorine, is specific to the mammalian M₁ muscarinic receptor (Roszkowski, 1961). This compound evoked a weak response with ACh2 membrane-potential-dependence and the response was blocked by 1 µmol l⁻¹ atropine and unaffected by 1 µmol l⁻¹ mecamylamine. No M₂- or M₃-specific agonists were available.

Activity of muscarinic compounds at the ACh1 receptor

Almost all of the muscarinic compounds, including those that were inactive against the muscarinic ACh2 response, blocked the nicotinic ACh1 response (Table 3). Of the muscarinic antagonists active on the ACh2 receptor, the non-subtype-selective compounds QNB, scopolamine and atropine were 10³-10⁴ times more active on the ACh2 receptor than on the ACh1 receptor, showing very clear discrimination between these Locusta migratoria nicotinic and muscarinic receptors. However, benactyzine, HHSiD and 4-DAMP showed an approximately 10-fold difference only. All four of the muscarinic antagonists inactive on the ACh2 receptor were quite strongly active on the ACh1 receptor. QNX, with an EC₅₀ value of approximately 20 nmol l⁻¹, rivalled the antagonistic effectiveness of the nicotinic antagonists mecamylamine and α-bungarotoxin. Only the M₁-selective compound pirenzepine was totally inactive against the ACh1 response.

Some of the muscarinic agonists were also active against the ACh1 response. For example, McN-A-343 induced desensitisation quite potently (EC₅₀ 11 µmol l⁻¹), while muscarine and oxotremorine were ineffective. Arecoline evoked the ACh1 response to an amplitude of about twice that of the ACh2 response evoked by the same application and recorded simultaneously. Taking into account the disparity in numbers of ACh1 and ACh2 receptors proposed above, this result is in accordance with the well-known low-potency action of arecoline at vertebrate nicotinic receptors (Bowman and Rand, 1980). A similar desensitising effect by pilocarpine was detected, probably reflecting a lower potency action of this compound on ACh1 receptors.

Effects of non-selective compounds

The compounds listed in Table 5 are acetylcholine, which does not distinguish between nicotinic and muscarinic receptors, and four other compounds that are selective for non-cholinergic receptors and channels. As expected, acetylcholine desensitised both the ACh1 and ACh2 responses.

Strychnine was without effect against the ACh2 response but was an extremely active antagonist at the ACh1 receptor, with an EC₅₀ value of 23 nmol l⁻¹, in the same activity range as mecamylamine, lobeline and α-bungarotoxin. δ-Phila...
toxin (Table 5) and many of its structural analogues block the ACh₁ response (J. A. Benson, L. Kaufmann, B. Hue, M. Pelhate, F. Schürmann, L. Gsell and T. Piek, in preparation). Bicuculline is a competitive antagonist at GABA_A receptors and picrotoxin is a blocker of GABA_A receptor-activated Cl⁻ channels. These compounds blocked the ACh₁ response equipotently (bicuculline EC₅₀ 31 µmol⁻¹; picrotoxin EC₅₀ 29 µmol⁻¹). The antagonistic actions of strychnine, bicuculline and picrotoxin were all independent of membrane potential (Benson, 1988b).

Discussion

We have shown that the response to acetylcholine in isolated, voltage-clamped, thoracic ganglion neuronal somata from Locusta migratoria consists of a fast, nicotinic (ACh₁) component and a slow, muscarinic (ACh₂) component (Benson and Neumann, 1987). In contrast to the vertebrate brain, nicotinic receptors are much more abundant in the insect brain and thoracic ganglia than are muscarinic receptors (Dudai and Ben-Barak, 1977; Breer, 1981), and a comparison of the doses of acetylcholine and muscarine required to evoke responses of similar magnitude suggests that this relationship may apply equally to individual somata (Figs 1 and 3).

Membrane-potential-dependence and ionic-dependence

The I–V curve for the ACh₁ response extrapolates to a reversal potential in the membrane potential range 0 to +20 mV. This value is in agreement with other studies of insect cholinergic responses, all of which indicate that the major charge carrier for the cholinergic current is Na⁺. Kerkut et al. (1969b) showed that the depolarising response to acetylcholine in cockroach neurones is substantially reduced in Na⁺-free saline and similar results were reported by Harrow et al. (1982) and David and Sattelle (1990). Reversal potential values for nicotinic acetylcholine responses, usually determined by extrapolation of current–voltage curves, have been reported for several insect neuronal preparations: —35 to —40 mV for neuronal somata in the cockroach sixth abdominal ganglion (Callec, 1974; Pitman and Kerkut, 1970) and the soma of the cockroach fast coxal depressor motoneurone D₁ (David and Pitman, 1982); —40 mV for the soma of the embryonic giant interneurone 2 (Blagburn et al. 1985); and +20 mV for dorsal unpaired median neurones in Schistocerca nitens (Goodman and Spitzer, 1980). The variation in these values may be due to the different salines used but all are consistent with activation of a predominantly Na⁺-mediated current by acetylcholine.

Pharmacology of the ACh₁ (nicotinic) response

The nicotinic cholinergic receptors of the vertebrates have been divided into two rather inhomogeneous classes, neuromuscular and ganglionic, based on location
and pharmacological profile. The sensitivity of insect neuronal nicotinic receptors to α-bungarotoxin (Schmidt-Neilsen et al. 1977; Dudai, 1978) distinguishes them from most vertebrate brain nicotinic receptors, which do not bind this toxin. In both binding (e.g. Jones et al. 1981) and electrophysiological (e.g. David and Sattelle, 1984; present work, Tables 1, 2) experiments, the low potencies of decamethonium, a neuromuscular agonist, and hexamethonium, a ganglionic antagonist, distinguish the insect receptor from its vertebrate counterparts.

Antagonists. The most active antagonist at the ACh1 receptor was PMNI, an example of the nitromethylene heterocycle insecticides (Soloway et al. 1979) that are rapidly emerging as an important new group of plant protection agents. These compounds have their site of action at the nicotinic receptor (Schroeder and Flattum, 1984; Harris et al. 1986) and they compete with α-bungarotoxin in insect neuronal membrane binding assays (Sattelle et al. 1989; I. Bermudez and J. A. Benson, unpublished observations). They are nicotinic agonists in cockroaches (e.g. Sattelle et al. 1989; Buckingham et al. 1989) and, at relatively high concentrations (threshold approximately 30 nmol l⁻¹), they also act as agonists at the ACh1 receptor in Locusta migratoria. However, we have shown that in Locusta migratoria they can be potent and selective (i.e. non-muscarinic) antagonists at concentrations several orders of magnitude below their agonist threshold (Benson, 1989b, 1990). There is no evidence of agonistic shifts in the baseline current at the concentrations of PMNI that result in 50% or more blockade of the ACh1 response in Locusta migratoria (Benson, 1989b, 1992a). PMNI is totally inactive at the muscarinic ACh2 receptor. Zwart et al. (1992) have recently confirmed the effects of PMNI at high concentrations on Locusta migratoria neuronal somata but report that this compound has much lower potency against vertebrate nicotinic receptors, particularly those of the neurones. They suggest that this differential sensitivity at the receptor level could provide a basis for the low mammalian toxicity of these insecticides.

The polypeptide α-bungarotoxin was a highly potent antagonist. Early studies localized α-bungarotoxin binding proteins, often showing nicotinic pharmacology, in a variety of insect neuronal tissues (Dudai, 1977) and these observations have been repeated and refined many times since (reviewed by Eldefrawi and Eldefrawi, 1988). This toxin is well-known as a competitive antagonist selective for the vertebrate neuromuscular junction cholinoreceptor. It has no effect on most vertebrate neuronal acetylcholine receptors. In contrast, it has frequently been shown to block the nicotinic cholinergic responses of insect neuronal somata (e.g. David and Sattelle, 1984). α-Bungarotoxin has been reported to bind irreversibly to its receptor in vertebrate muscle (Miledi and Potter, 1971) and on some insect neurones (e.g. David and Sattelle, 1984) but not others (Hall, 1980; Eldefrawi and Eldefrawi, 1980). Its effect on Locusta migratoria thoracic neuronal somata is readily, if comparatively slowly, reversed by superfusion of the preparation with control saline (Benson, 1988a) and is voltage-independent over the membrane potential range −40 to −100 mV (Benson, 1988a), as expected for an antagonist acting competitively at the acetylcholine recognition site.
A further difference between the ACh1 receptor and the classical vertebrate receptors was revealed by the action of lobeline, an alkaloid from Indian tobacco (Lobeliaceae). This compound is a potent agonist in mammalian systems with blocking activity that possibly results from desensitisation (Bowman and Rand, 1980). In contrast, at the ACh1 receptor, it shows only very weak agonism but is an antagonist with a potency rivalling that of α-bungarotoxin and mecamylamine. It was found to be a nicotinic antagonist in Periplaneta americana by Battersby and Hall (1985), but with a large difference in potency for Musca domestica and Periplaneta americana in [3H]α-bungarotoxin binding assays.

Mecamylamine, an antagonist selective for the vertebrate ganglionic nicotinic receptor, also blocked the ACh1 response with an EC₅₀ value (63 nmol l⁻¹) very similar to that for α-bungarotoxin. On the cockroach motoneurone Dᵢ, in situ, David and Sattelle (1984) reported an EC₅₀ value of 2.6 μmol l⁻¹, compared with 64 nmol l⁻¹ for α-bungarotoxin. Mecamylamine is always observed to be a poor displacer of radiolabelled α-bungarotoxin (e.g. Jones et al. 1981), probably because it acts as a channel blocker at a site different from the recognition site for competitive ligands. In Periplaneta americana, mecamylamine pretreatment does not protect the somal nicotinic receptor from blockade by α-bungarotoxin (David and Sattelle, 1984).

Trimethaphan camsylate (EC₅₀ 0.67 μmol l⁻¹), a vertebrate ganglionic blocker like mecamylamine, was about 10 times less active than mecamylamine. It is probably a rather unspecific blocker of ligand-binding-activated ion channels since it also blocks the open channels of glutamatergic receptors in locusts (Ashford et al. 1988). The remaining nicotinic antagonists exhibited comparatively low potencies, their EC₅₀ values covering the same range as the values for muscarinic compounds active at the nicotinic ACh1 receptor (see below).

In summary, the order of potency of the nicotinic antagonists at the ACh1 receptor was PMNI ≥ α-bungarotoxin (nm) ≥ lobeline ≥ mecamylamine (ga) > trimethaphan camsylate (ga) > chlorisondamine (ga) > d-tubocurarine (nm) > hexamethonium (ga) > gallamine triethiodide (nm) > tetraethylammonium (ga). This profile shows that the ACh1 receptor does not discriminate between antagonists selective for neuromuscular and ganglionic receptors in vertebrates. The EC₅₀ values of the two classes of compounds active at the ACh1 receptor are about equally distributed from highly potent to weak. In a comparable electrophysiological study on the Periplaneta americana neurone Dᵢ in situ, David and Sattelle (1984) determined a similar rank order of potency for nicotinic antagonists: α-bungarotoxin ≥ mecamylamine ≥ d-tubocurarine, with hexamethonium and gallamine triethiodide requiring more than 1 mmol l⁻¹ to block the response. For the gastric mill muscles of the crabs Cancer pagurus, C. irroratus and C. borealis (Marder and Paupardin-Tritsch, 1980a), the rank order of nicotinic antagonists was mecamylamine > trimethaphan camsylate > chlorisondamine > d-tubocurarine ≥ hexamethonium. α-Bungarotoxin was without effect.

Agonists. In vertebrates, nicotinic agonists in general are active at both the neuromuscular and ganglionic receptors. When bath-applied, all of the nicotinic
agonists reduced the ACh1 response evoked by acetylcholine in a dose-dependent manner (Table 2). The only simple criterion that distinguishes the reduction of the ACh1 response due to the combination of desensitization and receptor site competition, as for nicotine, from blockade at the acetylcholine recognition site or ion channel, is that desensitisation is accompanied by an agonistic shift in the resting current of the clamped neurone, with the threshold for this effect coinciding with or being lower than the threshold for reduction of the ACh1 response. For the agonists listed in Table 2, the EC50 values for desensitisation and blockade were high in comparison to the values for most of the antagonists described above (Table 1), and none of the agonists tested affected the ACh2 response when applied at 10 or 100 µmol l−1 (Table 2).

Nicotine and anabasine are alkaloids of similar structure from *Nicotiana* (cultivated tobacco), and both are potent agonists at the ACh1 receptor. Nicotine has a threshold of between 0.1 and 0.3 µmol l−1 and an EC50 value for desensitisation (0.5 µmol l−1) that is lower than that for anabasine (10 µmol l−1). The response to microapplication of nicotine was always larger and slower than that for an identical pulse of acetylcholine, an observation typical of arthropod neurones (e.g. crab stomatogastric neurones; Marder and Paupardin-Tritsch, 1978). A significant exception is the comparatively feeble response to nicotine recorded in a motor neurone, PPR, in the tobacco hornworm *Manduca sexta*, which feeds on *Nicotiana* (Trimmer and Weeks, 1989a). The considerable efficacy of nicotine in desensitising the ACh1 receptor was the main reason that acetylcholine was used in the study of the ACh1 response, rather than the more selective nicotine. Tetramethylammonium (EC50 62 µmol l−1) is a ganglionic compound that stimulates both nicotinic and muscarinic receptors in vertebrates (Bowman and Rand, 1980). However, in *Locusta migratoria* it was selective for the ACh1 receptors. It evokes a response in *Manduca sexta* PPR neurones similar to that of nicotine (Trimmer and Weeks, 1989a) and it mimics the nicotinic acetylcholine response in the stomatogastric ganglion neurones of the crab *Cancer pagurus* (Marder and Paupardin-Tritsch, 1978). DMPP (EC50 85 µmol l−1) strongly stimulates the adrenal medulla in vertebrates but is also active on the autonomic ganglia (Bowman and Rand, 1980). It is an effective agonist at α-tubocurarine-sensitive acetylcholine receptors in the mollusc *Aplysia californica* (Kehoe, 1972) and mimics the slow response to nicotine in the stomatogastric ganglion neurones of the crab *Cancer pagurus* (Marder and Paupardin-Tritsch, 1978). However, in contrast to its activity on *Locusta migratoria* somata, it was reported to give only a small response when applied at 10 mmol l−1 to the cockroach neurone D₁ (David and Sattelle, 1984).

The actions of suberyldicholine and decamethonium were complex. Both compounds are potent vertebrate neuromuscular agonists, and decamethonium is well-known as a 'depolarising blocker' (i.e. causing synaptic block by desensitisation of the neuromuscular nicotinic receptors) (del Castillo and Katz, 1955). However, in binding studies on *Drosophila melanogaster* neural extract, decamethonium is only very weakly active as a competitor with α-bungarotoxin,
weaker than the muscarinic agents pilocarpine or oxotremorine (Dudai, 1978). These compounds are without agonistic effect, in control saline, both on Locusta migratoria somata (Table 2) and on the stomatogastric ganglion neurones of the crab Cancer pagurus, at concentrations of up to 1mmol⁻¹ (Marder and Paupardin-Tritsch, 1978). At high concentrations, both compounds cause partial blockade of the ACh₁ response (EC₅₀ values approximately 1μmol⁻¹ and approximately 40μmol⁻¹, respectively) and of nicotinic cholinergic responses in the cockroach neurone Dₚ (EC₅₀ 2.8mmol⁻¹ for decamethonium; David and Sattelle, 1984). This blockade at high concentrations is probably the rather non-specific channel blocking effect also reported for vertebrates since it was not accompanied by any evidence of agonism. The unusual observations for the Locusta migratoria somata are that, when pulse-applied in the presence of acetylcholine in the bathing saline, both compounds evoked the ACh₁ current and the amplitude of the evoked current increased with the concentration of acetylcholine in the bathing saline (Benson, 1988a). The mechanism of this effect is unknown, but it is probably not due to saturation of cholinesterase since it could not be mimicked by addition of the cholinesterase inhibitor eserine to the control saline. The lack of agonistic effects by suberyldicholine and decamethonium distinguishes the ACh₁ receptor from the vertebrate neuromuscular nicotinic receptor, despite the observation that both receptor subtypes are highly sensitive to α-bungarotoxin.

Pyrantel and Levamisole are anthelmintics that act as agonists at the acetylcholine receptor on the muscle cells of the nematode Ascaris suum (Harrow and Gratian, 1985). The order of potency for Ascaris is Pyrantel>Levamisole>acetylcholine, with the agonist threshold for Pyrantel being between 10 and 100nmol⁻¹. When applied to Locusta migratoria somata, these compounds were low-potency blockers (Pyrantel EC₅₀ 36μmol⁻¹ and Levamisole EC₅₀ 38μmol⁻¹) and showed agonism only at concentrations above 30μmol⁻¹. They possess only weak insecticidal activities.

The EC₅₀ values given in Table 2 are based on the blocking and desensitising effects of the cholinergic agonists and disguise the varied modes of action of these compounds. Nicotine was the most potent agonist, followed by anabasine, tetramethylammonium and DMPP. Pyrantel and Levamisole were very weak agonists, and suberyldicholine and decamethonium were not agonistic.

Action of non-selective compounds. Strychnine has been used to distinguish between the vertebrate GABAₐ receptor and the glycine receptor, for which it is selective (e.g. Grenningloh et al. 1987), but it is also a vertebrate cholinergic blocker (Alving, 1961). Long ago Roeder showed that strychnine blocks a cholinergic synapse in the sixth abdominal ganglion of the cockroach (Roeder et al. 1947). It had no effect against the ACh₂ response but was extremely active at the ACh₁ receptor. At 10μmol⁻¹, it blocked the effect of acetylcholine and the excitatory junction potential (EJP) recorded from the stomach dorsal dilator muscle of the lobster Panulirus interruptus (Marder, 1976).

δ-Philanthotoxin 433, a polyamine and one of the toxic fractions from the
venom of the bee wolf *Philanthus triangulum* (Piek et al. 1988), blocks open channels at insect glutamatergic neuromuscular synapses (Clark et al. 1982; Benson et al. 1992). It also blocks nicotinic cholinergic neurotransmission in insects (Piek et al. 1984), and both this compound (Table 5) and many of its structural analogues block the ACh1 response with the same or higher potency as they do at the *Musca domestica* larva neuromuscular glutamate receptor (J. A. Benson, L. Kaufmann, B. Hue, M. Pelhate, F. Schürmann, L. Gsell and T. Piek, in preparation). Bicuculline and picrotoxin, blockers of vertebrate non-cholinergic neurotransmitter-evoked responses, were active against the ACh1 response but not the ACh2 response. The concentration range for these compounds was typical of channel blockers but higher than at their sites of selective action. Bicuculline is the diagnostic antagonist for GABA A receptors (Bowman and Rand, 1980). It appears to be inactive at most insect GABA A-type receptors but is a moderately effective blocker of the ACh1 response (Table 5; Benson, 1988c). Picrotoxin is a potent blocker of GABA A-receptor-activated Cl− channels both in vertebrates (Bowman and Rand, 1980) and in insects (Lees et al. 1987). The EC50 value for picrotoxin against the ACh1 response was 29 μmol L−1, and comparable concentrations also block cholinergic responses in non-insect arthropods. At 0.1–1 μmol L−1, picrotoxin blocked the effect of acetylcholine and the EJP recorded from the stomach dorsal dilator muscle of the lobster *Panulirus interruptus* (Marder, 1976) and at 50 μmol L−1 it blocked the acetylcholine-evoked increase in Na+ conductance in the paired gastric mill muscles of the crab *Cancer pagurus* (Marder and Paupardin-Tritsch, 1980b). Although moderately potent against the ACh1 response, none of these compounds had any effect on the ACh2 response. This probably indicates a fundamentally different channel structure and a different receptor superfamily for the ACh2 receptor.

**Somal versus synaptic insect nicotinic receptors**

The pharmacological profiles of the insect synaptic and somal nicotinic receptors are similar in some respects. Like the ACh1 response, insect nicotinic synapses are very sensitive to α-bungarotoxin (e.g. Sattelle et al. 1980), curare is much more effective as a blocker than atropine (e.g. Waldrop and Hildebrand, 1989) and transmission is blocked by gallamine triethiodide (Pitman and Kerkut, 1970). However, based on single-channel recordings from reconstituted *Locusta migratoria* nicotinic receptors in lipid bilayers, Tareilus et al. (1990) found evidence for two different receptor subtypes, which they suggest might represent synaptic and extrasynaptic nicotinic acetylcholine receptors. In contrast to the ACh1 receptor, the neuropile-derived, ‘synaptic’ receptors are more sensitive to suberyldicholine than to acetylcholine. In the absence of pharmacological data from the reconstituted somal receptors, it is not possible to be certain that they are the ACh1 receptors, but this seems very likely.

**The muscarine (ACh2) response**

The description of functional muscarinic receptors in insects has lagged behind
the elucidation of their pharmacology by biochemical methods. Trimmer and
Weeks (1987, 1988, 1989a,b) have reported a decrease in action potential threshold
in Manduca sexta motoneurones caused by muscarinic agents, and there is
evidence that muscarinic receptors inhibit acetylcholine release at the cercal
nerve–giant interneurone synapses of the cockroach Periplaneta americana (Hue
et al. 1989; Le Corronc et al. 1991). Gorczyca et al. (1991) were able to activate a
central pattern generator of the pharyngeal muscles of larval Drosophila melano-
gaster using muscarinic agonists and to block this response, as well as endogenous
activity, using muscarinic antagonists.

Membrane-potential-dependence and ionic-dependence

The current activated via the ACh2 receptor is inward at −30 mV and decreases
with hyperpolarisation to zero at −80 to −90 mV (Benson, 1988b). In Manduca
sexta motoneurone PPR, the current evoked by oxotremorine is at its maximum
near the normal resting potential of −50 mV and declines with hyperpolarisation
or depolarisation. The current is tetrodotoxin-insensitive but is abolished in the
absence of external Na+ (Trimmer and Weeks, 1991). In several crustacean
preparations, muscarine evokes responses that resemble the ACh2 current.
Marder and Paupardin-Tritsch (1978) described an inward current evoked by
microapplication of the muscarinic agonist acetyl-β-methylcholine to the somata
of stomatogastric ganglion neurones in the crab Cancer pagurus. This response
was blocked by 0.1–1 μmol l⁻¹ atropine and had a voltage-dependence very similar
to that of the ACh2 response, a decrease in amplitude with hyperpolarisation
levelling off at membrane potentials of between −80 and −90 mV. The same
neurones exhibit a predominant response to nicotine and acetylcholine that bears
many similarities to the ACh1 response (Marder and Paupardin-Trisch, 1978). A
similar muscarine-evoked current has been recorded in the cardiac ganglion
motoneurones of the lobster Homarus americanus, decreasing with hyperpolarisa-
tion and becoming zero but not reversing at −80 to −100 mV (Freschi and
Livengood, 1989). However, when these neurones are clamped at positive
potentials, the current is observed to reach a maximum at −30 to −10 mV and to
reverse at +20 mV. This current is carried largely by Na+ but is also dependent on
extracellular K+ concentration (Freschi and Livengood, 1989).

Pharmacology of the ACh2 (muscarinic) response

On pharmacological grounds, the mammalian muscarinic receptors have been
classified into at least three subtypes, neuronal-M1, cardiac-M2 and glandular-M3
(Doods et al. 1987; Giraldo et al. 1988; Mei et al. 1989). Molecular cloning studies
have revealed a family of five muscarinic receptor genes, m1–m5 (e.g. Peralta et al.
1987). The antagonist binding profiles of the expressed m1, m3 and m4 receptors
correspond best to the M1, M2 and M3 receptors (Mei et al. 1989). One insect
muscarinic receptor gene has been cloned and it codes for a receptor that is
comparatively similar in amino acid sequence to the m1, m3 and m5 vertebrate
muscarinic subtypes (Onai et al. 1989).
Dudai and Ben-Barak (1977) first demonstrated the presence of a distinct muscarinic binding receptor in an insect. Later studies suggested heterogeneity among the insect muscarinic binding sites, on the basis of Hill coefficients of less than 1 (Shaker and Eldefrawi, 1981; Aguilar and Lunt, 1984) and on linkage to different second messengers, cyclic AMP and phosphatidylinositol in particular (Trimmer and Berridge, 1985; Duggan and Lunt, 1986, 1988). Recently, a Drosophila melanogaster muscarinic receptor gene has been expressed in mammalian Y1 cells, where it mediates stimulation of phosphatidylinositol metabolism (Shapiro et al. 1989). Knipper and Breer (1988, 1989) provided pharmacological support for the existence of heterogeneity among functional muscarinic receptors in insects. Based on differences in pirenzepine affinity, they proposed that release of acetylcholine from locust ganglia synaptosomes was inhibited via presynaptic $M_2$ muscarinic autoreceptors coupled to a cyclic AMP second-messenger cascade. $M_1$ receptors seemed to be preferentially located in the cell body membrane fraction (Knipper and Breer, 1988). However, from the results presented in this paper, as well as from similar experiments on lobster cardiac neurones (Freschi, 1991), it seems that pirenzepine-sensitivity by itself is an insufficient basis for allocating arthropod muscarinic receptors to the vertebrate $M_1$ and $M_2$ categories, despite the pharmacological similarities between the pirenzepine-sensitive, somal ACh2 receptor and the vertebrate $M_1$ receptor.

**Antagonists.** QNB ($EC_{50}$ 4 nmol l$^{-1}$) and scopolamine ($EC_{50}$ 7 nmol l$^{-1}$) were the most potent of the muscarinic antagonists against the ACh2 response, with atropine, an alkaloid of structure very similar to that of scopolamine, slightly less potent (Table 3). QNB does not discriminate among the vertebrate muscarinic receptor subtypes, and the same appears to be true in the arthropods. For example, it also binds to muscarinic receptors in homogenates of whole bulb mite (Rhizoglyphus echinopus), which are effectively protected by pirenzepine ($M_1$) but not by methoctramine ($M_2$) (Huang and Knowles, 1990), a pharmacology different from the ACh2 profile. Like all of the muscarinic antagonists tested except pirenzepine, QNB, scopolamine and atropine, although highly selective for the ACh2 receptor, were moderate blockers of the ACh1 response, with $EC_{50}$ values of 20–45 μmol l$^{-1}$. The latter observation is in contrast to the report of Harrow and Sattelle (1983), who found that QNB at concentrations of up to 10 μmol l$^{-1}$ was without effect on the acetylcholine response recorded in the soma of the cockroach giant interneurone 2. This possibly reflects more the ready accessibility of the isolated Locusta migratoria soma in comparison with neuronal somata in situ, than a genuine difference in receptor pharmacology. The actions of QNB and atropine against the nicotinic response in the cockroach neurone $D_i$ in situ had $EC_{50}$ values of about 100 μmol l$^{-1}$ (David and Sattelle, 1984).

Pirenzepine is a tricyclic compound with a pronounced ability to discriminate between muscarinic receptors in different vertebrate tissues (Hammer et al. 1980). It binds with high affinity to the $M_1$ receptor and is quite potent as an ACh2 antagonist. The $M_1$ receptor seems to be linked to closure of $M^+$ K$^+$ channels (Brown and Adams, 1980). In contrast, the ACh2 current results from channels
opening (decrease in membrane resistance; Fig. 1B). Since pirenzepine was unique among the muscarinic antagonists tested in being without effect on the nicotinic ACh1 response, it should be the antagonist of first choice for discriminating between nicotinic and muscarinic mediation of physiological responses in the insect nervous system.

HHSiD has a high affinity for M₁ receptors in vertebrate neuronal tissues as well as for M₃ glandular and smooth muscle receptors in exocrine glands, but a much lower affinity for M₂ cardiac receptors (Mutschler and Lambrecht, 1984). It showed the same level of activity as pirenzepine against the ACh2 response (EC₅₀ values about 0.5 μmol L⁻¹). QNX, which is selective for M₁ receptors, was only very weakly active, at least 100 times less active than HHSiD and about 10,000 times less active than QNB, of which it is a structural analogue. There is thus no correlation between high affinity to M₁ receptors in vertebrates and efficacy at the ACh2 receptor. The very high potency of QNX against the ACh1 response has been discussed above.

AF-DX116 is a pyridobenzodiazepinone tricyclic compound that binds selectively to cardiac (M₂) receptors (Hammer et al. 1986). In contrast to pirenzepine, of which it is a structural analogue, it was without effect against the ACh2 response. The third M₂-selective compound was methoctramine, a tetra-amine, which is a competitive antagonist at vertebrate M₂ receptors with a potency similar to that of the non-selective compound atropine. It also interacts non-competitively with the nicotinic receptors of frog rectus abdominis muscle (IC₅₀ 0.23 μmol L⁻¹) (Melchiorre et al. 1987a, b). It was inactive against the ACh2 response, but, as in the frog, blocked the nicotinic response.

4-DAMP is an M₃-receptor-selective compound in vertebrates (Barlow et al. 1976, 1980), where it binds with 50-100 times greater affinity than does pirenzepine. This is in contrast to its potency against the ACh2 response, where 4-DAMP was only slightly less active than pirenzepine.

In summary, the order of potency of the muscarinic antagonists at the ACh2 receptor was: QNB > scopolamine > atropine > 4-DAMP (M₃) > benactyzine > HHSiD (M₁/M₃) > pirenzepine (M₁). QNX (M₁), AF-DX116 (M₂), gallamine triethiodide (M₂) and methoctramine (M₂) were almost or completely inactive. Two of the M₁ compounds were potent and one (QNX) was inactive. The three M₂ compounds were almost or completely inactive. Of the muscarinic-receptor subtype-selective compounds, 4-DAMP (M₃) was the most active and the only other M₃ compound tested (HHSiD) was almost equally active. The ACh2 receptor antagonist pharmacology thus does not correspond in detail to the M₁/M₂/M₃ classification. It seems to possess a mixture of M₁- and M₃-like properties and to differ most from the M₂ profile. In contrast, the presynaptic muscarinic receptors involved at the cockroach cercal afferent–giant interneurone synapse are blocked by the M₂ antagonists AF-DX116 and methoctramine, but not by the M₁ antagonists pirenzepine and 4-DAMP (Le Corronc et al. 1991). At the Manduca sexta sensory-to-motor synapse, scopolamine (0.1 μmol L⁻¹) and HHSiD (1 μmol L⁻¹) blocked the postsynaptic response, while pirenzepine was
effective only at concentrations above 10 \( \mu \text{mol l}^{-1} \) and 4-DAMP was without effect (Trimmer and Weeks, 1989b). These data from \textit{Locusta migratoria} somata and from cockroach and \textit{Manduca sexta} synapses are in broad agreement with the conclusions of Knipper and Breer (1988, 1989), discussed above, that \( M_1 \)-like receptors seem to be preferentially located on somata while \( M_2 \)-like autoreceptors exist presynaptically.

Freschi (1991) carried out an electrophysiological analysis of the response of the motor neurones of the cardiac ganglion of the lobster \textit{Homarus americanus} to the muscarinic agonist methacholine. The order of antagonist potency was atropine\( > \)pirenzepine\( \geq \)4-DAMP\( > \)methoctramine\( > \)HHSiD. AF-DX116 and gallamine triethiodide were inactive. Despite a similar high activity of atropine and pirenzepine, and low activity of AF-DX116 and gallamine triethiodide, this profile also differs from that of the ACh2 receptor, particularly in the higher sensitivity to methoctramine and lower sensitivity to HHSiD.

\textit{Agonists.} Unfortunately, agonists specific to particular muscarinic receptor subtypes are rare. The only one readily available is McN-A-343, which shows selectivity for \( M_1 \) receptors (Roszkowski, 1961). This compound was only feebly active on the isolated somata (Table 4). Similarly, at the presynaptic muscarinic receptors of the cockroach cercal afferent–giant interneurone synapse, McN-A-343 (\( EC_{50} \) 15 \( \mu \text{mol l}^{-1} \)) is much weaker than arecoline (\( EC_{50} \) 72 \( \mu \text{mol l}^{-1} \)) or oxotremorine (\( EC_{50} \) 4 \( \mu \text{mol l}^{-1} \)) (LeCorronc et al. 1991). At concentrations up to 3 \( \mu \text{mol l}^{-1} \), McN-A-343 was without detectable effect on \textit{Manduca sexta} sensory-to-motoneurone synapses (Trimmer and Weeks, 1989b), and it was inactive at the muscarinic receptors on the motoneurones of the lobster cardiac ganglion (Freschi, 1991).

\textit{Cross reaction of vertebrate cholinoreceptor-subtype-selective compounds between the ACh1 and ACh2 receptors}

A striking observation made during these experiments was that although only one nicotinic antagonist, lobeline, was active against the muscarinic ACh2 response, all of the muscarinic antagonists, with the single exception of pirenzepine, showed activity at the ACh1 nicotinic receptor, in some cases much more potently than against the ACh2 response. The ACh1 receptor appears to differ additionally from the vertebrate nicotinic subtypes in being unusually accessible to compounds highly selective for vertebrate muscarinic receptors.

\textit{The ‘mixed’ nicotinic–muscarinic cholinoreceptor}

Several early radiolabelled ligand binding studies revealed a receptor site, possibly a glycoprotein, with a ‘mixed’ nicotinic–muscarinic pharmacology (e.g. Eldefrawi and O’Brien, 1970). Since this site bound \(^3\text{H}\)nicotine or \(^3\text{H}\)decamethonium but not \(^3\text{H}\)a-bungarotoxin (Donnellan et al. 1975; Mansour et al. 1977), it does not correspond to either the ACh1 or the ACh2 receptor. Lapied et al. (1990) recently described a nicotine-evoked response in \textit{Periplaneta americana} DUM neuronal somata that consisted of two components, one of which appears to be a
genuine 'mixed' response blocked by \(d\)-tubocurarine (1 \(\mu\)mol l\(^{-1}\)), pirenzepine (10 nmol l\(^{-1}\)) and gallamine triethiodide (100 nmol l\(^{-1}\)). It was, however, also blocked by \(\alpha\)-bungarotoxin (100 nmol l\(^{-1}\)). The other component, although evoked by nicotine, was unaffected by the nicotinic and muscarinic agents tested, including \(\alpha\)-bungarotoxin.

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References


Locust neuronal soma cholinoreceptors


