J Comp Physiol A (2004) 190: 807–821
DOI 10.1007/s00359-004-0530-7

ORIGINAL PAPER

Daniel G. Wüstenberg · Bernd Grünewald

Pharmacology of the neuronal nicotinic acetylcholine receptor of cultured Kenyon cells of the honeybee, *Apis mellifera*

Received: 11 December 2003 / Revised: 29 April 2004 / Accepted: 30 April 2004 / Published online: 11 August 2004

Abstract We investigated the pharmacology of the nicotinic acetylcholine receptor of honeybee Kenyon cells, a subset of olfactory interneurons, which are crucial for olfactory learning and memory. Whole-cell currents were recorded using patch-clamp techniques. Pressure application of agonists induced inward currents in cultured Kenyon cells at holding potentials of –110 mV. Acetylcholine or carbamylcholine were full agonists, nicotine, epibatidine and cytisine were only partial agonists. Coapplications of these partial agonists with acetylcholine reduced the current amplitude. The most efficient antagonists were dihydroxy-β-erythroidine ($EC_{50} = 0.5$ pmol·l^{-1}) and methyllycaconitine ($EC_{50} = 24$ pmol·l^{-1}). The open channel blocker mecamylamine, d-tubocurarine and hexamethonium were rather weak blockers of the honeybee nicotinic response. Bath applications of the muscarinic antagonist atropine inhibited nicotinic currents dependent on concentration ($EC_{50} = 24.3$ μmol·l^{-1}). Muscarine, pilocarpine or oxotremorine (1 mmol·l^{-1}) did not induce any measurable currents. The non-cholinergic drugs strychnine, bicuculline and picrotoxin partially and reversibly blocked the acetylcholine-induced currents. Our results indicate the expression of only one nicotinic acetylcholine receptor subtype in cultured Kenyon cells. Muscarinic as well as non-cholinergic antagonists also inhibit the receptor function, distinguishing the honeybee nicotinic receptor from the "typical" nicotinic receptor of vertebrates and from many described insects receptors.

Keywords Dihydroxy-β-erythroidine · Insects · Methyllycaconitine · Mushroom body · Patch clamp

D. G. Wüstenberg · B. Grünewald (✉)
Institut für Biologie, Neurobiologie, Freie Universität Berlin,
Königin-Luise-Str. 28/30, 14 195 Berlin, Germany
E-mail: gruenewa@neurobiologie.fu-berlin.de
Tel.: +49-30-83854298
Fax: +49-30-83855455

Present address: D. G. Wüstenberg
Texas Medical School, University of Texas Houston,
6431 Fannin Street, Houston, TX 77030, USA

Abbreviations ACh: Acetylcholine · ATR: Atropin · BIC: Bicuculine · CCh: Carbamylcholine · CYT: Cytosine · DHE: Dihydroxy-β-erythroidine · DTC: d-Tubocurarine · EPI: Epibatidine · GABA: γ-Amino butyric acid · HEX: Hexamethonium · MEC: Mecamylamine · MLA: Methyllycaconitine · MUS: Muscarine · nAChR: Nicotinic acetylcholine receptor · NIC: Nicotine · OXO: Oxotremorine · PIC: Picrotoxin · STR: Strychnine

Introduction

Acetylcholine is the major excitatory transmitter within the insect central nervous system. Currents through neuronal nicotinic receptors or nicotinic acetylcholine receptors themselves (nAChR) have been described in a variety of insect species (*Acheta domestica*: Cayre et al. 1999; *Apis mellifera*: Goldberg et al. 1999; Déglise et al. 2002; Thany et al. 2003; *Drosophila melanogaster*: Albert and Lingle 1993; Lee and O'Dowd 2000; Rohrbough and Broadie 2002; *Locusta migratoria*: Tareilus et al. 1990: Benson 1992; Hermsen et al. 1998; *Manduca sexta*: Eastham et al. 1998; *Musca domestica*: Albert and Lingle 1993; *Periplaneta americana*: Beadle et al. 1988; Buckingham et al. 1997; *Schistocerca gregaria*: Suter and Usherwood 1985; Albert and Lingle 1993; reviews: Osborne 1996; Gundelfinger and Schulz 2000).

Immunocytochemical studies have shown that major parts of the olfactory pathway in the insect brain are cholinergic (Gorczyca and Hall 1984; Kreissl and Bicker 1989; Homberg 2002; Yusuyama et al. 2002). The olfactory pathway in insects comprises the central neuropil of the mushroom bodies. These paired structures receive cholinergic input from the antennal lobe and are involved in olfactory learning and memory formation in bees and fruit flies (Menzel et al. 1974; Heisenberg et al. 1985). In the honeybee brain each mushroom body is formed by about 170,000 intrinsic Kenyon cells. Primary cultures of neurons from mushroom body tissue can

therefore be used to study the properties of native currents on identified cells. Thus, the voltage-sensitive ionic currents of cultured Kenyon cells have already been analyzed in detail (Schäfer et al. 1994; Pelz et al. 1999; Grünewald 2003).

Kenyon cells in culture express nicotinic currents (*Acheta domesticus*: Cayre et al. 1999; *Apis mellifera*: Goldberg et al. 1999; Déglise et al. 2002; *Drosophila melanogaster*: Su and O'Dowd 2003). As a culture of identified cells, they provide an excellent preparation to overcome a difficulty of many studies of insect nicotinic currents: Although the basic physiological and pharmacological properties of the insect nicotinic currents have been analyzed, this available knowledge is still insufficient to allow a classification of insect nicotinic receptor subtypes. This is largely due to the fact that various different approaches (patch clamp, binding studies, genetics, behavioral pharmacology) were applied to many different preparations and mainly to unidentified neurons. There are only few examples of detailed physiological and pharmacological descriptions existing for a given neuron type or receptor (*Periplaneta americana*: Courjaret and Lapied 2001; *Schistocerca gregaria*: Marshall et al. 1990).

Pharmacological, biochemical and physiological approaches indicated the existence of different nicotinic receptor subtypes in insects (Beadle et al. 1988; Tareilus et al. 1990; Cheung et al. 1992; Leech and Sattelle 1992; Albert and Lingle 1993; Hermsen et al. 1998; van den Beukel et al. 1998). However, the various physiologically described currents cannot yet be associated to the various identified insect nAChR genes. A pharmacological classification may help to identify and characterize such different receptor subtypes, which were suggested also for honeybee Kenyon cells (Goldberg et al. 1999).

Pharmacological and physiological evidence suggested some similarities between vertebrate nAChR subtypes containing the α7-subunit and the honeybee nicotinic current (Goldberg et al. 1999). In both cases, a high Ca^{2+} permeability and sensitivity to the snake toxin α-bungarotoxine is found (Goldberg et al. 1999; Déglise et al. 2002). However, the pharmacological analyses remained preliminary and a thorough comparison is still missing. Since the vertebrate subtypes can be distinguished by their differing pharmacologies (Alkondon and Albuquerque 1993; Colquhoun and Patrick 1997; Cordero-Erausquin et al. 2000), we used nicotinic drugs that may prove possible similarities and differences between the insect and the vertebrate receptors such as muscular nAChRs, ganglionic nAChRs and central nervous nAChRs formed by α7-like subunits (Albuquerque et al. 1997; Colquhoun and Patrick 1997; Cordero-Erausquin et al. 2000).

Many cholinergic currents in insects also show a "mixed" muscarinic-nicotinic pharmacology (Grolleau et al. 1996; Buckingham et al. 1997; review: Osborne 1996) and nAChRs of locust neurons show a rather untypical sensitivity to substances, which are usually specific for non-cholinergic receptors of the same family such as $GABA_A$ or glycine-receptors (Benson 1988, 1992). Therefore, we also tested the efficiency of some non-nicotinic or non-cholinergic agonists and antagonists. The present work provides a thorough pharmacological characterization of the honeybee nicotinic current, which shares similarities with other insect and vertebrate neuronal receptors. However, it also shows several unusual features, which makes the honeybee nicotinic current rather special among the neuronal receptors described so far. The work also provides pharmacological tools that may allow to dissect the functional roles of the neuronal nAChR for the insect behavior (Lozano et al. 1996). Parts of this work were published previously in abstract form (Grünewald et al. 2000).

Materials and methods

Animals and cell preparation

Honey bee (*Apis mellifera L.*) pupae were collected from the comb between days 4 and 6 of the pupal development, which lasts 9 days under natural conditions.

Kenyon cells were dissected and cultured following a modified protocol published earlier (Kreissl and Bicker 1992). Brains were removed from the head capsule in a Leibovitz L15 medium (GIBCO BRL) supplemented with sucrose, glucose, fructose, and proline 42.0, 4.0, 2.5, and 3.3 g l^{-1}, respectively (500 mosmol, pH 7.2). The glial sheath was removed and the mushroom bodies were dissected out of the brains. After incubation (10 min) in a calcium-free saline solution to loosen cell adhesion (in mmol·l^{-1}: 130 NaCl, 5 KCl, 10 $MgCl_2$, 25 glucose, 180 sucrose, 10 HEPES; pH 7.2) mushroom bodies were transferred back to L15 preparation medium (two mushroom bodies per 100 µl) and dissociated by gentle trituration with a 100-µl Eppendorf pipette. Cells were then plated in aliquots of 10 µl on polylysine (polylysine-L-hydrobromide, MW 150–300 kDa; Sigma) coated Falcon plastic dishes and allowed to settle and adhere to the substrate for at least 10 min. Thereafter, the dishes were filled with approximately 2.5 ml of culture medium (13% (v/v) heat inactivated fetal calf serum (Sigma, St. Louis, MO., USA), 1.3% (v/v) yeast hydrolysate (Sigma), 12.5% (w/v) L-15 powder medium (GIBCO BRL), 18.9 mmol·l^{-1} glucose, 11.6 mmol·l^{-1} fructose, 3.3 mmol·l^{-1} proline, 93.5 mmol·l^{-1} sucrose; adjusted to pH 6.7 with NaOH; 500 mosmol) and were kept at 26°C in an incubator at high humidity. For measurements, cells were used between culture days 3 and 7. Processes of those cells chosen for recordings did not overlap with neighboring neurites.

Electrophysiological techniques

Whole-cell gigaohm seal recordings were performed at room temperature. Recordings were made using an

Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA) or an EPC9 amplifier (HEKA-electronics, Lamprecht, Germany). Pulse generation, data acquisition and analysis were carried out using a TL-1 interface in conjunction with pClamp programs (version 6.01, Axon Instruments) running on an AT-type microcomputer under DOS for experiments using the Axopatch 200A; for experiments with the EPC9, PULSE and PULSE-FIT (version 8.53, HEKA-electronics) software were used under Windows NT4.0. Currents were low-pass filtered with a four-pole Bessel (−3 dB at 0.5–1 kHz) filter and sampled at 2 kHz. Pipette offset potentials were nulled prior to seal formation; leakage currents were not subtracted. Series resistances ranged between 5 and 20 MΩ and were compensated at approximately 80%. Electrodes were pulled from borosilicate glass capillaries (1.5 mm o.d., 0.8 mm i.d., GB150-8P, Science Products, Hofheim, Germany) with a horizontal puller (DMZ-Universal Puller; Zeitz-Instrumente, München, Germany) and had tip resistances between 5 and 10 MΩ in standard external solution (see below). IGOR Pro, version 3.15 (Wavemetrics, Lake Oswego, Ore., USA), was used for data analyses.

Perfusion and pressure application

The bath was continuously perfused at flow rates of >4 ml min^{-1} with a standard external solution that consisted of (in mmol·l^{-1}): 130 NaCl, 6 KCl, 4 $MgCl_2$, 5 $CaCl_2$, 160 sucrose, 25 glucose, 10 HEPES-NaOH; pH 6.7, 520 mosmol l^{-1}. The internal solution contained (in mmol·l^{-1}): 115 potassium gluconate, 40 KF, 20 KCl, 3 $MgCl_2$, 5 K-BAPTA, 3 Na_2ATP, 0.1 Mg-GTP, 3 gluthatione, 120 sucrose and 10 HEPES-*bis*-TRIS; pH 6.7, 490 mosmol l^{-1} (all chemicals from Sigma, St. Louis, Mo., USA, unless otherwise stated).

The nicotinic agonists used were acetylcholine (ACh), nicotine (NIC), carbamylcholine (CCh), epibatidine (EPI), and cytisine (CYT). The muscarinic agonists used were pilocarpine (PIL), oxotremorine (OXO), and muscarine (MUS). All agonists were dissolved in standard external saline and were applied by pressure ejection, using a four channel pneumatic pump PPM-2 (List Medical systems, N.Y., USA). Four glass capillaries (borosilicate glass, 1.0 mm o.d., 0.58 mm i.d.; Clark Electromedical Instruments) were bundled with heat-shrinkable tubing and pulled on a modified Kopf vertical puller yielding tip diameters of 1–3 μm for each channel. Ejection times ranged between 500 ms and up to 5 s. The pipette tip was positioned at a distance of 10–50 μm from the cell upstream in the saline superfusion. The agonists were applied over the whole cell surface, which was checked previously by adding a few crystals of the dye, amaranth (Sigma), to the saline in the pipette and monitoring the diffusion of the saline pulse. Since the cell diameter is very small (7–10 μm), the agonist solution reaches all areas of the cell approximately at the same time. Each agonist was applied onto a given cell at least three times during each trial. The interval between agonist applications was 25 s to avoid any cumulative receptor desensitization. The mean amplitudes of agonist-induced currents were calculated by averaging the peak inward currents of the three applications. The antagonists, hexamethonium (HEX), d-tubocurarine (DTC), mecamylamine (MEC), methyllycaconitine (MLA), dihydroxy-β-erythroidine (DHE), atropine (ATR), strychnine (STR), bicuculline (BIC), picrotoxin (PIC) (all purchased from Sigma) were dissolved in standard external saline. Except for MEC, all antagonists were bath applied. Several pulses of ACh were applied before any of the antagonist to distinguish a potential rundown of the agonist-elicited current from the antagonist-induced inhibition. During antagonist applications, ACh was also applied several times to determine when the blocker reached its maximum effect. After this, the blocker was washed out with standard external saline for several minutes and ACh was repeatedly applied until either the full current amplitude was reached or no further recovery of the current could be detected. Subsequently, other blockers or several concentrations of a given blocker could be tested in most cells. MEC was pressure-applied like ACh. For this, one channel of the application pipette was filled with MEC and was pressure applied at the same time as ACh.

Dose-response curves

The dose-response curves were determined for four cholinergic agonists (ACh, CCh, NIC and EPI). The agonists were applied at various concentrations in alternation to a pulse of 200 μmol·l^{-1} ACh, a concentration that was found to elicit the maximum current in Kenyon cells (see Results). The peak current amplitude for each agonist application was measured. On a given cell up to three different concentrations of a given agonist could be tested. To establish complete dose-response relationships, several cells were examined using overlapping agonist concentrations and the current measurements of several cells were pooled (the number of cells tested is given in the figure legends). To eliminate the differences in current amplitudes between different cells, current amplitudes were normalized (I/I_{max}) to the response to 200 μmol·l^{-1} ACh (I_{max}) just prior to each application.

The effects of the antagonists (MLA, DHE, and ATR) were determined by comparing the peak current amplitudes elicited by 200 μmol·l^{-1} ACh (1 mmol·l^{-1} for ATR) in the presence of the bath applied blocker with those of the ACh pulse alone (prior to the antagonist application). Several antagonists at different concentrations were tested on a given cell. Between each antagonist application, ACh pulses were applied to test for the reversibility of the block and to control for current rundown. Data from several cells tested with overlapping concentrations were pooled. Current amplitudes were normalized as described above and the relative

amount of block ($1-I/I_{max}$) is plotted against the respective antagonist concentration.

The normalized peak current amplitudes were averaged and plotted against the concentration of the agonist or blocker. The curve was then fitted using a Hill equation:

$$I = I_{max} \times \frac{[agonist]^n}{k^n + [agonist]^n}$$

where, I_{max} is the maximal current, k the EC_{50}/IC_{50} value, and n the Hill-coefficient. The curve was constrained to be zero at a concentration, that was four orders of magnitude lower than the lowest tested agonist concentration. The derived Hill equation yielded the EC_{50} values and the Hill-coefficient of the dose-response relationship. The curves were fitted with Origin 6.0 (OriginLab, Northampton, MA, USA) running on a PC.

Data analyses and statistics

The peak current amplitudes of all recorded cells were pooled to determine the mean current amplitude of cultured Kenyon cells. To circumvent the effects of current rundown the first current elicited by a saturating ACh concentration (1 mmol·l^{-1}–200 μmol·l^{-1}) was used to determine the maximum current for a given cell. Current densities (pA/pF) were calculated to eliminate differences in current amplitudes due to different membrane areas (cell capacitances were derived from the compensation values of the patch amplifier). Differences between current amplitudes and densities expressed by cells after 3–7 days in culture or between cells taken from pupae of various stages (P4, P5 and P6) were tested using a *t*-test or an ANOVA. To test for the correlation of current amplitudes and densities with time in vitro or pupal stage, a linear correlation analysis was performed. Statistical analyses were performed using STATISTICA for Windows (version 5.5; StatSoft, Tulsa, OK, USA) on a PC.

Results

ACh-induced membrane currents

Pressure application of ACh (200 μmol·l^{-1}) induced inward currents at a command potential of –110 mV in over 90% of all recorded cells ($n = 368$, Fig. 1a, b). Recordings were performed on cells after 3–7 days in culture. Since the amplitude of the ACh-induced current varied over a range of 5–1,000 pA (mean peak amplitude 188 ± 129 pA, $n = 157$) we measured the current amplitudes and cell capacitances of the Kenyon cells after different days in vitro and of cells taken from different pupal stages.

Kenyon cells grow processes in culture, which increase in size with time spent in culture. This larger membrane area increased the mean cell capacitance. Thus, the cell capacitance was influenced by the age of cell culture, increasing from 2.9 ± 0.6 pF at day 3 to 5.1 ± 1.5 pF at day 7 (Table 1, ANOVA, $df = 79$, $F = 4.35$, $P < 0.05$). Accordingly, the mean peak amplitudes of the ACh-induced currents increased over time in culture ($I_{3d} = -103 \pm 82$ pA, $I_{7d} = -265 \pm 161$ pA, Table 1, ANOVA, $df = 79$, $F = 3.68$, $P < 0.05$).

The current densities (pA/pF) were similar for all cells measured at different days in vitro (mean –47 ± 39 pA/pF, ANOVA, $df = 79$, $F = 0.38$, $P = 0.8$; Table 1). Therefore, the increase of the current amplitudes was due to a larger membrane area of older and more extended cells. The capacitances, current amplitudes, or current densities measured from cells of different developmental stages (P4, P6) did not differ significantly (*t*-test; $P = 0.16$, 0.27 and 0.22, respectively; Table 2).

The peak current amplitudes of all recorded cells showed a Gaussian-like distribution (data not shown), indicating that Kenyon cells are physiologically a rather homogeneous neuron population with respect to the ACh currents. The variability of current amplitudes may be due to different expression levels of receptor proteins in the individual cells.

Pharmacology of the nAChR

We first tested the concentration dependency of currents induced by various agonists, ACh and CCh (Fig. 1), NIC and EPI (Fig. 2). Agonists were pressure applied at concentrations between 1 μmol·l^{-1} and 5 mmol·l^{-1}. All agonists elicited inward currents, however with different efficacies. CCh and ACh induced currents with the same maximum amplitude, although at different concentrations (ACh at > 100 μmol·l^{-1}, CCh at 5 mmol·l^{-1}, Fig. 1c, d). CCh was therefore a full agonist of the nicotinic receptor with a different dose-response relationship as compared to those of ACh-induced currents. The EC_{50} value differed ($EC_{50,ACh} = 6.7$ μmol·l^{-1}, $EC_{50,CCh} = 106$ μmol·l^{-1}), as well as the slope of the curve (Hill-coefficients, $h_{ACh} = 1.47 \pm 0.47$, $h_{CCh} = 0.56 \pm 0.08$). NIC and EPI were only partial agonists of the nAChR Kenyon cells. Both substances elicited only about a third of the maximal possible nicotinic response (~33% for NIC, ~31% for EPI). NIC and EPI also showed very similar Hill-coefficients ($h_{NIC} = 0.84 \pm 0.43$ and $h_{EPI} = 0.90 \pm 0.31$), indicating that their dose-response relationships have the same slope. This suggests the same mechanism of receptor activation for both substances. EC_{50} values were 0.88 μmol·l^{-1} for NIC and 21.5 μmol·l^{-1} for EPI. Pressure application of the vertebrate nicotinic agonist CYT (up to 5 mmol·l^{-1}) elicited only very small inward currents (Fig. 3c).

Partial agonists often act as inhibitors of receptor activation, because they compete with the agonist for the same binding site at the receptor. Another possible mechanism is an open channel block, when the agonist binds to a site in the open channel pore, thereby blocking the ion flux through the receptor. This, how-

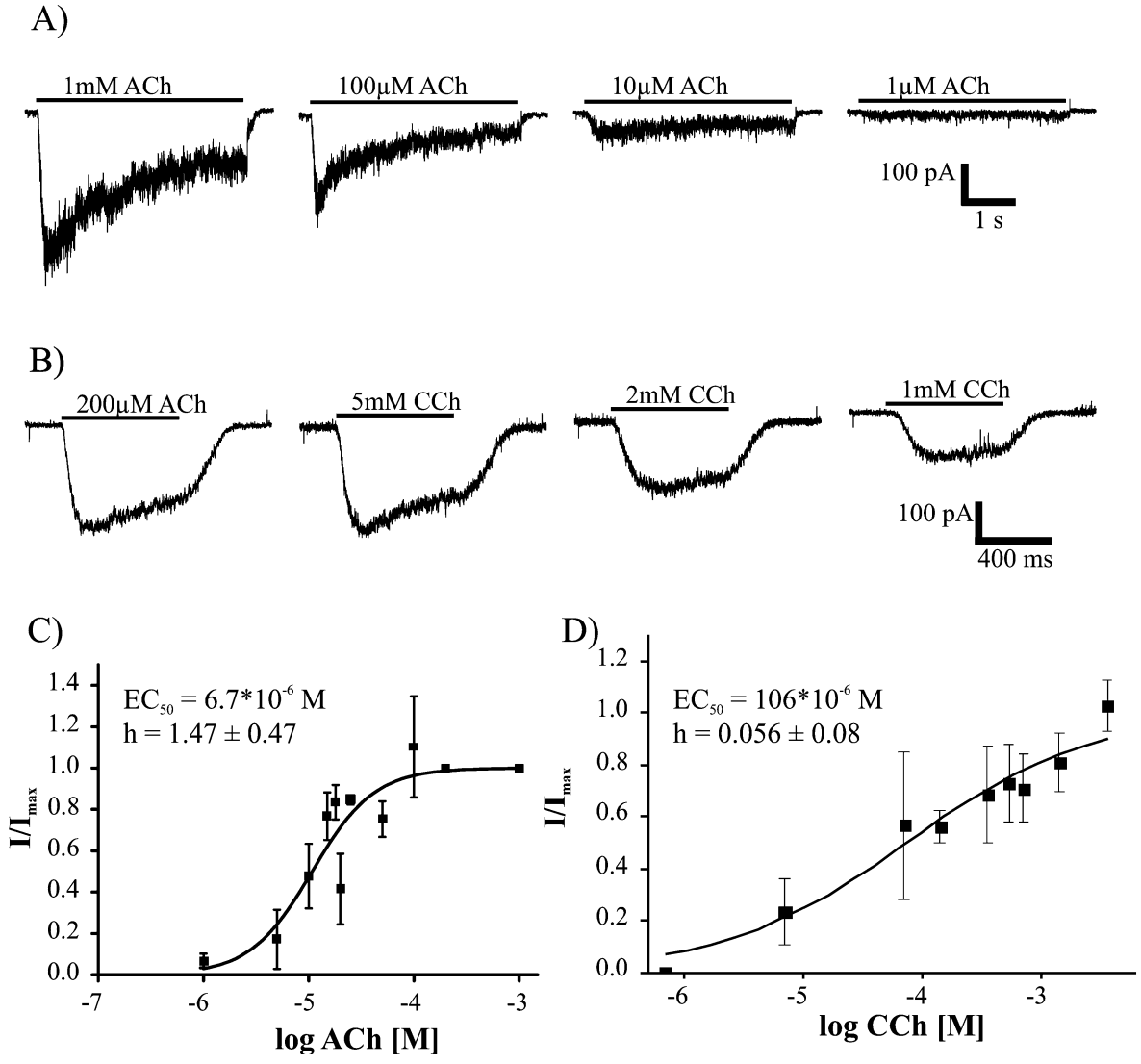


Fig. 1a–d Currents induced by pressure applications of acetylcholine (*ACh*) and carbamylcholine (*CCh*). **a** ACh induced rapidly activating inward currents at a command potential of –110 mV. The current amplitude is concentration-dependent between 1 µmol·l^{-1} and 1 mmol·l^{-1}, agonist application is indicated by a bar above the current trace (currents were measured on an individual cell, intervals between pulses are 25 s throughout). **b** Pressure applications of CCh-induced currents similar to those induced by ACh. However, higher concentrations of CCh are required to elicit currents of the same amplitudes (data from one typical cell). **c, d** Dose-response relationships of currents induced by ACh (**c**) or CCh (**d**). Peak current amplitudes were measured and normalized to the maximum curent elicited by 200 mmol·l^{-1} ACh (I_{max}). Plotted are the mean ± SD of the normalized currents (n = 23 for ACh and n = 16 for CCh. The data were fitted by a Hill function (see Materials and methods for details), EC_{50} values and Hill-coefficients are given

ever, would cause the dose-response curve of these partial agonists to be bell-shaped, because at higher concentrations of the agonist more and more of the open channels would be blocked. This phenomenon was not observed. To approach the mechanism of the partial activation by NIC and EPI, we coapplied NIC or EPI with 1 mmol·l^{-1} ACh (Fig. 3a, b). Coapplication of NIC or EPI reduced the amplitude of the ACh-induced current; the amount of current reduction depended on the concentration of the partial agonist. The same is true for the nicotinic agonist CYT (Fig. 3c). Thus, besides receptor activation, these partial agonists inhibit currents through the nAChR probably through a competitive mechanism.

Nicotinic blockers

In another series of experiments, we tested the effects of various nicotinic antagonists, HEX, MEC, DTC, DHE, MLA on the ACh-induced current (200 µmol·l^{-1}, Fig. 4). Each antagonist tested blocked the ACh-induced current in Kenyon cells, but with different potencies. DHE, MLA, MEC (in the order of potency) completely blocked the ACh-induced currents. For the two most efficient antagonists, DHE and MLA, dose-response relationships were established (Fig. 4b,

Table 1 Mean capacitances and mean maximal currents of cells that had grown in culture for different times (numbers are means ± SD)

Days in culture	Cell capacitance (pF)	Peak current (pA)	Current density (pA/pF)	*n*
3	2.9 ± 0.6	-103 ± 82	−37 ± 30	9
4	4.5 ± 1.6	-139 ± 86	−43 ± 52	13
5	3.9 ± 1.4	-161 ± 116	−46 ± 40	32
6	4.5 ± 1.4	-242 ± 168	−55 ± 39	16
7	5.1 ± 1.5	-265 ± 161	−51 ± 30	14
All cells	4.2 ± 1.5	-183.9 ± 135.0	−47 ± 39	84

Table 2 Mean capacitances and mean maximal currents of cells from different developmental stages (numbers are means ± SD)

Pupal stage	Cell capacitance (pF)	Peak current (pA)	Current density (pA/pF)	*n*
P4	4.0 ± 1.5	-153 ± 114	−41 ± 33	36
P6	4.3 ± 1.5	-200 ± 142	−51 ± 40	58
Sum	4.2 ± 1.5	-182 ± 133	−47 ± 38	95

$IC_{50,DHE} = 0.5$ pmol·l^{-1}, $IC_{50,MLA} = 24$ pmol·l^{-1}). MEC is known to block vertebrate nAChRs by binding in the open pore (open channel block). Similarly, in honeybee Kenyon cells MEC acted as a non-competitive nicotinic antagonist. When MEC is coapplied with ACh (200 μmol·l^{-1}), a brief current at the onset of the ACh application was measured, if the MEC-concentration was too low to block every opening channel immediately (10 nmol·l^{-1}, Fig. 4c). This indicates that the channel pore needs to be opened before the MEC can effectively block the current. Likewise, the channel pore remains open for a few milliseconds immediately after the offset of ACh application, because MEC is released from its binding site inside the pore keeping the channel permeable for a short while.

DTC and HEX were less potent nicotinic antagonists and showed only partial block (Fig. 4c). DTC (1 mmol·l^{-1}) blocked 90% of the current at ($n = 4$), whereas HEX (500 μmol·l^{-1}) blocked ~70% of the current ($n = 4$). The blockade of all antagonists was completely reversible after only a brief wash of 1–2 min.

Muscarinic agents

Several substances which act on muscarinic ACh receptors were tested for their ability to either elicit or inhibit currents through the nAChR of Kenyon cells. Such a mixed muscarinic/nicotinic pharmacology was reported for several insect receptors (e.g., Suter and Usherwood 1985; Pinnock et al. 1988; van den Beukel et al. 1998; van Eyseren et al. 1998). Pressure applications of the muscarinic agonists, MUS, PIL and OXO (at a concentration of 1 mmol·l^{-1}) did not induce any measurable currents in cells; the same cells did, however, show significant inward currents elicited by 200 μmol·l^{-1} ACh (Fig. 5a). In addition, co-applications of any of the muscarinic agonists with 200 μmol·l^{-1} ACh did not affect the nicotinic currents. These results indicate that there is no muscarinic contribution to the inward currents induced by the various agonists used. By contrast, bath applications of the muscarinic antagonist ATR efficiently inhibited ACh-induced currents in Kenyon cells (Fig. 5b). The ATR block was concentration-dependent between 10 μmol·l^{-1} and 10 mmol·l^{-1} with an IC_{50} of 24.3 μmol·l^{-1} (Fig. 5c). The block was competitive as it could be partially overcome by using higher ACh concentrations (3 mmol·l^{-1}, Fig. 5b).

Non-cholinergic modulators

We also tested several non-cholinergic substances, because findings in other insect systems, such as locusts and cockroaches, suggested effects of GABAergic agonists and antagonists onto ionotropic nicotinic receptors (e.g., Benson 1988, 1992; Waldrop 1993; van Eyseren et al. 1998). Glycine and $GABA_A$ receptors are two groups of ligand-gated ion channels that belong to the same superfamily as nAChRs. Pressure application of 1 mmol·l^{-1} glycine alone did not induce any detectable current in Kenyon cells (data not shown). By contrast, the glycine antagonist STR partially blocked the ACh-induced current reversibly (100 μmol·l^{-1}, $n = 3$, Fig. 6a). The inhibition was not just a reduction of the peak current amplitude, but differentially affected the phases of the ACh-induced current. STR strongly reduced the early phase of the current but the late phase was affected to a much lesser extent. Similarly, the GABAergic antagonists BIC (300 μmol·l^{-1}) and PIC (100 μmol·l^{-1}) also partially blocked the current elicited by 1 mmol·l^{-1} ACh and also affected preferably the fast transient component of the nicotinic current ($n = 2$ each, Fig. 6b, c).

Discussion

The pharmacology of the honeybee nicotinic current indicates a profile that is different from any of the identified vertebrate neuronal receptor subtypes. Muscarinic as well as non-cholinergic antagonists inhibit the receptor function, distinguishing the honeybee receptor from the "typical" nAChR of vertebrates and other insects.

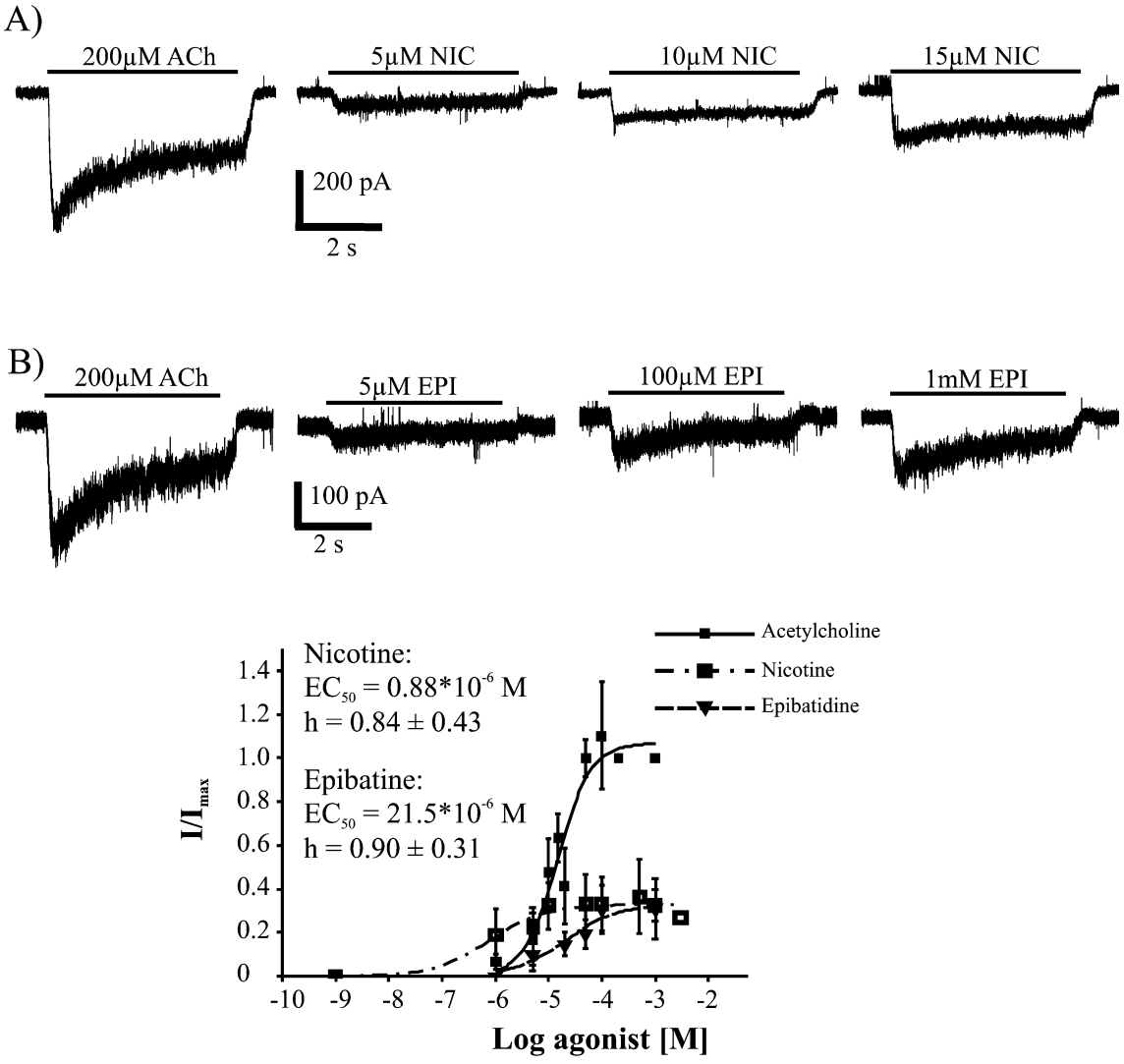


Fig. 2a–c NIC and EPI are partial agonists. **a** NIC elicits inward currents (command potential –110 mV, duration of application pulse indicated by the bar above the current trace) with peak amplitudes, which are lower than those of ACh (200 µmol·l^{-1}). Data were obtained from a single Kenyon cell. Intervals between pulses were 25 s. **b** EPI is also only a partial agonist at concentrations between 5 µmol·l^{-1} and 1 mmol·l^{-1}. It elicits inward currents (experimental conditions as in **a**) with similar peak amplitudes as NIC. **c** Dose-response relationships of EPI- and NIC-induced currents as compared to those of ACh. The mean peak currents ± SD, normalized to the current induced by 200 µmol·l^{-1} ACh (I_{max}) are plotted ($n = 11$ for NIC, $n = 11$ for EPI). The curve for ACh was taken from Fig. 1c. NIC- and EPI-induced current amplitudes saturated at < 200 µmol·l^{-1} for NIC (< 1 mmol·l^{-1} for EPI) reaching only 33% of the maximum current induced by 200 µM ACh. Curves were fitted with a Hill function

The nAChR is one of the best-known ionotropic receptors. Within the vertebrate nervous system, several different neuronal nAChRs are expressed subserving various functions. Most of the neuronal nAChR subtypes are presynaptic and modulate transmitter release (e.g., McGehee et al. 1995; reviews: Wonnacott 1997; Changeux et al. 1998; Alkondon et al. 1999). However, there are also reports about neuronal nAChRs mediating fast synaptic transmission (e.g., Alkondon et al. 1998). In the insect nervous system ACh is the major excitatory transmitter, but much less is known about insect as compared to vertebrate nAChRs (reviews: Osborne 1996; Gundelfinger and Schulz 2000). This is mainly due to the fact that it is difficult to express functional nAChRs in heterologous systems. Only nAChRs from the locust, *Schistocerca gregaria*, have been functionally expressed in frog oocytes (Marshall et al. 1990). In other cases, functional expression succeeded only when vertebrate and insect subunits were combined (e.g., Schulz et al. 1998). Insect nAChRs have therefore mostly been analyzed as native receptors in primary neuron culture and the subunit compositions of these nAChRs are yet unclear. Furthermore, most studies were performed on unidentified neurons or cells from a heterogeneous neuron population (Hermsen et al. 1998; Lee and O'Dowd 2000). Our study used Kenyon cell cultures from the honeybee mushroom body, because they comprise a physiologically homogenous cell population.

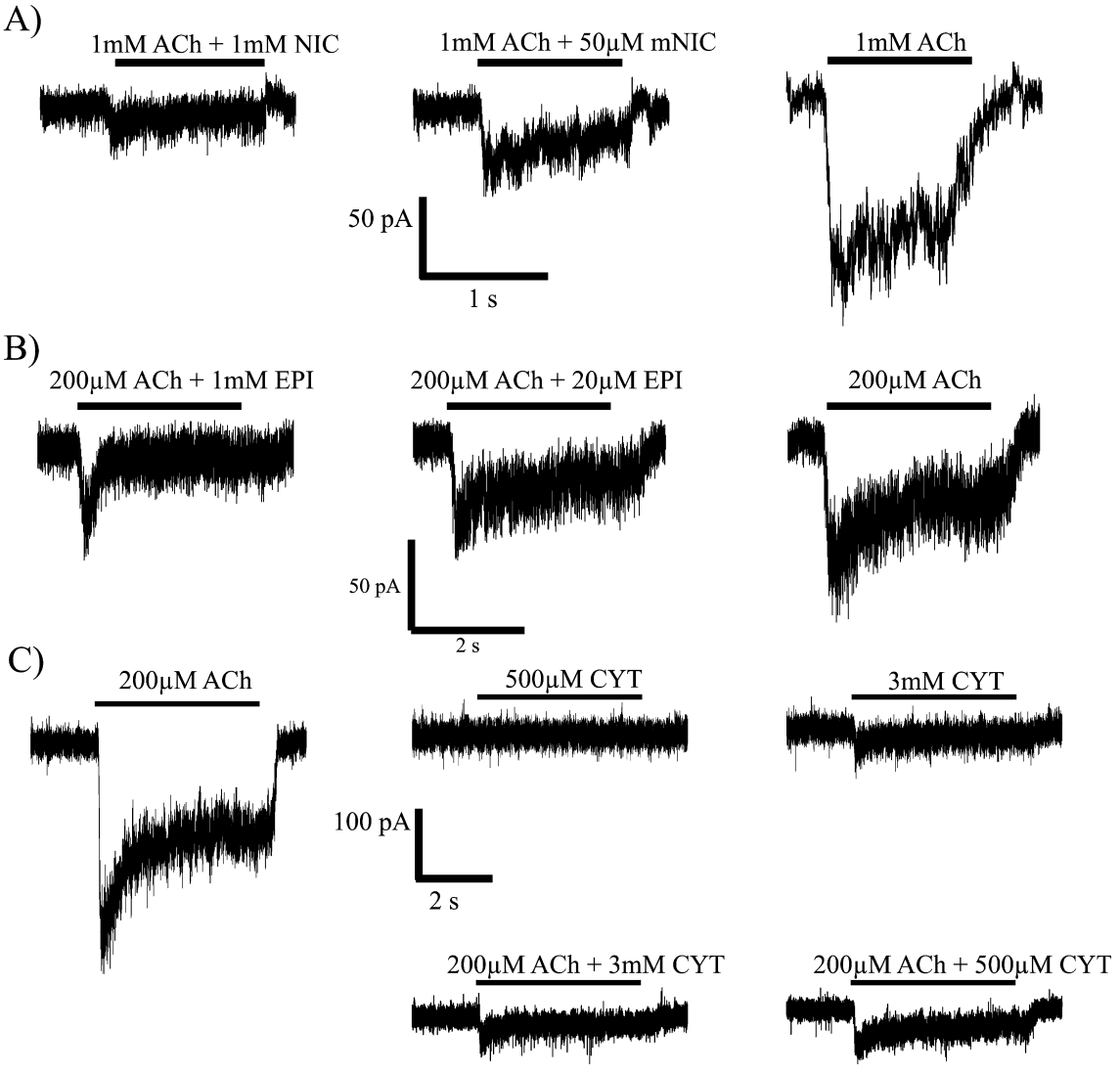


Fig. 3a–c Competitive block of the nAChR by NIC, EPI, and CYT. Coapplications of ACh with NIC (**a**), EPI (**b**) or CYT (**c**) reduced the amplitude of ACh-induced currents. NIC (1 mmol·l^{-1}) induced an inward current with a peak amplitude of 26 pA (17 pA for 50 µmol·l^{-1} NIC) in a given cell. The same cell responded to 200 µmol·l^{-1} ACh with a current of 131 pA peak amplitude. Coapplication of 1 mmol·l^{-1} or 50 µmol·l^{-1} NIC with 200 µmol·l^{-1} ACh reduced the current amplitude. Coapplication of epipatidine (1 mmol·l^{-1}) or CYT (500 µmol·l^{-1}, 3 mmol·l^{-1}) with ACh (200 µmol·l^{-1}) almost completely blocked current through the nAChR. Conditions were identical in all experiments: command potential was –110 mV, pulse durations are indicated

Pharmacology of the honeybee nAChR

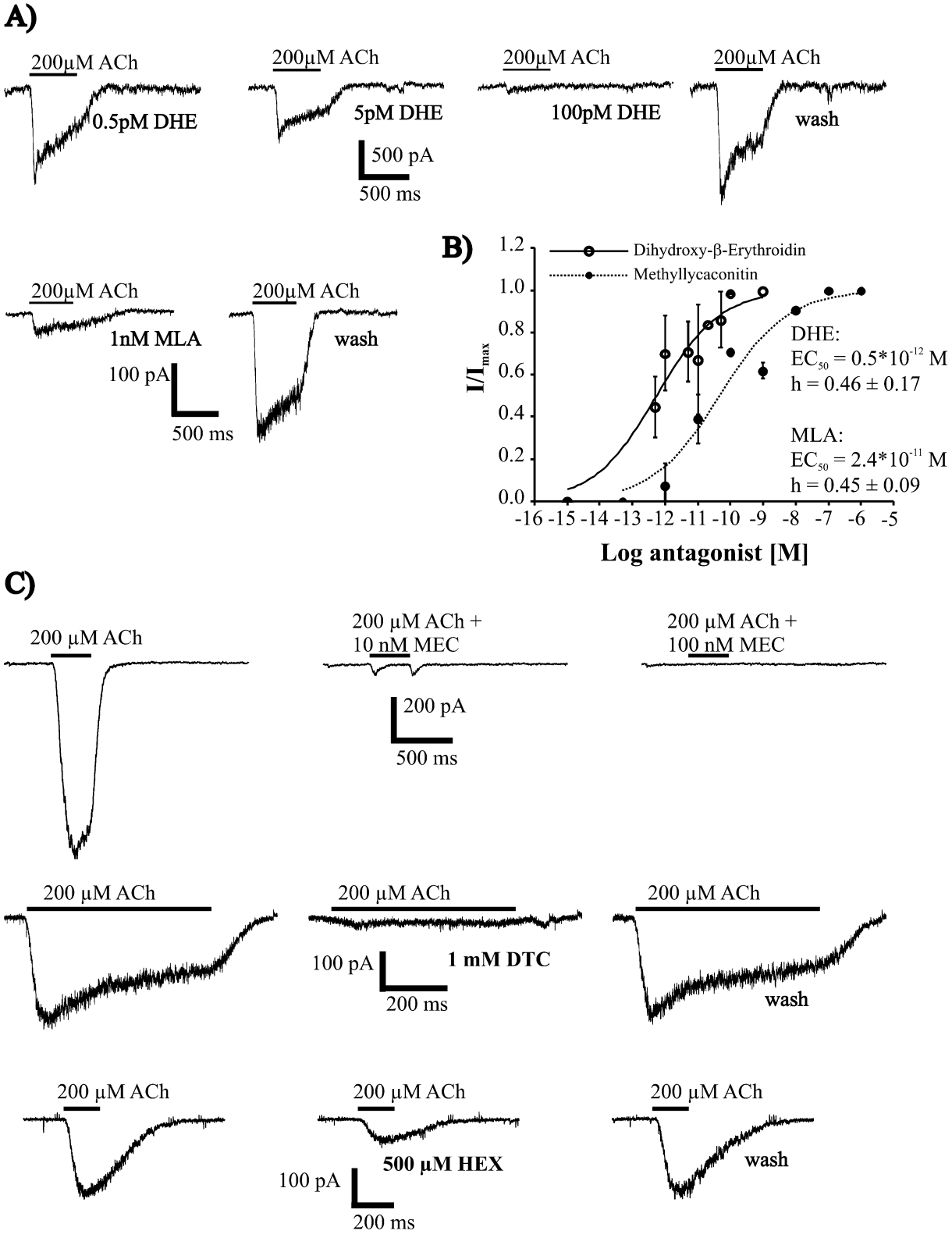
Agonists

The five nicotinic agonists tested during the present study are commonly used for discriminating between different vertebrate receptor subtypes. The agonists differed in their affinities to the honeybee nicotinic receptor. Based on the EC_{50} values, the agonist with the highest affinity was NIC, followed by ACh, EPI, CCh

►

Fig. 4a–c Antagonists of the honeybee neuronal nAChR. **a** Dihydroxy-β-erythroidine (*DHE*, *upper row*) and methyllycaconitine (*MLA*, *lower row*) are highly potent blockers of the nAChR. Currents were elicited by repeated pressure applications of 200 µmol·l^{-1} ACh (interval 25 s); blockers were applied by bath perfusion. After each application of a blocker the cells were washed with standard external saline for at least 2 min (perfusion flow rate 2–5 ml min^{-1}) before the next concentration was tested. DHE and MLA are readily reversible. **b** Dose-response relationships of current inhibition by MLA and DHE. Conditions as in (**a**); peak currents elicited by 200 µmol·l^{-1} ACh were measured at the antagonist concentrations indicated on the *X*-axis. For each application the peak current was normalized to the ACh-induced current (I_{max}) immediately prior to the blocker application. Plotted are the normalized mean peak currents ± SD ($1-I/I_{max}$); thus, a value of 1 means complete block of the ACh-induced current. Curves were fitted by a Hill function ($n=9$ for MLA, $n=8$ for DHE). Both dose-response curves are almost parallel, suggesting the same mechanism of block. **c** Blocking of receptor function by MEC (*upper row*), DTC (1 mmol·l^{-1}, *middle row*), or HEX (500 µmol·l^{-1}, *bottom row*). Currents were elicited by pressure application of 200 µmol·l^{-1} ACh. Pressure applied MEC completely blocked at a concentrations of 100 nmol·l^{-1}. At 10 nmol·l^{-1} MEC the cell showed a small inward current at the onset and offset of the pulse, indicating that MEC acts as an open channel blocker

and CYT. This is in general agreement with reports in other insects (*Locusta migratoria*: Tareilus et al. 1990; Hermsen et al. 1998; *Periplaneta americana*: David and Sattelle 1984; Pinnock et al. 1988; Courjaret and Lapied 2001). Other orders of affinity have also been reported (e.g., *Drosophila*: ACh = CCh > NIC, Albert and Lingle 1993). However, the comparison of the agonist rankings must be considered carefully because only very few of these studies tested whether the agonists used were partial or full agonists, i.e. the efficacy of the drug was not considered. Under some circumstances, this can lead to a wrong order of affinities (this is probably why



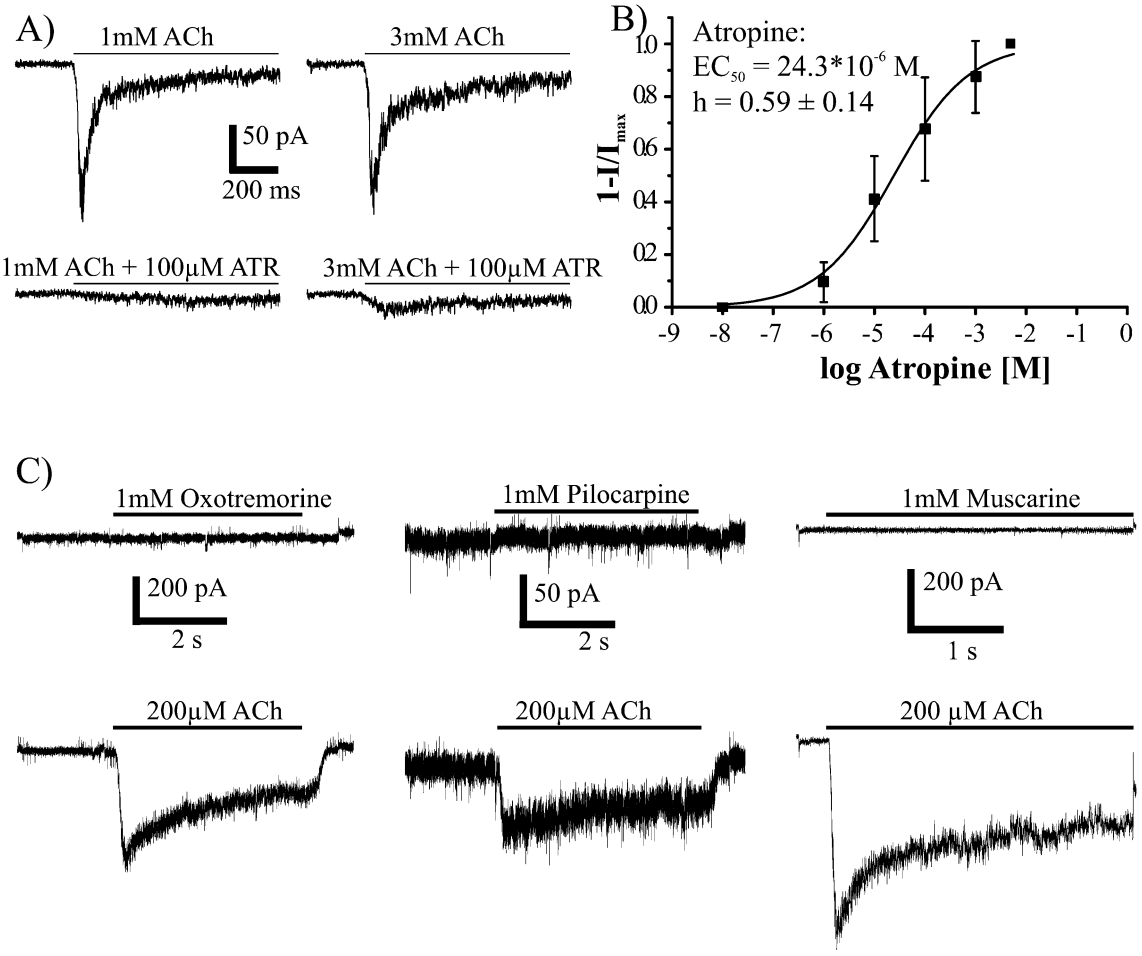


Fig. 5a–c Effects of muscarinic agents on the honeybee nAChR. **a** Pressure applications of muscarine (*MUS*), oxotremorine (*OXO*) or pilocarpine (*PIL*) (*top row*) induced no measurable current in Kenyon cells (command potential –110 mV, pulse duration indicated). The data were derived from three different cells. ACh (200 mmol·l^{-1}, *bottom row*) applied to the same cells after the respective muscarinic agonists induced inward currents. Note the different scale bars. **b** Atropine (*ATR*) inhibited ACh-induced inward currents in a concentration dependent manner. The currents elicited by 1 or 3 mmol·l^{-1} ACh are similar (*top row*). Bath applications of ATR (100 µmol·l^{-1}, *middle row*) block these currents, but at 3 mmol·l^{-1} ACh (*right*) the ATR block is attenuated, suggesting a competitive mechanism of inhibition. The block by 100 µmol·l^{-1} ATR is completely reversibly (*bottom row*). Data are from one cell. **c** Dose-response relationship of the ATR block (currents elicited by 1 mmol·l^{-1} ACh, $n=6$). Peak current amplitudes were measured and normalized as described above

Goldberg et al. (1999) give a different ranking than that resulting from the present study).

NIC is a high-affinity agonist of the honeybee nicotinic receptor ($EC_{50}=0.88$ µmol·l^{-1}) as in other insect neurons, where EC_{50} values range between 0.25 and 7.4 µmol·l^{-1} (Pinnock et al. 1988; Orr et al. 1997; Hermsen et al. 1998). Although NIC showed the lowest EC_{50} and, therefore, the highest affinity to the honeybee receptor of all tested agonists, it is only a partial agonist, whereas ACh and CCh show a lower affinity but are more efficacious, i.e., they can induce higher current amplitudes. Also EPI and CYT are only partial agonists and EPI showed a very similar Hill-coefficient ($h_{EPI}=0.9\pm0.3$, $h_{NIC}=0.8\pm0.4$), and an almost identical efficacy (31% versus 32% for NIC), indicating a similar mechanism of action of both agonists. EPI is the most efficacious agonist for neuronal vertebrate nAChRs (Albuquerque et al. 1997). CYT acts with high specificity at vertebrate receptors containing β4-subunits, but is less specific to those with β2-subunits (Albuquerque et al. 1997; Cordero-Erausquin et al. 2000). These agonists, however, did not discriminate between different nAChR subtypes in the honeybee brain. In cockroach neurons, CYT and a stereoisomer of EPI, (+)–EPI, were partial agonists of the nAChR (Orr et al. 1997). Whereas on locust thoracic ganglion neurons NIC and CYT acted as partial agonists, but were full agonists of the nAChR on other cells within the same preparation (Hermsen et al. 1998). The concentration-dependent inhibition of the ACh-induced current by each of the three partial agonists indicates a competitive mechanism of block. ACh and CCh are full agonists of the honeybee neuronal receptor. An EC_{50} value for ACh of 6.7 µmol·l^{-1} in the honeybee nicotinic current is similar to other insect ACh-currents varying between 11 and 59 µmol·l^{-1} (Pinnock et al. 1988; Albert

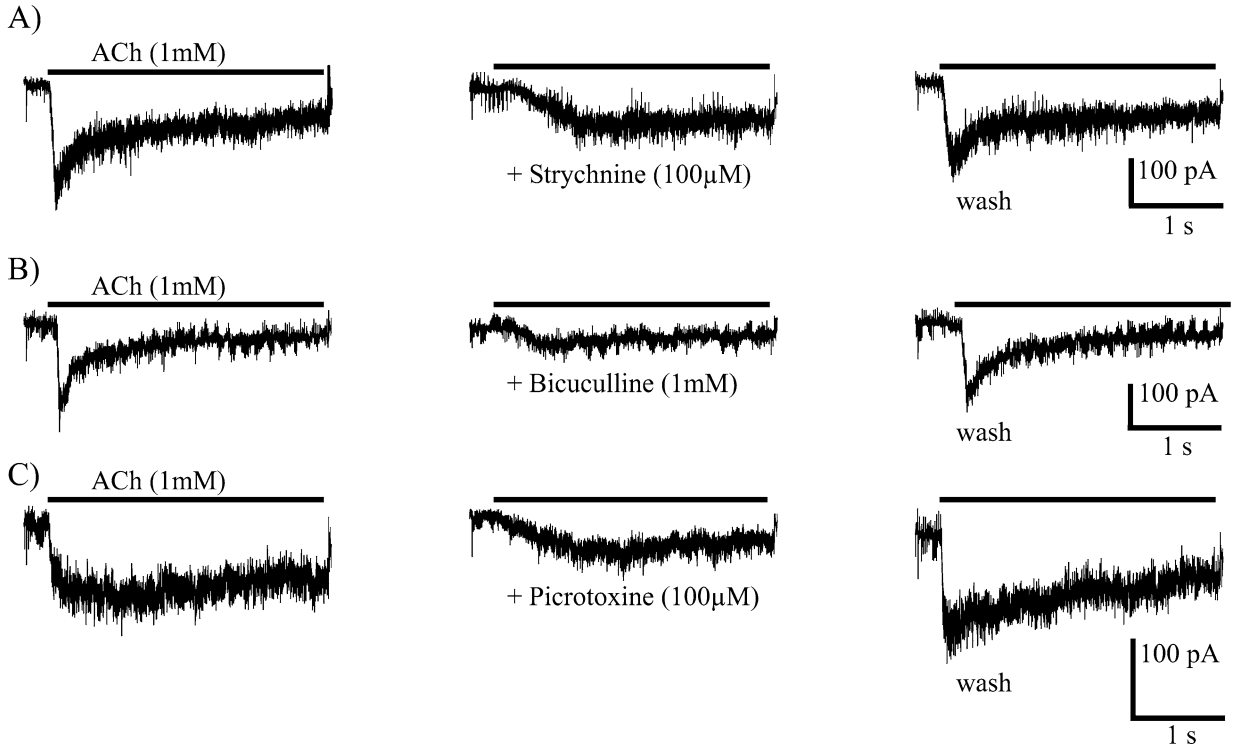


Fig. 6a–c Effects of non-cholinergic receptor inhibitors. **a** Bath application of the glycine receptor blocker strychnine (STR, 100 μmol·l^{-1}) reduced the transient component of the ACh-induced current (1 mmol·l^{-1}). Glycine (1 mmol·l^{-1}) did not induce any detectable current in Kenyon cells (not shown). **b** Bath application of the $GABA_A$-inhibitor bicuculine (*BIC*) (1 mmol·l^{-1}) partially blocked the ACh-induced current. **c** The GABA blocker, picrotoxin (*PIC*, 1 mmol·l^{-1}), blocked a similar amount of the ACh-induced current. All three inhibitors were readily reversible after several minutes of wash. Data are from three different cells; command potential was –110 mV

and Lingle 1993; Hermsen et al. 1998; van den Beukel et al. 1998; van Eyseren et al. 1998). Similarly, the EC_{50} value of 106 μmol·l^{-1} for CCh of the honeybee current is in the range of other reported values (Pinnock et al. 1988). Another interesting point was raised by the slopes of the dose-response relationships of the agonists. The two full agonists, ACh and CCh, differed clearly in their Hill-coefficients ($h_{ACh} = 1.5 \pm 0.5$, $h_{CCh} = 0.56 \pm 0.1$, Fig. 1). This finding is puzzling because of the equal efficiacy of both agonists. Hill-coefficients are expected to be lower for partial agonists than for full agonists, which may be explained by the simultaneous agonistic and antagonistic action of partial agonists on the receptor that leads to a less steep slope of the dose-response relationship and also to a reduced efficacy. Thus, the Hill-coefficients for EPI and NIC versus ACh are consistent with the theory. However, the different coefficients for ACh and CCh may indicate the expression of two different receptor subtypes with differential sensitivities to ACh and CCh, respectively.

Antagonists

The five different antagonists tested were chosen because they show some specificity for various subtypes in vertebrates. MEC and HEX are potent blockers of vertebrate neuronal nAChR, blocking the muscular subtype only weakly and vice versa for DTC (Paton and Zaimis 1952; Lukas 1989). MLA is a potent blocker of vertebrate nAChRs formed by α7-subunits (Ward et al. 1990) and DHE most efficiently inhibits nAChR subtypes that include the subunit combinations α3β2 and α4β2 (Alkondon and Albuquerque 1993; Dwoskin and Crooks 2001). In insects, such a classification of pharmacologically distinct subtypes does not yet exist, although there is evidence for a differential sensitivity of nAChR-subtypes (Hermsen et al. 1998; Courjaret and Lapied 2001).

DHE was the most potent antagonist of the honeybee nicotinic current, followed by MLA and MEC; HEX and DTC were very weak blockers. HEX blocked about half of the honeybee nicotinic current at 500 μmol·l^{-1}. It is a rather weak antagonist in various insect nAChR (*Drosophila melanogaster*: Albert and Lingle 1993; *Periplaneta americana*: Pinnock et al. 1988; Lummis et al. 1992). Only in cultured neurons from *Locusta migratoria* HEX had a higher potency ($EC_{50} = 19$ μmol·l^{-1}, Benson 1992). DTC blocked >90% of the honeybee ACh current only at 1 mmol·l^{-1} concentration, whereas in most insect preparations it is efficient at micromolar concentrations (David and Sattelle 1984; Cheung et al. 1992; Waldrop 1993; Albert and Lingle 1993; van den Beukel et al. 1998; Lee and O'Dowd 2000), though there are also reports of no or only weak antagonistic effects (Buckingham et al. 1997).

MEC is commonly used in concentrations of 10–1,000 $\mu mol \cdot l^{-1}$ for investigating insect nicotinic currents and it usually completely blocked the receptor function (*Periplaneta*: Pinnock et al. 1988; Cheung et al. 1992; Buckingham et al. 1997; *Schistocerca*: Marshall et al. 1990). This is also true for the honeybee nicotinic current and MEC was successfully used for behavioral pharmacological experiments (Lozano et al. 1996). By contrast, nicotinic currents in the cockroach, *Periplaneta americana*, are only partially blocked by MEC (Blagburn and Sattelle 1987; van Eyseren et al. 1998; Courjaret and Lapied 2001).

MLA was first described as a nicotinic antagonist of high insecticidal potency (Jennings et al. 1986). Consistently, in Kenyon cells MLA is a high-potency nicotinic blocker as in many other insect preparations (*Locusta*: van den Beukel et al. 1998; *Periplaneta*: Cheung et al. 1992; Orr et al. 1997; van Eyseren et al. 1998). In vertebrates, MLA specifically blocks $\alpha 7$ containing neuronal receptors (Alkondon and Albuquerque 1993; Alkondon et al. 1998). This points to an interesting pharmacological similarity between the insect receptor and the vertebrate $\alpha 7$ receptor. The vertebrate nAChR with $\alpha 7$-subunits is considered to be a phylogenetically primordial, and it may be closer related to invertebrate nAChRs than other subunits (Le Novère and Changeux 1995; Tsunoyama and Gojobori 1998: Gundelfinger and Schulz 2000).

DHE was the most potent nicotinic antagonist of all substances tested. Other than in vertebrates, where it is a commonly used antagonist mainly due to its specifity to $\alpha 3\beta 2$ and $\alpha 4\beta 2$ nAChRs (Alkondon and Albuquerque 1993; Dwoskin and Crooks 2001) DHE has been tested rarely in insects. One report studying affinity labeling showed a high affinity to nAChRs of *Schistocerca gregaria* (Macallan et al. 1988) but its potency to block ACh currents was not tested. The only physiological study so far reports a IC_{50}-value of 5.6 $\mu mol \cdot l^{-1}$, for DHE in a cockroach neuron (David and Sattelle 1984), a value much higher than for the Kenyon cell nAChR. Clearly, in honeybees DHE and MLA do not discriminate between different receptor subtypes, but rather act on the same receptor or on pharmacologically indistinguishable receptors.

The dose-response relationships for the antagonists MLA, DHE (and also ATR, see below) are spread over a range of seven orders of magnitude. The Hill-coefficients are close to each other ($h_{MLA} = 0.45 \pm 0.09$, $h_{DHE} = 0.46 \pm 0.17$, $h_{ATR} = 0.59 \pm 0.14$). This in agreement with the assumption that all antagonists bind to the same binding site of the receptor.

Two receptor subtypes?

Goldberg et al. (1999) suggested the existence of two different receptors and a detailed study in isolated cockroach DUM-neurons revealed three pharmacologically different nicotinic currents (Courjaret and Lapied 2001). The pharmacological data presented here are somewhat ambiguous concerning this issue. On the one hand, the consistency of the Hill-coefficients of the antagonists MLA, DHE and ATR suggests that Kenyon cells express only one receptor subtype. Three antagonists would probably show differential potency for different receptor subtypes, if they existed. In this case, a Hill fit that assumes only one receptor type would be skewed, changing the slope/Hill-coefficient of the curve. On the other hand, the different Hill-coefficients for ACh and CCh may indicate the expression of two different receptor subtypes with differential sensitivities to ACh and CCh, respectively. Another argument for the existence of several subtypes might be that all three of the tested non-cholinergic antagonists block preferentially the fast transient phase of the nicotinic response but have a less pronounced impact on the persistant phase of the current. Probably, genetic and molecular analyses may ultimately unravel whether honeybee neurons express more than one nAChR.

Non-nicotinic pharmacology

The neuronal nicotinic current of the honeybee shows some unusual pharmacological properties: sensitivity to ATR and to blockers of GABA and glycine receptors. The ACh receptor of Kenyon cells was blocked by ATR, while muscarinic agonists did not induce any currents. This finding partially contradicts previous results (Goldberg et al. 1999) that ATR did not affect the ACh-induced current. In our study, ATR competitively inhibited current through the nAChR. The reason for these contradictory results remains unclear, but it may be caused by differences in Kenyon cell culture conditions. Some other insect nAChRs are sensitive to ATR, including those expressed by cockroach DUM neurons (Buckingham et al. 1997), motoneurons (David and Sattelle 1984; Pinnock et al. 1988) and neurons from the locust thoracic ganglia (Suter and Usherwood 1985; Benson 1992; van den Beukel et al. 1998), where ATR blocked the nAChR in a non-competitive manner (Suter and Usherwood 1985). However, in many other studied insect preparations nicotinc currents are not ATR sensitive (*Acheta domestica* Kenyon cells: Cayre et al. 1999; *Drosophila melanogaster* embryonic cells: Albert and Lingle 1993; *Periplaneta americana* sensory neurons: Blagburn and Sattelle 1987; *Periplaneta americana* abdominal interneurons: Buckingham et al. 1997). Several insect receptors show a ''mixed pharmacology'' and were even classified as such (Osborne 1996), when muscarinic and nicotinic agents act on the same receptor (Lapied et al. 1990; Lapied and Hue 1991; Grolleau et al. 1996; Buckingham et al. 1997). ATR also blocks some vertebrate nAChR-subtypes (Zwart and Vijverberg 1997; Verbitsky et al. 2000; Elgoyhen et al. 2001; Parker et al. 2003).

The honeybee neuronal nicotinic current was also sensitive to non-cholinergic agents ($GABA_A$: PIC and

BIC; glycine receptors: STR), which is not too surprising before this background. Such non-nicotinic sensitivities are common to some insect nAChRs (Benson 1992; Waldrop 1993; van Eyseren et al. 1998) and have also been found in vertebrate receptors made up of α7-subunits (strychnine sensitivity: Peng et al. 1994; Matsubayashi et al. 1998) and α9-subunits (sensitivity to strychnine: Elgoyhen et al. 1994; bicuculline: Rothlin et al. 1999). A similar pharmacological profile has been found for crustacean muscular receptors that showed high sensitivity for DHE, MEC and the non-nicotinic inhibitor PIC—though not for α-BTX—(Marder and Paupardin-Tritsch 1980a, b) and neuronal receptors (Pfeiffer-Linn and Glantz 1990). Non-nicotinic inhibitors (BIC, PIC, STR) were also found to be efficient in mollusc neurons (Yarowsky and Carpenter 1978) which argues in favor of this pharmacology being more ancient than the typical pharmacology of vertebrate nAChRs.

Conclusion

The insect neuronal nicotinic currents are clearly based on nAChRs that have a rather particular pharmacology and physiology of its own. In some aspects, these receptors resemble the vertebrate neuronal receptors formed by α7, α9 or α10 subunits (desensitization, Ca^{2+} permeability, MLA sensitivity, non-nicotinic pharmacology). Interestingly, those vertebrate receptors are considered the phylogenetically old vertebrate receptors. This may suggest that parts of these pharmacological "oddities" are a common heritage from a primordial version of the receptor in a common ancestor of insects and vertebrates. Although the honeybee receptor is a typical insect neuronal receptor, it is not identical to any one of the identified insect receptors. Obviously, nAChRs in insects are as diverse as those in vertebrates, covering a variety of different pharmacological profiles and reflects the genetical diversity of insect nAChRs (Le Novère and Changeux 1995; Gundelfinger and Schulz 2000). It is as yet impossible to classify the insect nAChR subclasses according to their pharmacological profiles as for the vertebrate subtypes (for exceptions see David and Sattelle 1984; Benson 1992; Courjaret and Lapied 2001).

Our data together with earlier work (Goldberg et al. 1999; Déglise et al. 2002) are consistent with a role of the nAChR for fast excitatory synaptic transmission in the honeybee brain. It was shown that the olfactory pathway is mainly cholinergic (Kreissl and Bicker 1989). Furthermore, the present study provides potent pharmacological tools (DHE, MLA), which we currently use to dissect the functional role of cholinergic transmission during olfactory information processing and olfactory learning.

Acknowledgements The authors are grateful to Dr. Greg Phares for critically reading the manuscript, Dr. Randolf Menzel for his continuous support and Marion Ganz for technical assistance. The work was supported by the Deutsche Forschungsgemeinschaft, SFB 515/C5 to B.G. and Graduiertenkolleg 120 to D.G.W.

References

Albert JL, Lingle CJ (1993) Activation of nicotinic acetylcholine receptors on cultured *Drosophila* and other insect neurones. J Physiol (Lond) 463:605–630

Albuquerque EX, Alkondon M, Pereira EF, Castro NG, Schrattenholz A, Barbosa CT, Bonfante-Cabarcas R, Aracava Y, Eisenberg HM, Maelicke A (1997) Properties of neuronal nicotinic acetylcholine receptors: pharmacological characterization and modulation of synaptic function. J Pharmacol Exp Ther 280:1117–1136

Alkondon M, Albuquerque EX (1993) Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. J Pharmacol Exp Ther 265:1455–1473

Alkondon M, Pereira EF, Albuquerque EX (1998) alpha-bungarotoxin- and methyllycaconitine-sensitive nicotinic receptors mediate fast synaptic transmission in interneurons of rat hippocampal slices. Brain Res 810:257–263

Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX (1999) Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. J Neurosci 19:2693–2705

Beadle DJ, Horseman G, Pichon Y, Amar M, Shimahara T (1988) Acetylcholine activated ion channels in embryonic cockroach neurons growing in culture. J Exp Biol 109:115

Benson JA (1988) Bicuculline blocks the response to acetylcholine and nicotine but not to muscarine or GABA in isolated insect neuronal somata. Brain Res 458:65–71

Benson JA (1992) Electrophysiological pharmacology of the nicotinic and muscarinic cholinergic responses of isolated neuronal somata from locust thoracic ganglia. J Exp Biol 170:203–233

Blagburn JM, Sattelle DB (1987) Nicotinic acetylcholine receptors on a cholinergic nerve terminal in the cockroach, *Periplaneta americana*. J Comp Physiol A 161:215–225

Buckingham SD, Lapied B, Le Corronc H, Grolleau F, Sattelle DB (1997) Imidacloprid actions on insect neuronal acetylcholine receptors. J Exp Biol 200:2685–2692

Cayre M, Buckingham SD, Yagodin S, Sattelle DB (1999) Cultured insect mushroom body neurons express functional receptors for acetylcholine, GABA, glutamate, octopamine, and dopamine. J Neurophysiol 81:1–14

Changeux JP, Bertrand D, Corringer PJ, Dehaene S, Edelstein S, Lena C, Le Novere N, Marubio L, Picciotto M, Zoli M (1998) Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. Brain Res Rev 26:198–216

Cheung H, Clarke BS, Beadle DJ (1992) A patch-clamp study of the action of an nitromethylen heterocycle insecticide on cockroach neurones growing in vitro. Pestic Sci 34:187–193

Colquhoun LM, Patrick JW (1997) Pharmacology of neuronal nicotinic acetylcholine receptor subtypes. Adv Pharmacol 39:191–220

Cordero-Erausquin M, Marubio LM, Klink R, Changeux JP (2000) Nicotinic receptor function: new perspectives from knockout mice. Trends Pharmacol Sci 21:211–217

Courjaret R, Lapied B (2001) Complex intracellular messenger pathways regulate one type of neuronal alpha-bungarotoxin-resistant nicotinic acetylcholine receptors expressed in insect neurosecretory cells (dorsal unpaired median neurons). Mol Pharmacol 60:80–91

David JA, Sattelle DB (1984) Actions of cholinergic pharmacological agents on the cell body membrane of the fast coxal depressor motoneurone of the cockroach *Periplaneta americana*. J Exp Biol 108:119–136

Déglise P, Grünewald B, Gauthier M (2002) The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. Neurosci Lett 321:13–16

Dwoskin LP, Crooks PA (2001) Competitive neuronal nicotinic receptor antagonists: a new direction for drug discovery. J Pharmacol Exp Ther 298:395–402

Eastham HM, Lind RJ, Eastlake JL, Clarke BS, Towner P, Reynolds SE, Wolstenholme AJ, Wonnacott S (1998) Characterization of a nicotinic acetylcholine receptor from the insect *Manduca sexta*. Eur J Neurosci 10:879–889

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S (1994) Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79:705–715

Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J (2001) alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proc Natl Acad Sci U S A 98:3501–3506

Goldberg F, Grünewald B, Rosenboom H, Menzel R (1999) Nicotinic acetylcholine currents of cultured Kenyon cells from the mushroom bodies of the honey bee *Apis mellifera*. J Physiol (Lond) 514:759–768

Gorczyca M, Hall JC (1984) Identification of a cholinergic synapse in the giant fiber pathway of *Drosophila* using conditional mutations of acetylcholine synthesis. J Neurogenet 1:289–313

Grolleau F, Lapied B, Buckingham SD, Mason WT, Sattelle DB (1996) Nicotine increases $[Ca^{2+}]_i$ and regulates electrical activity in insect neurosecretory cells (DUM neurons) via an acetylcholine receptor with 'mixed' nicotinic-muscarinic pharmacology. Neurosci Lett 220:142–146

Grünewald B (2003) Differential expression of voltage-sensitive K^+ and Ca^{2+} currents in neurons of the honeybee olfactory pathway. J Exp Biol 206:117–129

Grünewald B, Wüstenberg DG, Menzel R (2000) Nicotinic receptors and modulations of voltage-sensitive ion channels in insect mushroom body neurons. Soc Neurosci Abstr 30:270–278

Gundelfinger ED, Schulz R (2000) Insect nicotinic acetylcholine receptors: genes, structure, physiological and pharmacological properties. In: Clementi F, Fornasari D, Gotti C (eds) Neuronal nicotinic receptors. Springer, Berlin Heidelberg New York, pp 497–521

Heisenberg M, Borst A, Wagner S, Byers D (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. J Neurogenetics 2:1–30

Hermsen B, Stetzer E, Thees R, Heiermann R, Schrattenholz A, Ebbinghaus U, Kretschmer A, Methfessel C, Reinhardt S, Maelicke A (1998) Neuronal nicotinic receptors in the locust *Locusta migratoria*. Cloning and expression. J Biol Chem 273:18394–18404

Homberg U (2002) Neurotransmitters and Neuropeptides in the brain of the locust. Microsc Res Tech 56:189–209

Jennings KR, Brown DG, Wright DP Jr (1986) Methyllycaconitine, a naturally occurring insecticide with a high affinity for the insect cholinergic receptor. Experientia 42:611–613

Kreissl S, Bicker G (1989) Histochemistry of acetylcholinesterase and immunocytochemistry of an acetylcholine receptor-like antigen in the brain of the honeybee. J Comp Neurol 286:71–84

Kreissl S, Bicker G (1992) Dissociated neurons of the pupal honeybee brain in cell culture. J Neurocytol 21:545–556

Lapied B, Hue B (1991) Sensitive nicotinic, mixed, and muscarinic receptors in isolated identified adult insect neurones. Pestic Sci 32:377–379

Lapied B, Le Corronc H, Hue B (1990) Sensitive nicotinic and mixed nicotinic-muscarinic receptors in insect neurosecretory cells. Brain Res 533:132–136

Le Novère N, Changeux JP (1995) Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. J Mol Evol 40:155–172

Lee D, O'Dowd DK (2000) cAMP-dependent plasticity at excitatory cholinergic synapses in *Drosophila* neurons: alterations in the memory mutant dunce. J Neurosci 20:2104–2111

Leech CA, Sattelle DB (1992) Multiple conductances of neuronal nicotinic acetylcholine receptors. Neuropharmacology 31:501–507

Lozano VC, Bonnard E, Gauthier M, Richard D (1996) Mecamylamine-induced impairment of acquisition and retrieval of olfactory conditioning in the honeybee. Behav Brain Res 81:215–222

Lukas RJ (1989) Pharmacological distinctions between functional nicotinic acetylcholine receptors on the PC12 rat pheochromocytoma and the TE671 human medulloblastoma. J Pharmacol Exp Ther 251:175–182

Lummis SC, Buckingham SD, Balk ML, Holyoke CW Jr, Sattelle DB (1992) Actions of a series of bisquaternary compounds on nicotinic acetylcholine receptors in insects: ligand binding and electrophysiological studies. Neuropharmacology 31:379–382

Macallan DR, Lunt GG, Wonnacott S, Swanson KL, Rapoport H, Albuquerque EX (1988) Methyllycaconitine and (+)-anatoxin-a differentiate between nicotinic receptors in vertebrate and invertebrate nervous systems. FEBS Lett 226:357–363

Marder E, Paupardin-Tritsch D (1980a) Picrotoxin block of a depolarizing ACh response. Brain Res 181:223–227

Marder E, Paupardin-Tritsch D (1980b) The pharmacological profile of the acetylcholine response of a crustacean muscle. J Exp Biol 88:147–159

Marshall J, Buckingham SD, Shingai R, Lunt GG, Goosey MW, Darlison MG, Sattelle DB, Barnard EA (1990) Sequence and functional expression of a single alpha subunit of an insect nicotinic acetylcholine receptor. EMBO J 9:4391–4398

Matsubayashi H, Alkondon M, Pereira EF, Swanson KL, Albuquerque EX (1998) Strychnine: a potent competitive antagonist of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal neurons. J Pharmacol Exp Ther 284:904–913

McGehee DS, Heath MJ, Gelber S, Devay P, Role LW (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. Science 269:1692–1696

Menzel R, Erber J, Masuhr T (1974) Learning and memory in the honeybee. In: Barton Browne L (ed) Experimental analysis of insect behaviour. Springer, Berlin Heidelberg New York, pp 195–217

Orr N, Shaffner AJ, Watson GB (1997) Pharmacological characterization of an epibatidine binding site in the nerve cord of *Periplaneta americana*. Pest Biochem Physiol 58: 183–192

Osborne RH (1996) Insect neurotransmission: neurotransmitters and their receptors. Pharmacol Ther 69:117–142

Parker JC, Sarkar D, Quick MW, Lester RA (2003) Interactions of atropine with heterologously expressed and native alpha 3 subunit-containing nicotinic acetylcholine receptors. Br J Pharmacol 138:801–810

Paton WD, Zaimis EJ (1952) The methonium compounds. Pharmacol Rev 4:219–253

Pelz C, Jander J, Rosenboom H, Hammer M, Menzel R (1999) I_A in Kenyon cells of the mushroom body of honeybees resembles shaker currents: kinetics, modulation by K^+, and simulation. J Neurophysiol 81:1749–1759

Peng X, Katz M, Gerzanich V, Anand R, Lindstrom J (1994) Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in *Xenopus* oocytes. Mol Pharmacol 45:546–554

Pfeiffer-Linn C, Glantz RM (1990) A neuronal nicotinic acetylcholine receptor in crayfish neurons. Neurosci Lett 120:234–236

Pinnock RD, Lummis SC, Chiappinelli VA, Sattelle DB (1988) Kappa-bungarotoxin blocks an alpha-bungarotoxin-sensitive nicotinic receptor in the insect central nervous system. Brain Res 458:45–52

Rohrbough J, Broadie K (2002) Electrophysiological analysis of synaptic transmission in central neurons of *Drosophila* larvae. J Neurophysiol 88:847–860

Rothlin CV, Katz E, Verbitsky M, Elgoyhen AB (1999) The alpha9 nicotinic acetylcholine receptor shares pharmacological properties with type A gamma-aminobutyric acid, glycine, and type 3 serotonin receptors. Mol Pharmacol 55:249–254

Schäfer S, Rosenboom H, Menzel R (1994) Ionic currents of Kenyon cells from the mushroom body of the honeybee. J Neurosci 14:4600–4612

Schulz R, Sawruk E, Mulhardt C, Bertrand S, Baumann A, Phannavong B, Betz H, Bertrand D, Gundelfinger ED, Schmitt B (1998) D alpha3, a new functional alpha subunit of nicotinic acetylcholine receptors from *Drosophila*. J Neurochem 71:853–862

Su H, O'Dowd DK (2003) Fast synaptic currents in Drosophila mushroom body Kenyon cells are mediated by α-bungarotoxin-sensitive nicotinic acetylcholine receptors and picrotoxin-sensitive GABA receptors. J Neurosci 23:9246–9253

Suter C, Usherwood PN (1985) Action of acetylcholine and antagonists on somata isolated from locust central neurons. Comp Biochem Physiol C 80:221–229

Tareilus E, Hanke W, Breer H (1990) Neuronal acetylcholine receptor channels from insects: a comparative electrophysiological study. J Comp Physiol A 167:521–526

Thany SH, Lenaers G, Crozatier M, Armengaud C, Gauthier M (2003) Identification and localization of the nicotinic acetylcholine receptor alpha3 mRNA in the brain of the honeybee, *Apis mellifera*. Insect Mol Biol 12:255–262

Tsunoyama K, Gojobori T (1998) Evolution of nicotinic acetylcholine receptor subunits. Mol Biol Evol 15:518–527

van den Beukel I, van Kleef RG, Zwart R, Oortgiesen M (1998) Physostigmine and acetylcholine differentially activate nicotinic receptor subpopulations in *Locusta migratoria* neurons. Brain Res 789:263–273

van Eyseren I, Tiaho F, Le Guen J, Guillet JC, Pichon Y (1998) Effects of nicotinic and muscarinic ligands on embryonic neurones of *Periplaneta americana* in primary culture: a whole cell clamp study. J Insect Physiol 44:227–240

Verbitsky M, Rothlin CV, Katz E, Elgoyhen AB (2000) Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. Neuropharmacology 39:2515–2524

Waldrop B (1993) Physiological and pharmacological properties of responses to GABA and ACh by abdominal motor neurons in *Manduca sexta*. J Comp Physiol A 174:775–785

Ward JM, Cockcroft VB, Lunt GG, Smillie FS, Wonnacott S (1990) Methyllycaconitine: a selective probe for neuronal alpha-bungarotoxin binding sites. FEBS Lett 270:45–48

Wonnacott S (1997) Presynaptic nicotinic ACh receptors. Trends Neurosci 20:92–98

Yarowsky J, Carpenter DO (1978) A comparison of similar ionic responses to gamma-aminobutyric acid and actylcholine. J Neurophysiol 41:531–541

Yusuyama K, Meinertzhagen IA, Schürmann F-W (2002) Synaptic organization of the mushroom body calyx in *Drosophila melanogaster*. J Comp Neurol 445:211–226

Zwart R, Vijverberg HP (1997) Potentiation and inhibition of neuronal nicotinic receptors by atropine: competitive and noncompetitive effect. Mol Pharmacol 52:886–895