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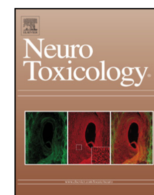

Full Length Article

Antagonist pharmacology of desensitizing and non-desensitizing nicotinic acetylcholine receptors in cockroach neurons☆

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A B S T R A C T

Two α-bungarotoxin-sensitive nicotinic acetylcholine (ACh) receptor subtypes in neurons of the American cockroach have been identified as desensitizing (nAChD) and selectively inhibitable with 100 nM imidacloprid, and non-desensitizing (nAChN) and selectively inhibitable with 100 pM methyllycaconitine. In this paper, the single-electrode voltage-clamp technique was used to measure concentration-response relations for the action of ACh and five antagonists on pharmacologically separated nAChD and nAChN receptors of acutely dissociated neurons from thoracic ganglia of the American cockroach. A dual bath and U-tube perfusion system was used to achieve rapid application of ACh in the continued presence of antagonists, which was essential to accurately measure inhibition by rapidly-reversible antagonists. ACh activated both receptors with an EC_{50} of 7 μM and the antagonist potencies were (nAChD/nAChN in nM): dihydro-β-erythroidine: 1.0/5.6, d-tubocurarine: 1000/34, condelphine: 0.39/0.65, phencyclidine: 74/980 and mecamylamine 47/1150. While each of these antagonists displayed some subtype selectivity, none are selective enough to be used as subtype-selective tools. These results bring to a total of 16 the number of nicotinic compounds that have been measured on nAChD and nAChN currents. Characterization of these receptors is important for understanding the role of nAChRs in the insect nervous system and the mechanism of action of insecticides.

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1. Introduction

Toshio Narahashi was a pioneer in the study of drugs, pesticides and toxins on nerves and ion channels, realizing early on that this knowledge would not only elucidate the mechanisms of intoxication, but also provide tools for the study of the nervous system (Narahashi, 1974; Narahashi and Herman, 1992a, 1992b). Nicotinic acetylcholine receptors (nAChRs) play a central role in fast excitatory synaptic transmission in the insect central nervous system (Gundelfinger and Schulz, 2000), and are also the targets of many drugs, toxins and three commercially-important groups of insecticides, the neonicotinoids (Nauen et al., 2001), the natural product spinosyns (Salgado et al., 1997) and the dithiolanes cartap and bensultap (Lee et al., 2003). This paper provides further characterization of known cholinergic drugs and toxins on nicotinic receptors of the American cockroach, in the hope that the results can be used as tools to better understand the receptors, their function in the nervous system and the mechanism of action of insecticides.

Two types of α-bungarotoxin (α-BGTX)-sensitive nicotinic receptors have been characterized with voltage-clamp measurements in neurons isolated from the CNS of adult cockroaches, *Periplaneta Americana* (Salgado and Saar, 2004). The desensitizing subtype, nAChD, becomes desensitized in the continued presence of agonists and binds agonists very strongly in the desensitized state, while the nondesensitizing subtype, nAChN, does not desensitize and is therefore continuously activated by agonists. Receptor binding studies indicate the presence of α-BGTX-selective and imidacloprid (IMI)-selective components (Nauen et al., 2001; Zhang et al., 2004). Compared to nAChD receptors, nAChN receptors have a much higher affinity for the antagonists α-BGTX and methyllycaconitine, and are expected to be measured specifically in receptor binding assays with $[^3H]$-α-BGTX (Zhang et al., 2004), while nAChD receptors would be specifically assayed in binding assays with $[^3H]$-imidacloprid (IMI) (Salgado and Saar, 2004). nAChD receptors appear to be the primary targets of

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neonicotinoids, while nAChN receptors are the targets of spinosyns (Salgado and Sparks, 2010).

Ten nAChR genes have been identified in insects, and alternative splicing and RNA editing creates even more diversity, (Lansdell and Millar, 2002; Grauso et al., 2002,Jones and Sattelle, 2010), so there is potential for many subtypes of nAChD and nAChN receptors. Using methods similar to the ones used in this paper, at least four pharmacologically distinct nAChD subtypes can be distinguished in stick insect neurons (Oliveira et al., 2011). Furthermore, two subtypes of α-BGTX-resistant nicotinic receptors have been identified in dorsal unpaired median neurons of the cockroach (Courjaret and Lapied, 2001; Lapied et al., 1990), although these are atropine-sensitive (David and Pitman, 1993), so were probably blocked by the inclusion of 1 μM atropine in our bath solution.

The present study was undertaken to further characterize the pharmacology of nAChD and nAChN receptors in neurons isolated from thoracic ganglia of the American cockroach. The work builds upon that of Salgado and Saar (Salgado and Saar, 2004), with the addition of a U-tube perfusion system to enable quantitative measurement of the effects of rapidly reversible agonists and antagonists. For each receptor, the agonist dose-response relation for ACh was measured, as well as dose-response relations for the competitive antagonists dihydro-β-erythroidine (DHBE), d-tubocurarine, condelphine, and the noncompetitive antagonists phencyclidine and mecamylamine.

2. Methods

The three thoracic ganglia of adult male American cockroaches, *Periplaneta americana*, were removed, desheathed with forceps and treated with collagenase (Sigma type I, 1 mg/ml) and Trypsin (1 mg/ml) in cockroach saline without $CaCl_2$ for 30 min at room temperature. The ganglia were then washed three times with normal cockroach saline (in mM, 150 NaCl, 3 KCl, 5 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.2) and triturated through a series of glass Pasteur pipettes that had been fire-polished to have progressively smaller tips. Neurons were allowed to settle and adhere for at least 30 min in the middle of 35 mm diameter polystyrene tissue culture dishes that were then placed on the stage of an inverted microscope.

Large neurons, 50–100 μm in diameter, were selected and impaled with 3 M KCl-filled microelectrodes of 15–25 Mohm resistance that were fabricated on a DMZ Universal Puller (Zeitz Instrument Co., Muenchen, Germany). The cells were voltage-clamped at the zero current potential, which was between −50 and −95 mV, with a SEC 05L single electrode voltage clamp amplifier (npi electronic GmbH, Tamm, Germany). The electrodes were optimally compensated, and a switching frequency of 25 kHz was used, with proportional-integral feedback, allowing excellent control of the membrane potential at all frequencies of interest (Polder and Swandulla, 1990). Voltage clamp currents were recorded with an ITC-16 computer interface and Pulse software (Heka elektronik, Lambrecht/Pfalz, Germany). Potency and slope values were determined from non-linear fits of log concentration-response data to the Hill Equation, using GraphPad Prism software (Graphpad Software, Inc., La Jolla, CA).

External solution flowed directly over the cell at a constant rate of 0.5 ml/min from the opening of a 500 μm internal diameter PTFE bath perfusion tube placed 800 μm from the cell. A U-tube was used for rapid, controlled application of ACh alone or with antagonists. The U-tube was a loop of fine polyethylene tubing (PE10, Becton Dickinson, Sparks, MD) with a single hole, made with a 0.2 mm insect pin, that was placed near the cell. External solution, containing ACh alone or with an antagonist, was fed through the tube by gravity from a container located 30 cm above the bath, and normally flowed to a waste container from the other end, which was located 30 cm below the bath, so that no solution flowed either in or out of the hole located by the cell. Closure of a computer-operated solenoid valve in the waste line allowed the U-tube solution to flow out of the hole located near the cell, displacing the stream of external solution that normally flowed over the cell from the bath perfusion system. In contrast to other studies (Zhao et al., 2003), the bath solution ran continuously, but was displaced away from the cell when the U-tube solution flowed. With this method, the external solution surrounding the cell could be completely exchanged with test solution within 30 ms. Test compounds were added to the bath and U-tube solutions simultaneously and the effect on the ACh-evoked current was monitored with a test pulse every 60 s until a steady-state effect of the treatment was achieved. Ascending concentration-response series were measured, followed by washout of the drug to test for rundown of the current. Under these conditions, single cockroach neurons could usually be studied for many hours, with nicotinic currents showing very little, if any, rundown in most cases.

In all experiments, 1 μM atropine was present in the bath to specifically block muscarinic ACh receptors. For measurements of nAChN currents, 100 nM IMI, which selectively desensitized nAChD receptors, was present in the external solution. For measurements of nAChD currents, 100 pM MLA, which specifically blocked nAChN receptors, was present (Salgado and Saar, 2004). At this low MLA concentration, the time constant for onset of block is 10–15 min, so 45 min are needed to achieve full block of nAChN current. To speed up equilibration, 1 nM MLA was added for 10 min and then replaced with 100 pM MLA, leaving a few minutes for the nAChD receptors to recover from the partial block.

All compounds were obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in external solution, except for phencyclidine, which was dissolved in and diluted in DMSO to 1000X the desired test concentration, which was then directly suspended in external solution, giving a final DMSO concentration of 0.1%, which had no effect on ACh-evoked currents.

3. Results

U-tube application of up to 300 nM ACh to isolated cockroach neurons produced no effect. In the presence of 100 pM MLA in the bath solution, which blocks nAChN receptors, concentrations of ACh between 1 and 100 μM evoked inward currents that increased in amplitude and decay rate. An exemplary family of ACh-evoked nAChN currents is shown on the right in Fig. 1. At 300 μM and above, the maximum current declined slightly and the decay rate continued to accelerate. This decrease in peak current and acceleration of decay appears to be due largely to faster desensitization, but could also be due partially to channel block, which is known to occur at 300 μM and higher concentrations of ACh (Sine and Steinbach, 1984).

An example of the nAChD current family is shown on the left in Fig. 1. In the presence of 100 nM IMI, ACh specifically activated nAChN receptors. The evoked current increased gradually as the ACh concentration increased from 1 μM to 30 μM, but did not increase further between 30 and 300 μM. At 1000 μM, the peak current was not decreased, but the current decayed during the first second of the pulse and increased after the pulse, indicating open-channel block by ACh. Average ACh dose-response relations for nAChD and nAChN currents are shown by the squares and circles, respectively in Fig. 1, with fits of the Hill equation. ACh had an EC_{50} of 7 μM for both receptors, with Hill coefficients of 1.4–1.7 (Fig. 1, Table 1).

In order to study the effects of rapidly reversible competitive antagonists, the agonist concentration was kept as low as possible, to minimize the effects of competition on the measurements. For nAChD, 2 μM ACh was used, while for the smaller nAChN currents, 3 μM ACh was used.

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