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Nornicotine application on cockroach dorsal unpaired median neurons induces two distinct ionic currents: Implications of different nicotinic acetylcholine receptors

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ABSTRACT

The goal of the present study is to examine the agonist action of nornicotine on insect nicotinic acetylcholine receptors. Using patch-clamp techniques on cockroach dorsal unpaired median neurons, we demonstrated that nornicotine induced two distinct ionic currents named types 1 and 2. We found that alpha-bungarotoxin induced a rapid desensitization of type 1 currents whereas type 2 was completely blocked. Interestingly, types 1 and 2 currents were not blocked by the muscarinic antagonist, pirenzepine but by co-application of 1 μM pirenzepine and 0.5 μM alpha-bungarotoxin, suggesting that muscarinic receptors modulated nornicotine-induced current amplitudes. In addition, type 1 current amplitudes were strongly reduced by 20 μM D-tubocurarine and 5 μM mecamylamine which blocked the previously identified alpha-bungarotoxin-insensitive nAChR1 and nAChR2 receptors. Co-application of alpha-bungarotoxin with D-tubocurarine or mecamylamine completely blocked all ionic currents. We propose that types 1 and 2 currents are associated to several nicotinic receptors subtypes, including nAChR1 and nAChR2 receptors. Finally, we conclude that nornicotine could be used as an agonist to identify distinct insect nicotinic receptors.

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Nornicotine (Nor) is a tobacco alkaloid and an active nicotine metabolite which is associated to physiological and behavioural effects [3,8,26]. Nor is present in the brain after exposure to nicotine [6,7,11,12,21], it increases dopamine release through nicotinic receptors activation [10,15] and functionally discriminates between different vertebrate nAChR subtypes [22]. Indeed, Nor acts as a potent partial agonist of homomeric $\alpha 7$ receptor while it is a relatively poor agonist for heteromeric $\alpha 3\beta 4$ and $\alpha 4\beta 2$ receptors, leading to the assumption that it could be used as a ligand to characterize nAChR subtypes [22]. Due to the difficulty to express functional nAChR subtype in heterologous system; the subunit composition of native insect nAChRs remains unknown. To date; only one combination using insect nAChR subunit is functional; *i.e.* *Schistocerca gregaria* $\alpha 1$ ($Sg\alpha 1$) subunit [17,20]. However; electrophysiological and binding studies performed on insect neurons revealed several nAChRs which could be classified as α -bungarotoxin (α Bgt)-sensitive and -insensitive [9,13]. The snake toxin α Bgt was commonly used to characterize vertebrate and invertebrate nAChR subtypes [10,13,14]. Thus; it was suggested that such as vertebrates, insect α Bgt-sensitive nAChRs

could include homomeric receptors [25]. In the cockroach *Periplaneta americana*; α Bgt-sensitive and -insensitive nAChR subtypes have been identified [4,5,9,16,23]. Two distinct α Bgt-insensitive nAChRs named nAChR1 and nAChR2 were found in the dorsal unpaired median (DUM) neurons. They differ in their pharmacological properties: nAChR1 was blocked by D-tubocurarine (dTC) and nAChR2 by mecamylamine (Mec) [5]. In addition, they are activated by different intracellular pathways involving calcium and second messengers such as PKA and two distinct PKCs [4,5,24]. The α Bgt-sensitive nAChRs include the 'mixed' nicotinic/muscarinic receptor which was blocked by α Bgt and the muscarinic antagonist, pirenzepine (Pzp) [9,16,18]. In the present study, using patch-clamp methods on DUM neurons, we evaluated for the first time the agonist action of the nicotine metabolite, nornicotine, on insect nAChRs.

Patch clamp recordings were performed on DUM neuron cell bodies isolated from the dorsal midline of the terminal abdominal ganglion of the nerve cord of adult male cockroach *Periplaneta americana*, following enzymatic treatment and mechanical dissociation as previously described [18]. Nor was applied by pneumatic pressure ejection (15 psig. Miniframe, Medical System Corporation, USA). The pressure ejection was made through a glass micropipette, resistance 1.8 M Ω , positioned in solution within 100 μm of the isolated cell body. Nor-induced currents were recorded using the patch-clamp technique in the whole-cell recording configuration

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under voltage-clamp mode with an axopatch 200B (Patch-clamp amplifier, Axon Instruments, Foster City, CA). Signals were digitized and acquired using a MiniDigidata 1440 analog-digital converter (Axon Instruments) and axoscope 10.2 software (Axon Instruments). The Petri dish containing isolated cell bodies was placed onto the inverted microscope (CK2: Olympus), and continuously bathed with the standard extracellular solution (in mM: NaCl 200, KCl 3.1, MgCl₂ 4, CaCl₂ 5, sucrose 50, HEPES 10, pH 7.4 adjusted with NaOH) using a gravity perfusion system positioned within 100 μm from the cell body. Patch pipettes (borosilicate glass capillary tubes: GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) were filled with internal solution containing (in mM): K-D-gluconic acid, 160; NaCl, 10; MgCl₂, 1; CaCl₂, 0.5; K-fluoride, 10; ATP Mg, 3; EGTA 10; HEPES, 20 and pH adjusted to 7.4 with KOH. Pipettes had resistances ranging from 1.2 to 1.4 MΩ. All compounds tested were purchased from Sigma Chemical Company (France). Statistical analysis was performed using Student's *t*-test. Statistical significance

was assessed by a standard Bonferroni's test using GraphPad Software. Results were expressed as means ± SEM. The dose–response curve was derived from the fitted curve following the equation:

$$y = I_{\min} + \frac{(I_{\max} - I_{\min})}{(1 + 10^{(\log EC_{50} - X)H})}$$

where *Y* is the normalized response, *I*_{max} and *I*_{min} are the maximum and minimum responses, '*H*' is the coefficient and EC₅₀, the concentration giving half the maximum response.

As illustrated in Fig. 1, application of Nor induced a transient inward current in cockroach DUM neurons, voltage clamped at –50 mV (membrane potential) [19]. We found that most of the recorded cells responded with high Nor currents (Type 1), while the other cells had lower current amplitudes (Type 2), with respect to the peak current amplitudes (Fig. 1A and B). In each condition, the EC₅₀ values were estimated at 19.6 μM and 10.4 μM for types 1 and

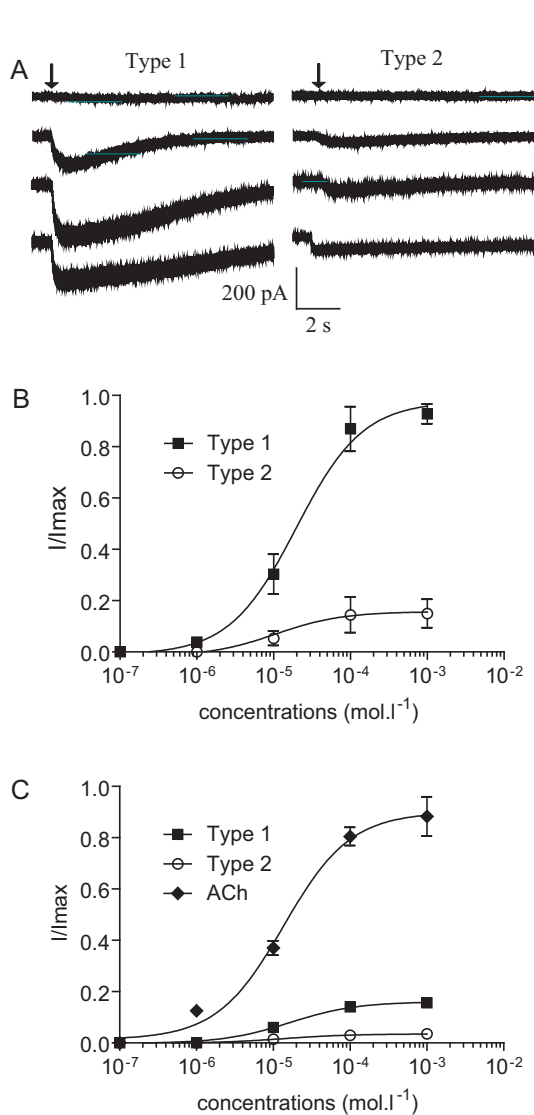


Fig. 1. Effect of Nor on DUM neuron. (A) Typical examples of DUM neuron responses after pressure application of 100 ms Nor at –50 mV holding potential. Currents from the upper trace correspond to 1 μM, 10 μM, 100 μM and 1 mM, respectively. Arrows indicate 150 ms pulse duration. (B) Concentration–response curves for types 1 and 2 currents. Responses were normalized to the maximum peak current elicited by 100 μM Nor. (C) Concentration response–curve comparing the agonist action of ACh and Nor (Types 1 and 2 currents). Current amplitudes were normalized to the maximum peak current amplitude elicited by 1 mM ACh. Data are mean ± SEM (N = 10). All currents are measured at –50 mV holding potential.

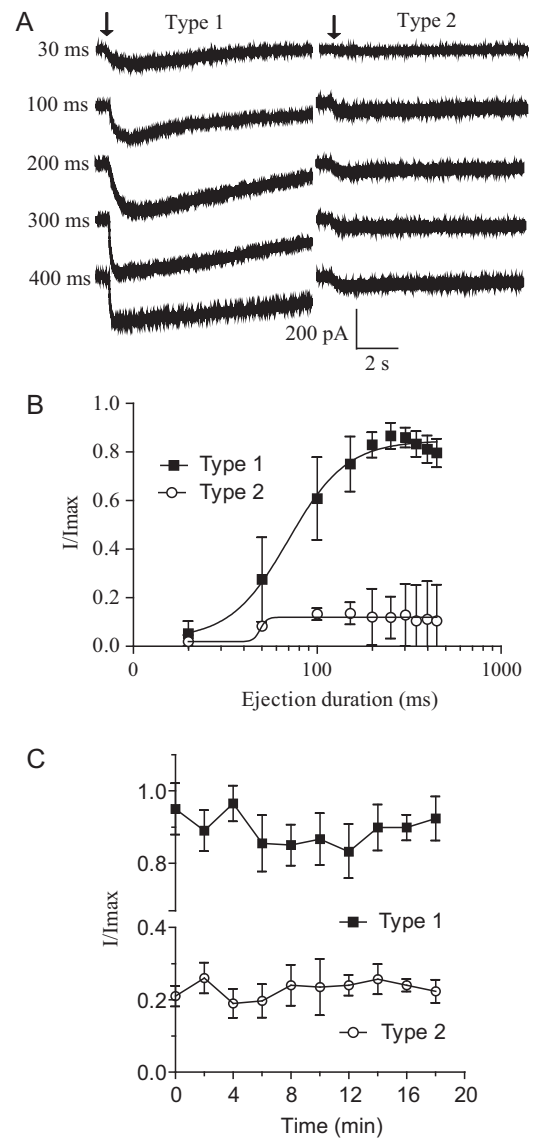


Fig. 2. Successive applications of Nor. (A) Typical examples of type 1 and type 2 current amplitudes at different pulse durations. Arrows indicated 150 ms pulse duration (100 μM Nor). (B) Comparison of type 1 and type 2 current amplitudes. 100 μM Nor was applied at different pulse durations (from 30 ms to 450 ms) and 2 min interval pulse. (C) Nor-induced current amplitudes (types 1 and 2) remained stable after the beginning of the first pulse (100 μM Nor, 150 ms pulse duration, 15 psig). Data are mean ± SEM (N = 10) and normalized to the maximum Nor-induced currents at –50 mV holding potential.

2 currents, respectively. Interestingly, the peak current amplitudes of ACh measured in the same condition demonstrated that Nor was a partial agonist of DUM neuron nAChRs (Fig. 1C). These kinds of profiles were previously demonstrated using honeybee antennal lobe neurons. Barbara et al. found that acetylcholine (ACh)-induced currents exhibited two distinct profiles for which some cells elicited low currents and others higher current amplitudes [2]. Unfortunately, the electrophysiological properties of these currents were not investigated, and only ACh-induced higher current amplitudes were examined. Here, the maximal Nor-induced current amplitude was -260 pA at 100 μ M and 150 ms pulse duration. We therefore used this concentration to study the agonist action of Nor on DUM neuron nAChRs and all currents were normalized to the Nor maximum response. The mean current amplitudes were -239.3 ± 8 pA and -44 ± 5 pA at 100 μ M, for types 1 and 2 currents. Then, to estimate the possible saturation or desensitization of ionic currents, 100 μ M Nor was applied with different pulse durations (from 30 ms to 450 ms with 2 min interval pulse), on the same cell [1]. In these conditions, we found a sigmoidal relationship between the ejection duration and the ionic current amplitude for type 1 whereas similar effect was not found with type 2 currents (Fig. 2A and B). These data were in general agreement with previous studies demonstrating that cockroach *Periplaneta americana* expresses two distinct α Bgt-sensitive receptors: desensitizing (nAChRD) and non-desensitizing (nAChRN) nicotinic receptors [23]. Indeed, nAChRD and nAChRN could be identified by their peak current amplitudes and we found that types 1 and 2 current amplitudes remained stable 20 min after successive applications of 100 μ M Nor (150 ms pulse duration with 2 min interval pulse) (Fig. 2C). Moreover, when Nor-induced currents were investigated using 0.5 μ M α Bgt, type 1 current amplitudes did not show any significant difference between

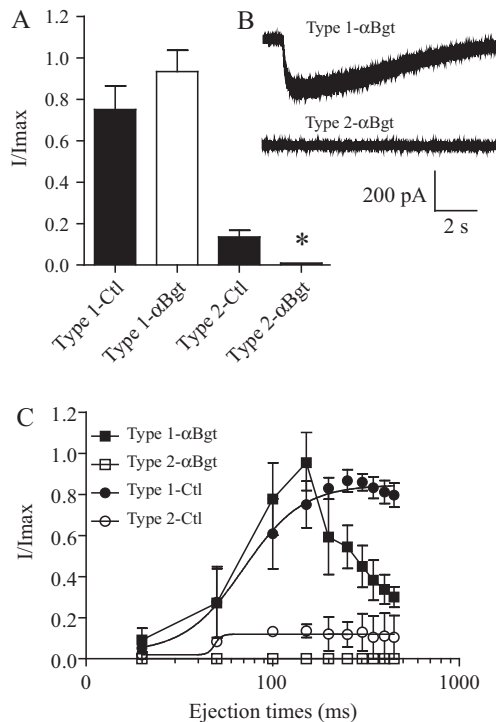


Fig. 3. Effect of 0.5 μ M α Bgt on Nor-induced currents. (A) Histogram illustrating types 1 and 2 current amplitudes (Ctl = control). Type 1 currents were not affected by α Bgt whereas type 2 currents were completely blocked. * $p < 0.05$ using Student's t -test ($N = 10$). Data are mean \pm SEM, normalized to the maximum Nor-induced currents at -50 mV holding potential and 150 ms pulse duration. (B) Example of Nor-induced currents under 0.5 μ M α Bgt. (C) Successive applications of 100 μ M Nor, using the same cells. Data are normalized using the same condition as indicated above.

control (-239.3 ± 8 pA) and α Bgt treated cells, whereas type 2 currents were completely blocked. Currents were -248 ± 16 pA and 0 pA for types 1 and 2, respectively (Fig. 3A and B). Interestingly, type 1 currents measured between 150 ms and 450 ms pulse durations showed a strong decrease of peak current amplitudes from -248 ± 16 pA to -90 ± 0.9 pA, indicating a rapid desensitization of nAChRs (Fig. 3C). According to our results, we propose that α Bgt affects the desensitization of type 1 currents whereas type 2 is completely blocked. As it was previously suggested that bath applied muscarinic antagonist such as Pzp could block the 'mixed' nicotinic/muscarinic receptors [18], 100 μ M Nor was applied under 1 μ M Pzp. Neither types 1 and 2 current amplitudes were affected by Pzp, demonstrating that Nor did not bind to DUM neuron muscarinic receptors. The type 1 and 2 Nor-induced currents under Pzp treatment were -266.8 ± 16 pA and -92 ± 13 pA, respectively (Fig. 4A). When Nor was co-applied with 1 μ M Pzp and 0.5 μ M α Bgt, all current amplitudes were strongly reduced to -78 ± 0.1 pA and 0 pA for type 1 and 2 (Fig. 4A). Thus, we propose that Pzp could inhibit the modulatory effect of muscarinic receptors resulting in a strong inhibition of type 1 currents. This hypothesis is supported by the finding that (1) this concentration of Pzp did not block Nor-induced currents, (2) the strong desensitization induced by α Bgt

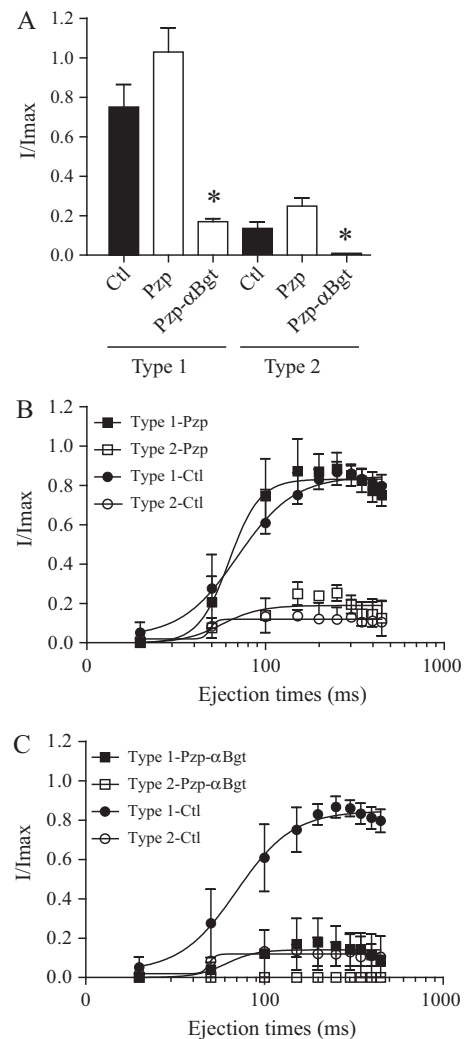


Fig. 4. Effect of 1 μ M Pzp on Nor-induced types 1 and 2 currents. (A) Significant decrease of Nor currents (100 μ M and 150 ms pulse duration) were found under co-application of Pzp and α Bgt ($N = 7$) compared to control (Ctl, $N = 10$). *Student's t -test, $p < 0.001$. (B) and (C) Repeated applications of Pzp and α Bgt.

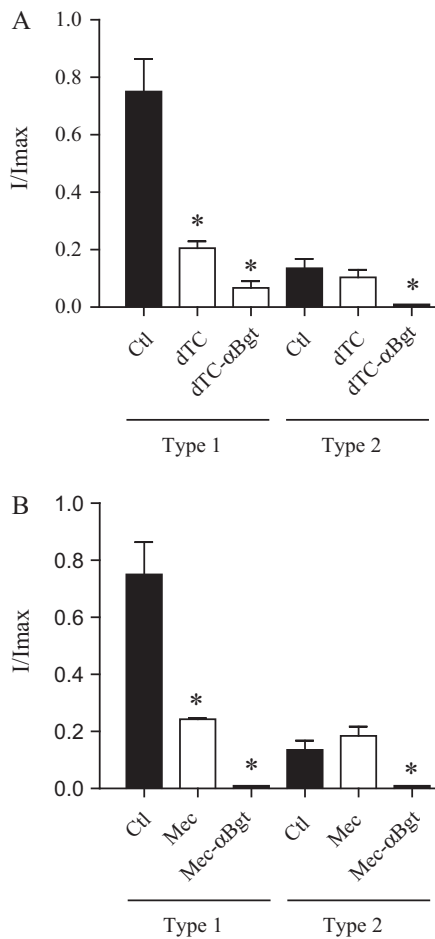


Fig. 5. Effect of dTC and Mec on Nor-induced current amplitudes. (A) Histograms illustrating the decrease of types 1 and 2 currents under dTC and bath application of both dTC and α Bgt. In each condition, $p < 0.001$ using Student's *t*-test, $N = 5$. (B) Similar decrease was found with $5 \mu\text{M}$ Mec or co-applications of Mec and α Bgt. *Statistical difference $p < 0.05$, $N = 5$. Ctl = control. Mean current amplitudes are plotted at -50 mV holding potential and 150 ms pulse durations.

(See Fig. 3C) was not observed under Pzp treatment (Fig. 4B) while repeated applications of Nor were completely blocked by both α Bgt and Pzp (Fig. 4C) and (3) this hypothesis was in accordance with previous studies demonstrating that the variability of the acetylcholine-induced currents [23] and nicotine-induced current amplitudes through activation of DUM neurons nAChR1 subtype could be modulated by muscarinic receptors [5]. Consequently, we suggested that Nor did not act as an agonist of the 'mixed' nicotinic/muscarinic receptor but that its agonist action was modulated by muscarinic receptors.

In a second set of experiments, we explored the agonist effect of Nor on α Bgt-insensitive nAChR1 and nAChR2 subtypes [4,5,24]. Nor was first applied with $20 \mu\text{M}$ D-tubocurarine (dTC) which blocked nAChR1 [4,5,24]. The relative current amplitude induced by $100 \mu\text{M}$ Nor was significantly reduced to $-61.7 \pm 1.7 \text{ pA}$ for type 1 currents whereas there was not significant effect on type 2 (current amplitudes were $-36.3 \pm 0.3 \text{ pA}$, Fig. 5A). As it appeared that type 1 currents were more sensitive to dTC compared to type 2, we proposed that DUM neurons contained two distinct nAChRs which were sensitive (type 1) and insensitive (type 2) to dTC. Thus, bath application of both $20 \mu\text{M}$ dTC and $0.5 \mu\text{M}$ α Bgt strongly reduced Nor-induced type 1 and 2 currents to $-22 \pm 3 \text{ pA}$ and 0 pA , respectively (Fig. 5A). These results demonstrated that Nor action was associated to α Bgt-insensitive nAChRs (*i.e.* type 1 currents) which were blocked by dTC but did not include nAChR1 subtype. The

Nor responses were further compared by examining effects on nAChR2 subtype. We first found that $5 \mu\text{M}$ Mec strongly reduced Nor-induced type 1 to $-54.4 \pm 4 \text{ pA}$ whereas co-application of Mec and α Bgt completely blocked all currents (Fig. 5B). In the light of our results and compared to previous electrophysiological studies, we propose that DUM neurons express nAChRs that are blocked by dTC and Mec but are distinct from the previously identified α Bgt-insensitive nAChR1 and nAChR2 subtypes [4,5]. Consequently, type 2 currents could be induced through activation of α Bgt-sensitive nAChRs while type 1 includes α Bgt-sensitive and -insensitive receptors. We have identified several nAChR subunits in the cockroach suggesting different nAChR subtypes (Unpublished data). Our future goal will be to investigate the subunits composition of these receptors and their pharmacological properties. Finally, in the present study we demonstrate that each of these currents can be selectively blocked providing evidence that they are induced by distinct nAChRs and therefore, Nor can be used as an agonist to characterize insect nAChR subtypes.

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