Nicotinic-acetylcholine receptors are functionally coupled to the nitric oxide/cGMP-pathway in insect neurons

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Abstract

In addition to their ionotropic role, neuronal nicotinic acetylcholine receptors (nAChRs) can influence second messenger levels, transmitter release and gene transcription. In this study, we show that nAChRs in an insect CNS control cGMP levels by coupling to NO production. In conditions that inhibit spiking, nicotine induced cGMP synthesis. This increase in cGMP was blocked by nicotinic antagonists, and by inhibitors of both nitric oxide synthase and soluble guanylyl cyclase. The nicotinic-evoked increase in cGMP was localized to specific NO-sensitive neurons in the CNS, several of which are identified motoneurons. Because NO production requires Ca²⁺, we investigated the effect of nicotinic stimulation on [Ca²⁺]i in cultured neurons. We found that activation of nAChRs increased [Ca²⁺], which was blocked by nAChR antagonists. Nicotinic stimulation of neurons in the isolated CNS in low-Na⁺, also evoked increases in [Ca²⁺] independent of fast changes in voltage. In addition, approximately 10% of the nicotinic-evoked [Ca²⁺] increase in cultured neurons persisted when voltage-gated Ca²⁺ channels were blocked by Ni²⁺. Under the same conditions, nicotinic stimulation of cGMP in the CNS was unaffected. These combined results suggest that nicotinic stimulation is coupled to NOS potentially by directly gating Ca²⁺.

Keywords: calcium, cGMP, nicotinic acetylcholine receptors, nitric oxide, phosphodiesterase inhibitors, soluble guanylyl cyclase.


Nitric oxide (NO) has diverse physiological functions in vertebrates (Denninger and Marletta 1999) and invertebrates (Martinez et al. 1997). In the vertebrate CNS, NO is implicated in long-term depression and potentiation (Prast and Philippu 2001), and the generation of rhythmic motor patterns associated with locomotion (McClean and Sillar 2000). NO also plays a role in the invertebrate CNS, where it mediates anterograde transmission (Jacklet 1995; Park et al. 1998; Koh and Jacklet 1999), and is essential for normal olfaction (Elphick et al. 1995; Gelperin et al. 1996; Nighorn et al. 1998; Nighorn et al. 2001) and development (Gibbs and Truman 1998; Champlin and Truman 2000; Gibson and Nighorn 2000). Many of these actions are mediated by NO-induced activation of soluble guanylyl cyclase (sGC, Denninger and Marletta 1999).

In the mammalian CNS, neuronal NOS (nNOS) contains a PDZ protein motif important for its subcellular localization and coupling to NMDA receptors (Brenman et al. 1996; Brenman and Brecht 1997), and NO production in glutamatergic synapses is stimulated via an NMDA increase in calcium (Ca²⁺) (Schuman and Madison 1994; Brenman and Brecht 1997). Recently, it has been shown that NO-induced cGMP synthesis is localized to cholinergic regions of the rat brain (de Vente et al. 2001). Neuronal nicotinic acetylcholine receptors (nAChRs) in the brain have relatively high Ca²⁺ permeability and have been implicated in the modulation of synaptic transmission (Rathouz et al. 1995; Sivilotti and Colquhoun 1995; Jones et al. 1999; Shoop et al. 2001). It has been suggested that nAChR-mediated synaptic modulation in the CNS could involve NOS activation (Prast and Philippu 2001) and there is preliminary evidence that nicotinic stimulation can trigger NO synthesis in the rat hippocampus (Fedele et al. 1998; Smith et al. 1998).
The insect nervous system is useful for examining the central role of nAChRs, where they are found at very high density and mediate most primary sensory transmission. Moreover, it has been shown that nicotine induces Ca\(^{2+}\) changes in Kenyon cells isolated from cricket (Cayre et al. 1999) and honeybee (Bicker 1996), cockroach DUM neurons (Grolleau et al. 1996), and isolated thoracic ganglia neurons of the locust (Oertner et al. 1999). Insects are also suitable model systems for studying the NO/cGMP-pathway (Bicker 2001). For example, in Manduca, NO-producing and -responding neurons have been identified (Zayas et al. 2000b), a Ca\(^{2+}\)-dependent isofrom of NOS (MsNOS) and two sGC genes have been cloned and their expression mapped in the adult olfactory system (Nighorn et al. 1998).

In this study, we test the role of nAChRs in NO-signaling in the CNS. We show that stimulation of nAChRs activates the NO/cGMP-pathway in the larval CNS of Manduca. This nicotine-induced cGMP synthesis is localized to specific neurons of the ventral nerve cord. In addition, we show that nAChR stimulation increases intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\text{c}\)). These findings suggest that nAChRs are coupled to the NO/cGMP-pathway, which may have important functional implications for the neuromodulatory role of this family of receptors in the CNS.

**Materials and methods**

**Reagents and pharmacological agents**

All reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

**Experimental animals**

Larvae of the tobacco hornworm, *Manduca sexta*, were reared at 27°C on an artificial diet (modified from, Bell and Joachim 1978) in a light-dark cycle of 17 h : 7 h. Larvae were anesthetized by chilling for 30 min at 4°C. The nervous system was then exposed by making an incision along the dorsal midline, removing the gut and pinning the larvae in an elastomer-lined dish containing cold insect saline (Miyazaki 1980; Trimmer and Weeks 1989). The connectives between the ganglia were removed and the ganglia were dissected in insect saline (Miyazaki 1980; Trimmer and Weeks 1989). The connectives between the ganglia were removed and the ganglia were incubated in Hank's Ca\(^{2+}\)- and Mg\(^{2+}\)-free balanced salt solution (Life Technologies, Rockville, MD, USA) containing 0.5 mg/mL collagenase and 2 mg/mL dispase (Boehringer Mannheim, Mannheim, Germany) for 30 min at room temperature followed by 5 min at 37°C. The tissue was then dispersed by 3 Biochemical analyses

**3',5'-Cyclic guanine monophosphate assays and immunocytochemistry**

Nerve cords (abdominal ganglia to 1 terminal) were removed from fifth instar larvae and treated with collagenase (1 mg/mL) for 15 min in phosphate-buffered saline (PBS) then washed for 5 min in PBS. After washing, nerve cords were incubated in saline with, or without, 10 \(\mu\)M 1H-(1,2,4)-oxadiazolo[4,3-a]quinolin-1-one (ODQ; Calbiochem, San Diego, CA, USA) in dimethyl sulfoxide (< 1%). After 30 min the nerve cords were transferred to low-Na\(^{+}\) saline containing 1,4-dihydro-5-(2-propanoxyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one (zaprinast, 500 \(\mu\)M; Calbiochem) or nicotinic agonists for 15 min. Because zaprinast is insoluble in solutions with \(\text{NiCl}_2\), it was replaced with sildenafil citrate (Pfizer, Inc., Sandwich, UK) in these experiments, as noted in the figures. Treatments were terminated by individually placing each nerve cord in an Eppendorf tube containing 50 \(\mu\)L of acidified ethanol (0.1 \(\mu\)L HCl) to stop the reaction. The nerve cords were homogenized with an additional 200 \(\mu\)L of acidified ethanol and centrifuged for 5 min at 4000 \(g\). The supernatant was retained and the pellet washed with an additional 250 \(\mu\)L of acidified ethanol, which was pooled with the supernatant and stored at \(-80°C\). Samples were freeze-dried in a SpeedVac (Savant Instruments, Inc., Holbrook, NY, USA), and reconstituted in 150 \(\mu\)L EIA (enzyme-linked immunoassay) buffer (0.02 \(\mu\)M NaPO\(_4\), 0.15 \(\mu\)M NaCl, 1 \(\mu\)M Na\(_2\)EDTA, 0.1% bovine serum albumin, pH 7.4) and 50 \(\mu\)L was assayed in duplicate with a cGMP EIA (Kingan et al. 1997). Assay plates were coated with goat anti-rabbit antibodies (0.5 \(\mu\)g/well; American Qualex, CA, USA). Rabbit anti-cGMP antiserum (STI-Signal Transduction Products, CA, USA) was used at a 1 : 20 000 dilution and the cGMP-HP-R (horseradish peroxidase) conjugate (American Qualex) was used at a 1 : 16 000 dilution. A typical nonacetylated assay gave a standard curve with a 0.1 ratio of bound cGMP-HP-R in the presence of unlabeled cGMP to bound cGMP-HP-R in the absence of unlabeled cGMP (B/B\(_0\)) of 1 pmol cGMP and a 0.9 B/B\(_0\) of 4 fmol.

The protocol for cGMP immunocytochemistry was as described by Ewer et al. (1994) and Morton (1996). Nerve cords were removed from fifth instar larvae, collagenased and treated as described above. Treatment was terminated by overnight fixation in 4% paraformaldehyde in PBS. Nerve cords were washed in PBST (0.3% Triton-X 100 in PBS) and treated with 0.5 mg/mL collagenase (type IV) for 30 min, washed again in PBST, blocked in 20% donkey serum in PBST, and incubated in sheep anti-cGMP antiserum (courtesy of Dr J. de Vente) at 1 : 10 000 in PBST/20% donkey serum for 48 h at 4°C. Nerve cords were washed again and incubated in a peroxidase-labeled donkey anti-sheep antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1 : 1000 in PBST overnight at 4°C, washed in PBST, PBS and visualized with 0.5 mg/mL diaminobenzidine and 0.01% hydrogen peroxide with 0.036% nickel chloride. Neurons that stain using this technique are referred to as cGMP immunoactive (cGMP-IR).

Optical sections of wholemount ganglia were captured using an Olympus MagnaFire SP digital camera mounted on an Olympus BX40 microscope (Olympus America, Melville, NY, USA). Optical sections were modified adjusting the contrast, brightness, highlights and shadow in Paint Shop Pro 5.03 (Jasc Software Inc., Minnetonka, MN, USA).

**Primary neuronal cell culture**

The isolation and maintenance of Manduca neurons in culture followed the protocol of Prugh et al. (1992). Second and third day fifth instar larva were cold anesthetized and the abdominal ganglia were dissected in insect saline (Miyazaki 1980; Trimmer and Weeks 1989). The connectives between the ganglia were removed and the ganglia were incubated in Hanks Ca\(^{2+}\)- and Mg\(^{2+}\)-free balanced salt solution (Life Technologies, Rockville, MD, USA) containing 0.5 mg/mL collagenase and 2 mg/mL dispase (Boehringer Mannheim, Mannheim, Germany) for 30 min at room temperature followed by 5 min at 37°C. The tissue was then dispersed by
Neuronal cell cultures on individual glass slides were incubated with saline. Relative Ca\(^{2+}\) levels were monitored ratiometrically at 350 and 380 nm excitation wavelengths at 1 Hz (Ionoptix, Inc., Milton, MA). The absolute Ca\(^{2+}\) concentration was not determined. Only cells that showed a response to treatments were included in the analysis. The amplitude of the change in ratio 100 s prior to a response (baseline) and 150 s after the onset of the response to treatment were measured off-line. These values were averaged over all the cells within a treatment for statistical comparison.

Ca\(^{2+}\) monitoring from individual neurons was performed in isolated abdominal segments 3 or 4 from the fifth instar larva. To facilitate electrode penetration and saline access, the dorsal sheath was softened using collagenase (type IA) and removed as described previously (Weeks and Jacobs 1987). Intracellular recordings were made with borosilicate glass electrodes backfilled with 20 mM FURA-2 pentapotassium salt (Molecular Probes) dissolved in electrode solution (200 mM potassium acetate, 50 mM KCl, 5 mM Hepes). Electrodes were beveled to improve dye injection and had resistances of 40–60 MΩ. FURA-2 was pressure injected with a Picospritzer (General Valve Corp., Fairfield, NJ, USA). Neurons were visualized and relative Ca\(^{2+}\) levels were monitored as described above. Intracellular voltage was recorded on VCR tape (Vetter Corporation, Rebersberg, PA, USA), and analyzed with Dataq Instruments DI-720 acquisition system and Windaq/Pro waveform recording software (Dataq Instruments, Inc., Akron, OH, USA).

Statistical analysis

Data sets were transformed to the natural logarithms to normalize distribution and homogenize within group variances. Statistical comparisons were performed using ANOVA. Planned comparisons between specific treatments were performed using linear contrast (Moore and McCabe 1993) unless specified otherwise in the text.

Results

The activation of sGC by NO is independent of spike activity

Treatment of nerve cords with a phosphodiesterase (PDE) inhibitor and NO donors produced a significant increase in cGMP levels (Fig. 1a, one-way ANOVA, \(F_{3,19} = 18.9, p < 0.0001\)). Incubation with the cGMP-PDE selective inhibitor, zaprinast was sufficient to raise cGMP basal levels. Zaprinast incubation for 5 min followed by application of the NO donor, NOC-7 (0.1 mM) for 15 min evoked a four-fold increase in cGMP. Furthermore, treatment with zaprinast and NOC-7 in low-Na\(^+\) saline evoked an increase in cGMP that was significantly greater than zaprinast treatment alone. Inhibiting spiking did not have a significant effect on the NO-evoked response, which suggests this is a direct effect of NO on specific targets (see cGMP-IR below). Pre-treatment with the sGC inhibitor, ODQ blocked the NO-evoked increase in cGMP in non-spiking conditions.

Similarly, treatment with the cGMP-PDE inhibitor, sildenafil citrate (sildenafil; Pfizer, Inc.), NOC-7 and ODQ produced significantly different cGMP levels (Fig. 1b). Pre-treatment with 10 \(\mu\)M sildenafil increased baseline cGMP levels 15-fold, and was three-fold greater when treated with 0.1 mM NOC-7. Pre-incubating with ODQ for 30 min blocked the NO-evoked increase.

Nicotine evokes an increase in cGMP

To investigate the role of nAChRs in NO release we stimulated whole abdominal nerve cords with nicotinic
agonists in low-Na\(^+\) saline and measured cGMP levels as an indirect correlate of NO-production. When we treated isolated nerve cords with zaprinast, and cholinergic agonists or antagonists, there were significant differences in cGMP levels (Fig. 2a, one-way ANOVA, \(F_{1,37} = 9.1, p < 0.0001\)).

![Fig. 2](image)

**Fig. 2** Stimulation of nAChRs evokes an increase in cGMP that is dependent on NOS and sGC. Levels of cGMP (fmol/nerve cord [mean ± SEM]) were measured by enzyme immunoassay from Manduca abdominal nerve cords treated with or without nicotine (0.1 mM) in the presence of 0.5 mM zaprinast and 126 mM NMDG. (a) Baseline levels of cGMP in the presence of zaprinast and NMDG were unaffected by the nAChR antagonist, mecamylamine or the mAChR antagonist, scopolamine. Treatment with nicotine alone evoked a significant increase in cGMP levels \((F_{1,37} = 17.2, +p < 0.0001)\). The nicotine-evoked increase was significantly decreased with mecamylamine in a concentration-dependent manner \((\text{mecamylamine: 0.1 mM, } F_{1,37} = 4.3, +p = 0.044; 0.5 \text{ mM, } F_{1,37} = 35.0, **+p < 0.0001;\) and 1 mM, \(F_{1,37} = 33.6, ***+p < 0.0001)\). Scopolamine had no effect on the nicotine-evoked increase, which was significantly greater than scopolamine treatment alone \((F_{1,37} = 14.9, @+p = 0.001)\). (b) Baseline levels of cGMP were significantly decreased when treated with either the NOS inhibitor, \(\text{L-NNA (1 mM, } F_{1,45} = 12.7, *+p = 0.001)\) or the sGC inhibitor, ODQ \((10 \mu M, F_{1,45} = 6.2, \|+p = 0.016)\). As in Fig. 2a, 0.1 mM nicotine evoked a significant increase in cGMP \((F_{1,45} = 98.0, +p < 0.0001)\) that was significantly greater than treated with \(\text{L-NNA in a concentration-dependent manner (1 mM, } F_{1,45} = 12.4, *+p = 0.001;\) and 5 mM, \(F_{1,45} = 170.9, **+p < 0.0001)\). Pre-treatment with 10 \(\mu M\) ODQ significantly blocked the nicotine-evoked increase in cGMP \((F_{1,45} = 165.6, @+p < 0.0001)\). Nicotine treatment (0.1 mM) for 15 min in the presence of 0.5 mM zaprinast and low-Na\(^+\) evoked a significant increase in cGMP. The nAChR antagonist, mecamylamine significantly decreased nicotine stimulation and its effect was concentration-dependent. Nicotine treatment in the presence of the muscarinic antagonist, scopolamine \([0.1 \text{ mM, a dose sufficient to block muscarinic receptors in Manduca; see Trimmer 1994}]\), produced a significant increase in cGMP, which was not significantly different from nicotine stimulation alone.

Furthermore, treatment with nicotine, ODQ, and the NOS inhibitor, \(N^\alpha\text{-nitro-L-arginine (L-NNA)}\), produced significant differences in cGMP levels (Fig. 2b, one-way ANOVA, \(F_{6,45} = 59.5, p < 0.0001\)). Baseline levels of cGMP were significantly decreased when treated with either \(\text{L-NNA or ODQ}\). Consistent with Fig. 2a, nicotine evoked an increase in cGMP significantly greater than control. The increase evoked by nicotine was significantly inhibited in the presence of \(\text{L-NNA in a dose-dependent fashion, and was blocked by ODQ}\).

**Nicotine evoked cGMP-IR**

We have previously shown that when Manduca abdominal nerve cords are treated with NO donors, cGMP-IR is increased in segment-specific neurons (Zayas et al. 2000b). Additional neurons stain if the NO treatment is in the presence of cGMP-selective PDE inhibitors such as zaprinast (Zayas et al. 2000a). These same neurons are cGMP-IR in low-Na\(^+\) saline suggesting that this is a direct effect and not a result of increased spike frequency (Zayas et al. 2000a). Therefore, we decided to investigate if the nicotine-evoked increase in cGMP levels was confined to specific neurons and neuronal processes.

Whole nerve cords in low-Na\(^+\) saline (control) or treated with 0.5 mM zaprinast were incubated for 20 min or for 5 min before adding 0.1 mM nicotine to the bath for 15 min. Treatment was terminated by placing nerve cords in 4% paraformaldehyde and processing them for cGMP-IR as described in the methods. There was no consistent staining in control nerve cords (Fig. 3, control). Treatment with zaprinast alone increased cGMP-IR in specific neurons. A subset of stained neurons was identified based on their neuroanatomy, axon branching pattern and physiological properties as the dorsal cluster motoneurons, intersegmental motoneurons and motoneuron-12 (Fig. 3, zaprinast). These are motoneurons that control larval prolegs and abdominal muscles and have been well described previously (Weeks and Truman 1984; Levine and Truman 1985). Treatment with nicotine increased the staining of the neurons detected with zaprinast alone, and produced cGMP-IR in additional neurons and neuronal processes (Fig. 3, zaprinast and nicotine). The same staining pattern was observed in identical experiments substituting zaprinast with 0.1 mM sildenafil \((n = 3, \text{ data not shown})\).
Stimulation of nAChRs increases \( [\text{Ca}^{2+}]_i \)

Because the activity of NOS is \( \text{Ca}^{2+} \) dependent, we examined the role of nAChRs in \( \text{Ca}^{2+} \) signaling. Cultured neurons were loaded with FURA PE3 for 90 min and washed with saline. Changes in the 350/380 fluorescence ratio were used to monitor \( [\text{Ca}^{2+}]_i \) changes in randomly chosen neurons (141 of 300 neurons responded to nicotinic stimulation). Bath application of acetylcholine (ACh) caused a transient increase in \( [\text{Ca}^{2+}]_i \) (Fig. 4a). The evoked increase was dose-dependent and significantly reduced when treated with mecamylamine (Figs 4a and b; \( p < 0.0001 \), Tukey–Kramer multiple comparisons test). Treatment with carbachol in the presence of the muscarinic antagonist, scopolamine, epibatidine, or nicotine also caused a transient increase in \( [\text{Ca}^{2+}]_i \) (data not shown). In addition, brief pulses of nicotine from a micropipette onto single neurons resulted in rapid transient increases in \( [\text{Ca}^{2+}]_i \) that persisted when neurons were treated with tetrodotoxin (data not shown; \( p < 0.0001 \), paired Student’s \( t \)-test, \( n = 27 \) responses including tetrodotoxin-treated neurons). To confirm that changes in \( [\text{Ca}^{2+}]_i \) evoked by cholinergic agents were directly mediated by a nicotinic-like AChR we performed experiments in the presence of AChR antagonists. \( [\text{Ca}^{2+}]_i \) changes evoked by 100 \( \mu \text{M} \) ACh were reduced in the presence of 10 \( \mu \text{M} \) mecamylamine but not 10 \( \mu \text{M} \) scopolamine (Fig. 4c; \( p < 0.001 \), Student’s \( t \)-test). Nicotine-evoked (0.5 mM) changes in \( [\text{Ca}^{2+}]_i \) were also significantly reduced in the presence of 10 \( \mu \text{M} \) mecamylamine (Fig. 4d; \( p < 0.001 \), Student’s \( t \)-test). These experiments demonstrate that nAChR stimulation is sufficient to increase \( [\text{Ca}^{2+}]_i \) in isolated Manduca neurons.

We further investigated nicotinic-evoked \( [\text{Ca}^{2+}]_i \), responses \textit{in situ} to substantiate our observations in isolated neurons. Individual neuronal cell bodies on the dorsal surface of abdominal ganglia 3 or 4 were impaled with beveled glass electrodes backfilled with FURA-2. Neurons were randomly chosen from a cluster of intersegmental motoneurons that project posteriorly to the contralateral neuropil, and stimulated with a puffer electrode backfilled with 1 mM nicotine placed adjacent to the neuropil. Once intracellular recordings and dye injections were successfully achieved, we tested the responsiveness of neurons in normal saline. Nicotine application (0.5–5 s) resulted in membrane depolarization (mean \( \pm \text{SEM} \) membrane potential changed from \( -43.2 \pm 6.0 \) to \( 26.0 \pm 2.3 \) mV; \( n = 3 \)) and a transient increase in \( [\text{Ca}^{2+}]_i \) (Fig. 4e; control). To examine the effect of nAChR stimulation on \( [\text{Ca}^{2+}]_i \), independent of action potentials, low-\( \text{Na}^+ \) saline was perfused 5–10 min until excitatory activity was

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eliminated and current injection could not evoke action potentials. In eight of 12 neurons tested, nicotine evoked an increase in $[\text{Ca}^{2+}]_i$ in low Na$^+$ (Fig. 4e). The voltage response was inconsistent but in three of the stimulated neurons, nicotine evoked a two-fold increase in $[\text{Ca}^{2+}]_i$ (mean ratio changed from 2.77 ± 1.15 to 7.06 ± 3.81, $n = 6$ individual responses from three neurons) with a small change in voltage ($-46.0 \pm 3.8 \text{ mV}$ to $-42.3 \pm 3.8 \text{ mV}$, $n = 6$). Typically, injecting enough current to depolarize the cell 5 mV did not result in $[\text{Ca}^{2+}]_i$ increases. Thus, the observed increase in $[\text{Ca}^{2+}]_i$ is likely to be independent of the voltage and possibly gated through the nAChR receptor itself.

To identify the source of $\text{Ca}^{2+}$ in the cholinergic responses we blocked voltage-gated $\text{Ca}^{2+}$ channels (VGCC) using divalent cations as described in Hayashi and Levine (1992). The increase in $[\text{Ca}^{2+}]_i$, evoked by ACh was reduced in the presence of 5 mM NiCl$_2$ (Ni$^{2+}$ Fig. 5a; $p < 0.001$, Tukey–Kramer multiple comparisons test). Despite the large reduction of $[\text{Ca}^{2+}]_i$ transients in Ni$^{2+}$, a substantial response always persisted (14% of initial response) even in the
Fig. 4 Nicotinic AChR-evoked increases in [Ca\(^{2+}\)]. (a) Treatment of isolated neurons with acetylcholine (ACh; gray bars) evoked a transient increase in [Ca\(^{2+}\)] that was dose-dependent (\(\mu M\): 5, 10 and 100). ACh (100 \(\mu M\)) treatment in the presence of the nAChR antagonist, mecamylamine (100 \(\mu M\); black bar) did not evoke the rapid elevation in [Ca\(^{2+}\)] observed when treated with ACh alone. Trace is representative of experiments summarized in (b). (b) Treatment of neurons with ACh (Ni\(^{2+}\), forward hatched bar). Analysis of the mean change in ratio for all responses (bottom) showed that treatment with ACh in the presence of 100 \(\mu M\) mecamylamine (*\(p < 0.001\), Tukey–Kramer multiple comparisons test). (c) Treatment with 100 \(\mu M\) nicotine (gray bars) evoked a rapid increase in [Ca\(^{2+}\)] that was reduced in the presence of 5 \(\mu M\) NiCl\(_2\) (Ni\(^{2+}\)). Under these treatment conditions, mecamylamine (*\(p < 0.001\), Tukey–Kramer multiple comparisons test). (d) Treatment with 0.5 \(\mu M\) nicotine induced changes in [Ca\(^{2+}\)] that were also reduced in the presence of 10 \(\mu M\) mecamylamine. The mean change in ratio for all responding neurons was significantly decreased with mecamylamine (*\(p < 0.001\), paired \(t\)-test). (e) Stimulation of nAChRs by pressure application of nicotine (gray bar) evoked a transient increase in [Ca\(^{2+}\)] that was dose-dependent (\(\mu M\)): 5, 10 and 100). After saline wash, treatment of the same neurons with ACh in the presence of 10 \(\mu M\) scopolamine evoked an [Ca\(^{2+}\)] increase. Analysis of the mean change in ratio for all responding neurons showed the mecamylamine block was significant (*\(p < 0.001\), paired \(t\)-test). (d) Treatment with 0.5 \(\mu M\) nicotine induced changes in [Ca\(^{2+}\)] that were also reduced in the presence of 10 \(\mu M\) mecamylamine. The mean change in ratio for all responding neurons was significantly decreased with mecamylamine (*\(p < 0.001\), Tukey–Kramer multiple comparisons test). Calibration bar = 200 s.

Fig. 5 The nAChR-evoked increase in [Ca\(^{2+}\)] is partially blocked by Ni\(^{2+}\). (a) Treatment with 100 \(\mu M\) ACh (gray bars) evoked a rapid increase in [Ca\(^{2+}\)] that was reduced in the presence of 5 \(\mu M\) NiCl\(_2\) (Ni\(^{2+}\), forward hatched bar). Analysis of the mean change in ratio for all responses (bottom) showed that treatment with Ni\(^{2+}\) significantly reduced but did not abolish the ACh-evoked Ca\(^{2+}\) responses (*\(p < 0.001\), Tukey–Kramer multiple comparisons test). Calibration bar = 200 s. (b) Similarly, treatment with 100 \(\mu M\) ACh evoked an increase in [Ca\(^{2+}\)] that persisted in the presence of the muscarinic antagonist, scopolamine (10 \(\mu M\)). Under these treatment conditions, Ni\(^{2+}\) also reduced the [Ca\(^{2+}\)] response (*\(p < 0.0001\), Tukey–Kramer multiple comparisons test).

Discussion

Previously we demonstrated that the NO/cGMP-pathway is coupled to the cholinergic system via muscarinic receptors (Trimmer and Qazi 1996). In this study, we have shown that ACh also stimulates cGMP in the CNS of Manduca via activation of nAChRs and NOS. NO directly evokes cGMP...
The increase in \([\text{Ca}^{2+}]\) is unlikely to be due to muscarinic receptors because it is blocked by nicotinic and not muscarinic antagonists (Fig. 4). Furthermore, separate experiments from our laboratory showed that responses to muscarinic receptors were stereotypically distinct and long-lasting (Trimmer and Qazi 1999). Nonetheless, scopolamine treatment had a significant effect on the ACh-evoked response on experiments presented in this paper (Fig. 5b; \(p < 0.05\), Tukey–Kramer multiple comparisons test). These data fit with our observations that muscarinic stimulation can also decrease \([\text{Ca}^{2+}]\), of Manduca neurons in culture (unpublished results). We are currently investigating in detail the effect and mechanism of muscarinic stimulation on \([\text{Ca}^{2+}]\). The nicotinic evoked change in \([\text{Ca}^{2+}]\) is mostly blocked by \(\text{Ni}^{2+}\) (Fig. 5), which suggests that it is mediated by VGCCs. \(\text{Ni}^{2+}\) never blocked the entire response even at a concentration sufficient to block all \(\text{Ca}^{2+}\) currents (Hayashi and Levine 1992), but the remaining \(\text{Ca}^{2+}\) response was prevented when the external \(\text{Ca}^{2+}\) was lowered to 0.1 mM and stimulated with a greater ACh dose (data not shown). This suggests that the nicotinic-evoked elevation in \([\text{Ca}^{2+}]\) is dependent on extracellular \(\text{Ca}^{2+}\). We did not test other
organic blockers because there is evidence that at least Manduca motoneurons are insensitive to compounds such as conotoxin and dihydropyridine (Hayashi and Levine 1992). However, our results are consistent with experiments in chick ciliary ganglion neurons where stimulation of nAChRs induces a rapid elevation in \([Ca^{2+}]_i\), that is completely dependent on extracellular \(Ca^{2+}\) (Rathouz et al. 1995).

Studies on ACh stimulated whole cell currents in honeybee Kenyon cells suggest that some insect receptors are quite permeable to \(Ca^{2+}\) (Goldberg et al. 1999). Bicker (1996) had previously shown that transmitter-induced changes in cytoplasmic \([Ca^{2+}]_i\), during nerve depolarization are correlated to NO production in locust Kenyon cells. Here we show that nicotinic stimulation evokes an increase in \([Ca^{2+}]_i\), independent of fast changes in voltage, and that NO-dependent and nicotine evoked cGMP production do not require depolarization or VGCCs. In addition, when \(Ca^{2+}\) was omitted from the saline nicotine failed to evoke an increase in cGMP (data not shown). Thus, we have hypothesized that nicotinic-evoked changes in \([Ca^{2+}]_i\), are directly gated by nAChRs in Manduca. There is evidence from other insect systems that cholinergic signaling increases \([Ca^{2+}]_i\), independent of voltage changes. In neurons dissociated from the locust thoracic ganglia, carbachol stimulates an increase in \([Ca^{2+}]_i\), when the membrane potential is voltage clamped (Oertner et al. 1999). In blowfly lobula plate tangential cells, carbachol stimulates \([Ca^{2+}]_i\), signals that are voltage-independent and are thought to be mediated by \(Ca^{2+}\) entry through nAChRs (Oertner et al. 2001). In addition, the Manduca \(\alpha\)-subunit, MARA1 can be down-regulated using double-stranded RNA interference. This treatment abolishes most \(Ca^{2+}\) responses and attenuates the small number of \(Ca^{2+}\) responses that remain (Vermehren et al. 2001). Because the normal nicotinic cGMP response in whole nerve cords is unaffected by Ni\(^{2+}\) and low-Na\(^+\) saline, this small \(Ca^{2+}\) response must be capable of eliciting NO production (Fig. 6). This implies that MsNOS is coupled to nAChRs. However, MsNOS does not have the amino terminal PDZ domain found in other nNOS isoforms (Nighorn et al. 1998); hence any association with nAChRs must be mediated by unknown mechanisms. It would be interesting to study the association of nAChRs with MsNOS as a parallel to the coupling of vertebrate NMDA receptors to nNOS (Brennan and Bredd 1997).

Nicotinic AChRs are implicated in many brain functions but their precise cellular role in the CNS is unclear. Although there are examples that AChRs can mediate postsynaptic mechanisms (for review see Jones et al. 1999) nAChRs are primarily thought to act presynaptically and to have a modulatory role. There are examples of presynaptic nAChRs in insects (Blagburn and Sattelle 1987a,b) and it is possible that the nicotine-evoked cGMP production is a consequence of ACh acting presynaptically on NO-producing neurons. Our finding that a nAChR activates the NO/cGMP-pathway has important implications for the functional role of NO-signaling and suggests a biochemical contribution by nAChRs to signal integration in the CNS.

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