Nerve Terminal Nicotinic Cholinergic Receptors on Excitatory Motoneurons in the Myenteric Plexus of Guinea Pig Intestine

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) localized to excitatory longitudinal muscle motoneurons were studied in segments of guinea pig ileum maintained in vitro. Longitudinal muscle contractions caused by the nAChRs agonists, dimethylphenylpiperazinium (DMPP), nicotine, and cytisine were measured using isometric strain gauge transducers. In normal Krebs’ solution, the nAChR agonists caused concentration-dependent biphasic contractions with a rank order potency of DMPP > cytisine = nicotine. Contractions caused by DMPP and nicotine were inhibited more than 80% by tetrodotoxin (TTX, 0.3 μM). Responses caused by DMPP were inhibited in a concentration-dependent manner by the competitive nAChR antagonist dihydro-β-erythroidine (pA2 = 5.4). In the presence of scopolamine (1 μM) to block muscarinic cholinergic receptors, the nAChR agonists caused longitudinal muscle contractions that were monophasic and smaller in amplitude than those present in the absence of scopolamine. With scopolamine present, the agonist rank order potency was nicotine = DMPP > cytisine. Contractions caused by DMPP and nicotine (each at 100 μM) were reduced by TTX by only 52 ± 7 and 59 ± 6%, respectively. Noncholinergic contractions caused by DMPP and nicotine were blocked by the neurokinin-1 receptor antagonist, CP 96,345-1 (0.3 μM). Dihydro-β-erythroidine also inhibited noncholinergic contractions caused by DMPP with a pA2 value of 5.4. It is concluded that nAChRs are localized to the somatodendritic region of excitatory longitudinal muscle motoneurons. There are also nAChRs localized to the nerve terminals of these neurons where agonists can cause noncholinergic contractions via a TTX-insensitive mechanism.

Acetylcholine (ACh) acting at nicotinic cholinergic receptors (nAChRs) is the principal excitatory neurotransmitter in the myenteric plexus. Intracellular electrophysiological studies have shown that fast excitatory postsynaptic potentials recorded from myenteric neurons are at least partly inhibited by nAChR antagonists such as hexamethonium (Nishi and North, 1973; Hirst et al., 1974; Galligan and Bertrand, 1994). Excitatory and inhibitory intestinal motor reflexes caused by distention of the gut wall or mucosal stimulation are also inhibited by hexamethonium (Nishi and North, 1973; Hirst et al., 1974; Galligan and Bertrand, 1994). Finally, studies in vivo have shown gastrointestinal motility is inhibited by nAChR antagonists (Sarna et al., 1981; Al-Saffar, 1984; Galligan et al., 1986). These observations highlight the central role of nAChRs in the control of gastrointestinal motor function.

Data from molecular biological and functional studies indicate that there are multiple subtypes of neuronal nAChRs (Sargent, 1993; Boyd, 1997). These receptor subtypes are composed of different combinations of α and β subunits, and specific subunit composition gives these receptors unique pharmacological and functional properties (Colquhoun and Patrick, 1997). However, little is known about the molecular composition, pharmacological properties, or subcellular distribution of enteric nAChRs. Recent, immunohistochemical studies using antibodies that recognize α3, α5, and β4 subunits (MAb-35) or the α7 subunit have localized nAChR immunoreactivity to nerve cell bodies, dendrites and nerve terminals in enteric ganglia (Kirchgesnner and Liu, 1998). The traditional model of enteric ganglionic neurotransmission has the nAChR localized to the somatodendritic region of the neuron where it is in a position to mediate fast excitatory neurotransmission (Töröcsik et al., 1991). The localization of nAChRs on the somatodendritic region of enteric neurons is consistent with this model (Kirchgesnner and Liu, 1998). However, it has also been established that nAChRs can be localized on nerve endings where these receptors function to cause release of neurotransmitter (McGehee et al., 1995; Gray et al., 1996; Wonnacott, 1997). Agonists acting at these receptors cause transmitter release using a calcium-dependent but action potential-independent mechanism as agonist induced transmitter release from preparations containing...

ABBREVIATIONS: ACh, acetylcholine; nAChR, nicotinic cholinergic receptor; DHβE, dihydro-β-erythroidine; DMPP, dimethylphenylpiperazinium; NKA, neurokinin A; NK-1, neurokinin-1; SP, substance P; TTX, tetrodotoxin.
Materials and Methods

Tissue Preparation. All animal use protocols were approved by the All University Committee on Animal Use and Care at Michigan State University. Male guinea pigs (300–450 g) were anesthetized via halothane inhalation and then were stunned and the carotid arteries were severed. A segment of distal ileum was removed from the animal and placed in Krebs’ solution of the following composition: NaCl, 117 mM; KCl, 4.7 mM; CaCl₂, 2.5 mM; MgCl₂, 1.2 mM; NaH₂PO₄, 1.2 mM; NaHCO₃, 25 mM; and glucose, 11 mM. The Krebs’ solution was oxygenated continuously with 95% O₂, 5% CO₂. Whole segments of ileum cut to 3 cm in length were mounted in Plexiglas tissue holders and the tissue and holder were placed in jacketed baths (20-ml volume). One end of the segment was attached to a screw at the base of the holder using 4-0 silk suture whereas the other end was attached to a force transducer (FT03C; Grass Instruments, Quincy, MA) also using 4-0 silk suture. Tissues were placed under 10 mN of tension and were allowed to equilibrate for 30 min before starting experimental protocols.

Agonist-Concentration Response Curves. The nAChR agonists, cytisine, nicotine, and dimethylphenylpiperazinium iodide (DMPP) were added to the baths in volumes of 20 to 60 μl. Concentration-response curves were obtained by measuring the peak contraction caused by each concentration of agonist. Successive agonist concentrations were applied at 20-min intervals and each concentration was applied for 1 min. The order in which concentrations of agonist were applied was randomized among tissues.

Antagonist Studies. In all tissues, a control concentration-response curve for an agonist was obtained before testing the effects of an antagonist. Increasing concentrations (10, 30, and 100 μM) of the competitive nAChR antagonist, dihydro-β-erythroidine (DHβE), was incubated with the tissues for 20 to 30 min before obtaining a subsequent agonist concentration-response curve. A control curve and curves in the presence of increasing concentrations of antagonist were obtained in each tissue. The DHβE Kᵦ value was determined using the method of Arunlakshana and Schild (1959). Studies of noncholinergic contractions caused by nAChR agonists were accomplished by adding 1 μM scopolamine to the Krebs’ solution. The neurokinin-1 (NK-1) receptor antagonist CP 96,345–1 (Snider et al., 1991) was used to demonstrate that the noncholinergic contractions were mediated by a tachykinin peptide. Concentration-response curves for nAChR agonists were obtained first in the presence of scopolamine and a second agonist concentration-response curve was obtained in the presence of scopolamine and 0.3 μM CP 96345–1. CP 96345–1 was incubated with the tissues for 20 min before agonist addition.

Statistics. Agonist concentration-response curves from individual preparations were fit using the logistic function:

\[ Y = \left( Y_{\text{min}} - Y_{\text{max}} \right) \left( 1 + \left( \frac{1}{\text{EC}_{50}} \right)^m \right)^{-1} + Y_{\text{max}} \]

where “\( Y_{\text{min}} \)” and “\( Y_{\text{max}} \)” are minimum and maximum responses, respectively, “\( \text{EC}_{50} \)” is the half-maximal effective concentration, and “\( m \)” is the slope factor. Agonist mean \( \text{EC}_{50} \) values and 95% confidence intervals were determined from individual fits. In studies in which complete concentration-response curves were not obtained, agonist response amplitudes in the absence and presence of an antagonist were compared using the univariate procedure from SAS/STAT version 6.12 (SAS Institute Inc., Cary, NC) to test for normality and for subsequent comparisons using the sign test. Antagonist \( K_{\text{B}} \) values were determined from Schild plots. Schild regressions were constructed from plots of the ratio of individual agonist \( \text{EC}_{50} \) values obtained in the absence and presence of antagonist. When the slope of the regression did not differ from unity, it was constrained to a value of -1 and the \( K_{\text{B}} \) was determined from the x-intercept (pA₂ value). Data are mean values ± S.E. “\( N \)” values refer to the number of tissues from which data were obtained.

Results

Agonist Concentration-Response Curves. Nicotine, DMPP, and cytisine caused concentration-dependent contractions of whole ileal preparations. The contractions were biphasic with an initial, rapidly developing peak contraction followed by a slower developing but longer lasting secondary contraction. Nicotine caused the most prominent secondary contraction (Fig. 1). When the peak contraction was measured, the agonist rank order potency was DMPP > cytisine = nicotine (Fig. 2, Table 1).

To verify that the peak contraction caused by the nAChR agonists was due to stimulation of myenteric neurons, the effect of TTX (0.3 μM) on responses caused by DMPP and nicotine was tested. In eight preparations from four animals, TTX completely blocked the contraction caused by 3 μM DMPP and reduced contractions caused by 10 and 30 μM DMPP by 86 ± 3 and 87 ± 8%, respectively (Fig. 3). TTX also completely blocked contractions caused by 10 μM nicotine and reduced contractions caused by 30 and 100 μM nicotine by 81 ± 6 and 74 ± 5% respectively (Fig. 3).

As DMPP was the most potent agonist tested here, the competitive nAChR antagonist DHβE was used in an attempt to block contractions caused by this agonist. DHβE (10, 30, and 100 μM) caused concentration-dependent rightward shifts in the DMPP concentration-response curve (Fig.

Fig. 1. Representative traces of longitudinal muscle contractions caused by increasing concentrations of nAChR agonists. Each agonist elicited a biphasic response. A, contractions caused by DMPP at the indicated μM concentrations. B, contractions caused by nicotine at the indicated μM concentrations. C, contractions caused by cytisine at the indicated μM concentration. Scale bars are approximately 10 mN and 1 min.
Schild regression of the data obtained from these rightward shifts yielded a $pA_2$ value of 5.4 ± 0.1 and a $K_B$ value of 3.8 μM (Fig. 4B).

To study excitation of noncholinergic excitatory motoneurons by nAChR agonists, 1 μM scopolamine was added to the Krebs' solution for all of these studies. DMPP, nicotine, and cytisine caused concentration-dependent contractions of the longitudinal muscle (Fig. 5). The maximum contractions caused by each agonist in the presence of scopolamine were less than half the amplitude caused by the same agonist concentrations in normal Krebs' solution (Fig. 6; compare with Fig. 2). DMPP and cytisine were approximately 8- and 2.5-fold less potent in causing noncholinergic contractions compared with contractions studied in normal Krebs' solution (Table 1). Nicotine was equipotent in eliciting contractions in normal Krebs' solution and in scopolamine-containing Krebs' solution (Table 1). The agonist rank order potency for noncholinergic contractions was DMPP > nicotine > cytisine (Fig. 6, Table 1). Because the peak amplitude of the contractions caused by cytisine was relatively small (see Fig. 6), characterization of responses caused by cytisine was not attempted.

To verify that contractions caused by nicotine and DMPP were neurally mediated, the effects of these antagonists were tested in the presence of the combined application of 1 μM scopolamine and 0.3 μM TTX. It was found that contractions caused by 10 μM nicotine and DMPP completely blocked by TTX (Fig. 7). However, TTX inhibited contractions caused by 100 μM DMPP and nicotine by less than 52 ± 7% ($n = 16$).

### TABLE 1

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Normal Solution</th>
<th>Scopolamine-Containing Solution</th>
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<tbody>
<tr>
<td>DMPP</td>
<td>2.5 (2–3)</td>
<td>21 (16–26)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>27 (16–39)</td>
<td>21 (10–32)</td>
</tr>
<tr>
<td>Cytisine</td>
<td>26 (15–37)</td>
<td>68 (45–91)</td>
</tr>
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**Noncholinergic Contractions.** To study excitation of noncholinergic excitatory motoneurons by nAChR agonists, 1 μM scopolamine was added to the Krebs' solution for all of these studies. DMPP, nicotine, and cytisine caused concentration-dependent contractions of the longitudinal muscle (Fig. 5). The maximum contractions caused by each agonist in the presence of scopolamine were less than half the amplitude caused by the same agonist concentrations in normal Krebs' solution (Fig. 6; compare with Fig. 2). DMPP and cytisine were approximately 8- and 2.5-fold less potent in causing noncholinergic contractions compared with contractions studied in normal Krebs' solution (Table 1). Nicotine was equipotent in eliciting contractions in normal Krebs' solution and in scopolamine-containing Krebs' solution (Table 1). The agonist rank order potency for noncholinergic contractions was DMPP = nicotine > cytisine (Fig. 6, Table 1). Because the peak amplitude of the contractions caused by cytisine was relatively small (see Fig. 6), characterization of responses caused by cytisine was not attempted.

To verify that contractions caused by nicotine and DMPP were neurally mediated, the effects of these antagonists were tested in the presence of the combined application of 1 μM scopolamine and 0.3 μM TTX. It was found that contractions caused by 10 μM nicotine and DMPP completely blocked by TTX (Fig. 7). However, TTX inhibited contractions caused by 100 μM DMPP and nicotine by less than 52 ± 7% ($n = 16$).
tissues from 5 animals) and 58 ± 5% (n = 18 tissues from 5 animals), respectively. Noncholinergic contractions caused by 300 μM nicotine were reduced by only 33 ± 5% (n = 14 tissues from 5 animals) by TTX (Fig. 7).

Because DMPP was the most potent agonist in causing noncholinergic contractions, the effects of DHβE on responses caused by DMPP were studied. Increasing concentrations (10, 30, 100 μM) of DHβE produced successive rightward shifts in the DMPP concentration-response curve (Fig. 8A). Schild regression of these rightward shifts yielded a DHβE Kᵦ value of 3.8 μM (pA₂ = 5.4; Fig. 8B).

Substance P (SP) and/or neurokinin A (NKA) can act at NK-1 receptors to cause noncholinergic contractions of the intestine. Therefore, the NK-1 receptor antagonist, CP 96,345–1 (0.3 μM) was used to block NK-1 receptors. Noncholinergic contractions caused by DMPP and nicotine were inhibited by the NK-1 receptor antagonist (Fig. 9).

**Discussion**

Synaptic interactions between myenteric nerves that result in normal contractions and relaxations of gastrointestinal smooth muscle are complex and involve multiple transmitters and synaptic mechanisms (Kunze and Furness, 1999). Most studies of these synaptic events have focused on postsynaptic mechanisms. However, data from the present study indicate that presynaptic receptors may be an additional site at which neurotransmitters can modulate the activity of other neurons. The data presented here indicate that there are presynaptic nAChRs on nerve terminals that release tachykinins to cause contractions of longitudinal smooth muscle.
Fig. 8. DHβE competitively inhibits noncholinergic contractions caused by DMPP. A, DMPP concentration-response curves in the absence (○) and presence of the indicated concentrations (●, 10 μM; ▲, 30 μM; ▼, 100 μM) of DHβE. Increasing concentrations of DHβE cause successive rightward shifts in the DMPP concentration response curves. Data are mean ± S.E. and were obtained from six preparations from two animals; 1 μM scopolamine was present throughout. B, Schild regression of the rightward shifts in the DMPP concentration-response curves caused by DHβE shown in A. Points are individual log dose ratio-1 values from each preparation. Solid line is the best fit (slope constrained to −1) and the dotted lines are the 95% confidence limits of the fit. The pA2 value was 5.4.

Presynaptic nAChRs. In normal Krebs’ solution, nicotine, DMPP, and cytisine caused longitudinal muscle contractions that were nerve-mediated as they were blocked by TTX. These data are consistent with established enteric circuitry in which motoneurons in the gastrointestinal tract express somal nAChRs (Bornstein et al., 1994). The contraction caused by each nAChR agonist was also due largely to excitation of cholinergic motoneurons as agonist-induced contractions were inhibited by the muscarinic cholinergic receptor antagonist scopolamine. However, each nAChR agonist induced a residual contraction that persisted in the presence of scopolamine. The noncholinergic contractions are due to release of the tachykinin peptides, SP and NKA (Grider, 1989; Holzer, 1989; Yunker and Galligan, 1996). This observation was confirmed in the present study when it was shown that the noncholinergic contractions caused by nicotine and DMPP were blocked by the NK-1 receptor antagonist CP 96,345–1. However, it was also shown that contractions caused by nicotine and DMPP were incompletely inhibited by TTX. It is unlikely that the concentration of TTX used was insufficient to block nerve-mediated responses as electrically-evoked contractions of the nerve muscle preparations of guinea pig ileum are completely blocked by the concentration of TTX (0.3 μM) used here (see Yunker and Galligan, 1996 for example). Therefore, DMPP and nicotine were acting via a TTX-resistant mechanism to cause noncholinergic contractions.

In the peripheral and central nervous systems, nAChRs are localized to nerve terminals or near the nerve terminal (Wonnacott, 1997). Agonists acting at these receptors cause transmitter release (McGehee et al., 1995; Gray et al., 1996; MacDermott et al., 1999). The actions of nAChR agonists have been concluded to be at the nerve terminal as transmitter release caused by these agonists is resistant to TTX. The data from the present study indicate that presynaptic nAChRs may be localized to nerve terminals releasing SP/NKA onto the longitudinal muscle in guinea pig ileum. It is important to note that the excitatory motoneurons innervating the longitudinal muscle layer contain both choline acetyltransferase and SP/NKA (Costa et al., 1996; Kunze and Furrness, 1999). This suggests that ACh and SP/NKA are released from the same nerve endings and implies that presynaptic nAChRs may function as facilitatory autoreceptors responding to ACh released from the same nerve terminals (MacDermott et al., 1999). Peptides are contained in large dense core vesicles that are generally localized away from the active zone regions of the synapse (Edwards, 1998). Neuropeptides are released at extrasynaptic regions where they modulate neurotransmission. Immunohistochemical studies using antibodies raised against nAChRs have demonstrated that nAChR-immunoreactivity is present in nerve terminals in enteric ganglia; this is consistent with a presynaptic localization of some nAChRs in the gut (Kirchgessner and Liu, 1998). Presynaptic nAChRs localized away from the active zone region near the dense core vesicles may be a mechanism by which peptide release could be enhanced from nerve terminals during periods of high frequency stimulation when...
here indicate that there are also nAChRs localized to the nerve endings of the longitudinal muscle motoneurons. To determine whether the somatodendritic and nerve terminal receptors were similar, the competitive nAChR antagonist DHβE was used to inhibit these responses. Schild regression of the parallel shifts in DMPP concentration-response curves yielded similar pA$_2$ values for both the total and noncholinergic contractions caused by DMPP. These data indicate that nerve terminal and somatodendritic nAChRs are the same or if they are different, DHβE does not discriminate between these receptors.

**Conclusions.** In guinea pig ileum myenteric plexus, excitatory longitudinal muscle motoneurons express nAChRs on the somatodendritic region and on nerve terminals. Nerve terminal nAChRs function to stimulate release of SP/NKA as mediators of noncholinergic contractions of the longitudinal muscle layer (Fig. 10). The nerve terminal nAChRs may act as facilitatory autoreceptors responding to ACh coreleased from the same nerves releasing SP/NKA.

**References**


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