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 nAChR 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 M). 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 recorded 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 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 (Kirchgessner 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 (Kirchgessner 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.
intact nerves is resistant to tetrodotoxin (TTX) or nAChR agonists can release transmitter from synaptosomal preparations (Wonnacott, 1997). These data may be important for understanding the actions of nAChR agonists on the gastrointestinal tract as some contractile responses caused by these drugs are partly resistant to TTX (Romano, 1981; Maggi et al., 1985; Börjesson et al., 1997) and nicotine can release transmitter from myenteric synaptosomes (White, 1982).

The purpose of the present study was to examine the actions of nAChR agonists on longitudinal muscle contractions of guinea pig ileum. These experiments were designed to determine whether nAChRs were localized exclusively to the somatodendritic region or to nerve terminals as well.

**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; CaCl$_2$, 2.5 mM; MgCl$_2$, 1.2 mM; NaH$_2$PO$_4$, 1.2 mM; NaHCO$_3$, 25 mM; and glucose, 11 mM. The Krebs’ solution was oxygenated continuously with 95% O$_2$, 5% CO$_2$. 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 $K_B$ 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:

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

where “$Y_{\text{min}}$” and “$Y_{\text{max}}$” are minimum and maximum responses, respectively, “EC$_{50}$” is the half-maximal effective concentration, and “$m$” is the slope factor. Agonist mean 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 $K_B$ values were determined from Schild plots. Schild regressions were constructed from plots of the ratio of individual agonist 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 $K_B$ was determined from the x-intercept (pA$_2$ 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.
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).

**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% ($n = 16$).

**TABLE 1**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Normal Solution</th>
<th>Scopolamine-Containing Solution</th>
</tr>
</thead>
<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>
</tbody>
</table>

4A). 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).
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 Kᵦᵦ 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.
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.
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 pA₂ 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.

Fig. 10. nAChRs localized to the somatodendritic and nerve terminal regions of excitatory motoneurons mediate noncholinergic contractions of the longitudinal muscle layer. A. DMPP, nicotine, and cysteine act on somatodendritic and nerve terminal nAChRs to cause release of ACh and SP/NKA. As scopolamine was present, ACh can not activate muscarinic receptors (M) but SP/NKA can activate NK-1 receptors localized to the longitudinal smooth muscle. B, in the presence of TTX, DMPP, nicotine, and cysteine can activate somatodendritic nAChRs, but action potentials initiated in the somatodendritic region can not propagate to the nerve terminal. DMPP and nicotine cause longitudinal muscle contractions by activating nerve terminal nAChRs to cause release of ACh and SP/NKA. As ACh and SP/NKA are colocalized in longitudinal muscle motoneurons, nerve terminal nAChRs may function as autoreceptors for ACh released from these nerve endings (?).

Concentrations of ACh near the neuroeffector junction would be high.

Pharmacological Properties of Myenteric Nicotinic Receptors. The subunit composition of neuronal nAChRs determines the functional and pharmacological properties of the receptor (Colquhoun and Patrick, 1997). In normal Krebs’ solution or in scopolamine-containing solutions, cysteine was either equipotent with or less potent than nicotine or DMPP in causing longitudinal muscle contractions. For receptors containing β4 subunits, cysteine is the most potent agonist (Luetje and Patrick, 1994; Papke and Heinemann, 1994) so it is unlikely that the nAChR(s) involved in the responses studied here contained β4 subunits. Immunohistochemical studies of myenteric neurons using an antibody that recognizes the α3, α5, and β4 subunits (MAb-35; Vernallis et al., 1993) showed immunoreactivity in neurons that also contain calretinin, a marker for excitatory longitudinal muscle motoneurons (Brookes et al., 1992; Kirchgessner and Liu, 1998). Data from the present pharmacological studies suggest that nAChRs containing β4 subunits are not expressed by excitatory longitudinal muscle motoneurons and that MAB-35 immunoreactivity indicates the expression of either (α3, α5, or both of these subunits in excitatory longitudinal muscle motoneurons.

Motoneurons in the myenteric plexus are S type neurons (Bornstein et al., 1994). One property of S neurons is that they receive fast nicotinic excitatory input mediated via nAChRs located on the somatodendritic regions of the neuron (Nishi and North, 1973; Hirst et al., 1974; Galligan and Bertrand, 1994). The nAChR agonists used here caused both cholinergic and noncholinergic contractions in part by activating these somatodendritic receptors. The data presented

References


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