Presynaptic nicotinic acetylcholine receptors in the myenteric plexus of guinea pig intestine

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Schneider, David A., and James J. Galligan. Presynaptic nicotinic acetylcholine receptors in the myenteric plexus of guinea pig intestine. Am J Physiol Gastrointest Liver Physiol 279: G528–G535, 2000.—Presynaptic nicotinic acetylcholine receptors (nAChRs) were studied in myenteric plexus preparations from guinea pig ileum using intracellular electrophysiological methods. Microapplication of nicotine (1 mM) caused a biphasic depolarization in all AH neurons (n = 30) and in 36 of 49 S neurons. Cytisine (1 mM) caused fast depolarizations in S neurons and no response in AH neurons. Mecamylamine (10 μM) blocked all responses caused by nicotine and cytisine. TTX (0.3 μM) blocked slow excitatory synaptic potentials in S and AH neurons but had no effect on fast depolarizations caused by nicotine. Nicotine-induced slow depolarizations were reduced by TTX in two of twelve AH neurons (79% inhibition) and four of nine S neurons (90 ± 12% inhibition). Slow nicotine-induced depolarizations in the remaining neurons were TTX resistant. TTX-resistant slow depolarizations were inhibited after neurokinin receptor 3 desensitization caused by senktide (0.1 μM); senktide desensitization inhibited the slow nicotine-induced depolarization by 81 ± 5% and 63 ± 15% in AH and S neurons, respectively. A low-calcium and high-magnesium solution blocked nicotine-induced slow depolarizations in AH neurons. In conclusion, presynaptic nAChRs mediate the release of substance P and/or neurokinin A to cause slow depolarizations of myenteric neurons.

Presynaptic receptors; neurokinin receptor 3; myenteric neurons

PRESYNAPTIC REGULATION of neurotransmitter release is an important mechanism governing synaptic transmission in the central and peripheral nervous systems (28). In the enteric nervous system (ENS), there are many receptor-mediated presynaptic mechanisms for inhibition of transmitter release but only presynaptic 5-hydroxytryptamine (5-HT)4 receptor has been shown to facilitate enteric neurotransmission (19, 32). However, in the central nervous system and in other autonomic ganglia, presynaptic nicotinic acetylcholine receptors (nAChRs) function to facilitate or induce transmitter release (1, 12, 26, 27, 39). Although there is evidence for presynaptic nAChRs on the nerve terminals of longitudinal muscle motoneurons in guinea pig intestine (9), it is not known if presynaptic nAChRs are present in enteric ganglia.

The fast sodium channel blocker TTX has been used to distinguish the cellular location of presynaptic receptors. For example, the TTX sensitivity of nAChR-mediated release of transmitter from synaptosomes distinguishes two populations of presynaptic nAChRs (24, 39). One population of presynaptic receptors is located on nerve terminals, and transmitter release mediated by these receptors is resistant to blockade by TTX. Stimulation of presynaptic receptors increases transmitter release either by causing direct depolarization of the nerve terminal or by directly gating calcium entry into nerve terminals (39). Transmitter release mediated by preterminal nAChRs is abolished by TTX as sodium-dependent action potentials propagated along the axon are required for preterminal receptors to mediate depolarization and calcium entry into nerve terminals (39). Studies of the TTX sensitivity of a variety of responses induced by nAChR agonists have provided mixed results as to the existence of presynaptic nAChRs in the ENS. ACh release induced by nAChR agonists is blocked by TTX in the ENS (10, 37), but other nAChR-induced responses in gastrointestinal tissues are TTX resistant (2, 3, 9, 10, 34, 38). Furthermore, recent immunohistochemical studies (20, 31) of myenteric neurons of the guinea pig ileum have demonstrated that nAChRs may be located on nerve terminals. The experiments conducted here were designed to determine if there are functional presynaptic nAChRs located on or near the nerve terminals of myenteric neurons in the guinea pig ileum. The results indicate that pharmacologically distinct populations of nAChRs are present in the myenteric plexus, some of which induce TTX-resistant, noncholinergic slow excitatory postsynaptic potentials (sEPSPs).

MATERIALS AND METHODS

Animal use and tissue preparation. Male albino guinea pigs (250–450 g) obtained from the Michigan Department of Public Health (Lansing, MI) were used. The care and use of these animals were approved by the All-University Commit-
tery on Animal Use and Care at Michigan State University. Animals were killed by exsanguination after general anesthesia induced by inhalation of halothane (Halocarbon Laboratories, River Edge, NJ). A segment of ileum was removed and placed into a dissection bath lined with Sylgard 184 elastomer (Dow Corning, Midland, MI) and filled with oxygenated Krebs solution maintained at 37°C. Individ-
ual myenteric ganglia and interconnecting fiber tracts were visualized at \times 200 magnification with Hoffman Modulation Contrast optics (Modulation Optics, Greenvale, NY).

Intracellular recording technique. Membrane potential was recorded from individual myenteric neurons using an Axoclamp-2A electrometer (Axon Instruments, Foster City, CA) and current-clamp technique. Recordings were obtained using glass microelectrodes filled with 2 M KCl and tip resistances of 80–120 MΩ. Data were sampled at 2 kHz, filtered at 5 kHz using a four-pole, low-pass Bessel filter (Warner Instruments, Hamden, CT), and digitally converted, monitored, and stored using acquisition and analysis software (Axotape, version 2.0.2 and Axoscope, version 7.0; Axon Instruments) and a desktop computer.

Experimental procedures. Myenteric neurons were impaled, and the resting membrane potential was measured 10–20 min after initial impalement to ensure stability of the recording. In some experiments, a constant hyperpolarizing current was passed through the recording microelectrode to facilitate a stable impalement and to minimize the number of action potentials occurring during drug- or nerve-stimulated depolarizations. Minimizing action potential discharge allowed more accurate measurements of the peak depolarization caused by drugs and nerve stimulation. Myenteric neurons were categorized as AH neurons if a single somal action potential was followed by an afterhyperpolarization \( \approx 4 \) mV in amplitude and \( \approx 4 \) s in duration. S neurons were categorized as those cells in which single electrical stimuli applied to interganglionic connectives elicited a fast excitatory postsynaptic potential (fEPSP) (14). Neurons for which these data were not complete were considered uncategorized. Synaptic responses were elicited using a Krebs solution-filled glass stimulating microelectrode positioned over an interganglionic fiber tract. Electrical stimuli were provided by a Grass S44 stimulator and stimulus isolation unit (SIU 5, Grass Instruments, Quincy, MA). Focal stimulation with single, 0.5-ms pulses (10–150 V) was used to induce fEPSPs at a stimulus rate of 0.3 Hz. Brief trains of stimuli (10 Hz for 300–800 ms) were used to evoke sEPSPs. The presence of fEPSPs and sEPSPs was tested by stimulating only one interganglionic fiber tract.

Local application of agonists was accomplished by microejection from the tip of a micropipette (30- to 40-μm tip diameter) placed within 50 to 150 μm of the impaled neuron. Agonists were applied using short pulses of nitrogen gas (3 to 35 ms, 10 psi) using a Picospritzer II (General Valve, Fairfield, NJ). Antagonists and the neurokinin receptor 3 (NK3) agonist senktide were superfused at 3–5 ml/min for a minimum of 5 min; there was a 30-s lag time for drugs to reach the recording chamber.

Statistical analysis. Responses were recorded as changes in membrane potential (mV) relative to the resting membrane potential. Descriptive and analytical statistics were computed using SAS, version 6.12 (SAS Institute, Cary, NC). The assumptions of equal variance and normal distribution were tested using chi-square residual analysis and the Wilk-Shapiro test, respectively. Maximum likelihood analysis was used to determine appropriate transformations for statistical modeling. A repeated-measures ANOVA was used to determine the effect of specific antagonists on agonist-induced responses. When statistical differences were detected, a Bonferroni t-test or specific linear contrasts were used to separate the means. A paired t-test was used to compare the responses induced by nicotine and cytisine within the same neuron. Significant differences were declared when \( P < 0.05 \). All data are presented as means ± SE.

RESULTS

Responses of myenteric neurons to microejection of nicotine or cytisine. Membrane potential responses to microejection of nicotine (1 mM) or cytisine (1 mM) were recorded from 124 myenteric neurons (30 AH, 67 S, and 27 unclassified neurons). The initial resting membrane potentials of AH, S, and unclassified neurons were: \(-70 ± 2, -54 ± 4, \) and \(-60 ± 6 \) mV, respectively. Nicotine caused a biphasic response in 87 of 94 (93%) myenteric neurons. The first phase was a fast depolarization, whereas the second phase was either slow depolarization (65 neurons, 75%) or hyperpolarization (22 neurons, 25%) (Fig. 1, A–C). The slow hyperpolarization in myenteric neurons that occurs after nAChR activation has been described previously [37] and will not be discussed further here. A monophasic fast depolarization occurred in 3 S (amplitude = 28 ± 9 mV) and 3 unclassified (amplitude = 32 ± 7 mV) neurons, and one S neuron did not respond to nicotine. Microejection of Krebs solution onto the ganglion or microejection of nicotine onto the surrounding smooth muscle did not affect the membrane potential of any neuron.

The time course of the nicotine-induced fast depolarization was similar for AH and S neurons with a rise time of 0.7 ± 0.5 s. However, the amplitude of the fast depolarization in AH neurons (14.5 ± 2 mV, \( n = 28 \)) was significantly smaller than that recorded from S neurons (21 ± 2 mV, \( n = 25, P < 0.05 \)). Although the
amplitudes of the nicotine-induced slow depolarization were similar in AH (15 ± 1 mV) and S (16 ± 1.5 mV, P > 0.05) neurons, the time course of the slow depolarization was different (compare Fig. 1, A and B). The time to reach the peak of the slow depolarization in AH neurons was 25 ± 3 s but was only 5 ± 3 s in S neurons (P < 0.0001). In addition, the half duration of the slow depolarization was 56 ± 11 s in AH vs. 15 ± 2 s in S neurons (P < 0.03).

Cytisine (1 mM) did not cause a slow depolarization in any neuron, whereas in the same neurons nicotine caused both fast and slow depolarizations (Fig. 2). In 10 of 12 AH neurons, cytisine (1 mM, 5- to 15-ms pulse duration) did not cause a fast depolarization, whereas in two AH neurons longer duration applications of cytisine caused a only small-amplitude fast depolarization (Fig. 2). In 14 of 20 S neurons (Fig. 1D), cytisine induced a fast depolarization (26 ± 3 mV) that was followed by hyperpolarization (−4.5 ± 1.5 mV), whereas the remaining six S neurons did not respond at all to cytisine. All responses caused by nicotine and cytisine were blocked by the nAChR antagonist mecamylamine (10 μM) (Fig. 3).

Responses to stimulation of interganglionic connec-
tives. Focal stimulation of a single interganglionic fiber tract induced a fEPSP in 57 of 57 (100%) S neurons and a sEPSP in 45 of 57 (79%) S neurons. In AH neurons (n = 30), focal stimulation never induced a fEPSP but always induced a sEPSP. Focal stimulation induced a sEPSP in 17 of 21 (81%) unclassified neurons. A slow depolarization in response to nicotine was observed in 63 of 73 (86%) neurons in which focal stimulation induced a sEPSP. Microejection of nicotine never induced slow depolarization in neurons in which focal stimulation did not induce a sEPSP. Input resistance changes were measured in four AH neurons during sEPSPs and nicotine-induced slow depolarizations. In these neurons, input resistance increased from a resting level of 212 ± 54 to 331 ± 74 MΩ at the peak of the sEPSP. In the same neurons, the nicotine-induced slow depolarization was associated with an increase in input resistance from a resting level of 234 ± 48 to 337 ± 87 MΩ at the peak of the nicotine response.
Presynaptic nAChRs. The effect of TTX (0.3 μM) on nicotine-induced slow depolarizations was tested in 9 S neurons and 12 AH neurons. In four S neurons the nicotine-induced slow depolarization was inhibited by 90 ± 12% by TTX, whereas in two AH neurons, TTX inhibited this response by 79%. TTX inhibited the nicotine-induced slow depolarization in the remaining AH and S neurons by only 24 ± 7% and 18 ± 8%, respectively. These data indicate either that nicotine was causing the slow depolarization by direct activation of nAChRs on the somatodendritic region of the neuron or that nicotine was causing TTX-resistant release of a mediator of the slow depolarization. To test the possibility that a tachykinin peptide acting at NK₃ receptors was a mediator of the nicotine-induced slow response, the NK₃ agonist senktide was used to desensitize NK₃ receptors. Senktide (0.3 μM) applied by superfusion caused a slowly developing depolarization that returned to baseline during continued application. Senktide-induced desensitization of NK₃ receptors inhibited the slow depolarization caused by subsequent nicotine application in all AH neurons by 81 ± 5% (n = 12) (Fig. 4A). In S neurons in which the nicotine-induced slow depolarization was TTX resistant, NK₃ receptor desensitization reduced the slow response by 63 ± 15% (n = 5) (Fig. 4B). Data summarizing the effects of sequential superfusion of TTX (0.3 μM) and the NK₃ receptor agonist senktide (0.1 μM) on fast and slow depolarizations induced by nicotine are summarized in Fig. 5. These data show that in all AH neurons (n = 12) (Fig. 5A) and in a subset of S neurons (n = 5) (Fig. 5B), in the presence of TTX, NK₃ receptor desensitization blocks the nicotine-induced slow depolarization in a reversible manner. Although the slow response was inhibited by NK₃ receptor desensitization, the fast nicotine-induced depolarization was not inhibited during NK₃ receptor desensitization. In fact, in AH neurons, the nicotine-induced fast depolarization was increased in amplitude during senktide superfusion (P < 0.05) (Fig. 5A). In a second subset of S neurons (n = 4), TTX inhibited the nicotine-induced slow depolarization by >90%. After TTX was washed out and the nicotine slow depolarization returned to the pre-TTX level, it was found that NK₃ receptor desensitization reduced the slow response in these neurons by only 23 ± 8% (Fig. 5C).

In a separate experiment, the effect of senktide-induced desensitization on responses to microejection of senktide or 5-HT was determined in the presence of TTX (0.3 μM). Microejection of senktide (0.3 μM) induced a depolarization in AH neurons (n = 3) that lasted several minutes. Only AH neurons (n = 5) in which microejection of 5-HT (1 mM) induced biphasic depolarization were used for comparison with nicotine-induced responses. Depolarization induced by microejection of senktide was abolished during senktide-induced desensitization, whereas the amplitudes of the...
biphasic depolarization induced by 5-HT were not changed (Fig. 6).

**Effects of low-calcium and high-magnesium solution.** The effect of a low-calcium (1.2 mmol/l) and high-magnesium (12 mmol/l) Krebs solution on the nicotine-induced slow depolarizations recorded from AH neurons (n = 6) was determined. Superfusion with low-calcium and high-magnesium Krebs solution depolarized AH neurons. Despite this complication, superfusion with low-calcium and high-magnesium Krebs solution abolished sEPSPs and the slow, but not the fast, depolarization induced by nicotine (Fig. 7). The control amplitudes for fast and slow depolarizations were 15 ± 4 and 15 ± 3 mV, respectively. After superfusion with the low-calcium and high-magnesium solutions, the fast and slow depolarization amplitudes were 10 ± 3 (P > 0.05) and −1 ± 2 mV (P < 0.05), respectively.

**DISCUSSION**

**TTX-resistant responses.** Because nAChRs are ligand-gated ion channels, responses mediated directly at nAChRs have short latencies and durations (8). Consistent with this expectation, nicotine-induced fast depolarizations recorded from myenteric neurons were due to a direct action of nicotine at somatodendritic nAChRs localized to impaled neurons. However, the time course of nicotine-induced slow depolarizations suggests that this response is mediated indirectly through the action of a slow excitatory neurotransmitter. The slow responses could be due to activation of somatodendritic nAChRs on presynaptic neurons with subsequent action potential-dependent release of slow excitatory neurotransmitters (17, 35, 37). Indeed, TTX inhibited the nicotine-induced slow depolarization in some S neurons. However, the slow depolarization induced by nicotine was resistant to TTX in AH neurons and in a subset of S neurons. Therefore, release of the slow transmitter was action potential independent and nAChRs mediating release of the slow transmitter must be near the release site.

A low-calcium and high-magnesium Krebs solution was used to reduce calcium entry into nerve terminals to test the hypothesis that nicotine acts near nerve terminals to release a slow neurotransmitter. In AH neurons, superfusion with low-calcium and high-magnesium Krebs solution abolished both slow synaptic transmission induced by focal stimulation of interganglionic connectives and the slow (but not the fast) depolarization caused by nicotine. Superfusion with low-calcium and high-magnesium Krebs solution produced a depolarization of AH neurons that mimicked the sEPSP. Depolarization under these conditions is mediated by inhibition of a resting calcium-activated potassium conductance that contributes to the resting membrane potential of AH neurons (11, 30). Despite this complication, the nicotine-induced slow depolarization in AH neurons was blocked. We conclude that the nicotine-induced slow depolarization is blocked by low-calcium and high-magnesium solutions because nicotine-induced transmitter release is blocked.

**Mediator of slow depolarizations induced by nicotine.** The typical response to microejection of nicotine was biphasic; the slow response to nicotine was always depolarizing in AH neurons but either depolarizing or hyperpolarizing in S neurons. Hyperpolarization after nicotinic depolarization has been studied previously and is mediated by a calcium-sensitive potassium conductance (36). An ACh-induced biphasic depolarization has been described in myenteric neurons previously and is similar to that induced by nicotine (29). In contrast with our findings, however, the ACh-induced slow depolarization was blocked by muscarinic receptor antagonists. The slow depolarizations reported here are not mediated by muscarinic receptors because they 1) persisted in the presence of scopolamine, 2) are induced by nicotine, and 3) are blocked by the nAChR.
antagonist mecamylamine. Therefore, we designed experiments to test the hypothesis that the nicotine-induced slow depolarization is mediated by nAChRs on neurons releasing a noncholinergic neurotransmitter.

Several transmitters mediate slow excitatory neurotransmission in the myenteric plexus, including ACh (29), tachykinin peptides (8), and 5-HT (25). Nicotinic ACh receptor-mediated release of ACh has been demonstrated (10, 35, 37) but is unlikely to have participated in nicotine-induced slow depolarization as described above. However, postsynaptic activation of NK₃ receptors by nAChR-mediated release of tachykinin peptides (15, 16) could contribute to the slow depolarization in myenteric neurons. Activation of NK₃ receptors is a mechanism of slow excitatory neurotransmission in the myenteric plexus, and we used continuous senktide superfusion to induce selective NK₃ receptor desensitization. Selective NK₃ receptor desensitization was confirmed by demonstrating that the response to microejection of senktide was completely inhibited during senktide superfusion, whereas slow depolarizations caused by 5-HT were unaffected. In addition, fast depolarizations caused by nicotine and 5-HT were also unaffected during senktide superfusion. However, nicotine-induced slow depolarizations were blocked during senktide superfusion in all AH neurons and a subset of S neurons. Therefore, the TTX-resistant slow depolarization induced by nicotine in AH neurons is dependent on NK₃ receptor activation. In some S neurons, the nicotine-induced slow response was TTX sensitive and the slow depolarization induced by nicotine was less affected by NK₃ receptor desensitization. It is likely that in these neurons the slow depolarization induced by nicotine is mediated by neurotransmitters in addition to tachykinin peptides acting at NK₃ receptors.

Pharmacology of nAChR-mediated responses. The pharmacology of nAChRs of different subunit combinations has been described previously (1, 5, 23, 27). Comparing responses induced by cytisine to those induced by nicotine may discriminate receptors differing in β-subunit composition (4, 5, 23, 33). Cytisine produces responses similar to nicotine when nAChRs contain the β₄-subunit, but cytisine is a weak agonist at nAChRs containing the β₂-subunit. Comparisons made in the present study reveal two notable findings: 1) AH neurons respond to nicotine but not to cytisine, and 2) fast depolarizations induced by nicotine and cytisine in S neurons were identical, whereas cytisine never caused a slow depolarization. The equivalence of fast depolarizations induced by cytisine and nicotine in S neurons suggests that the nAChR(s) mediating this response contains the β₄-subunit. In contrast, only nicotine induced fast depolarizations in AH neurons, suggesting that nAChRs in AH neurons do not contain β₄-subunits. Because cytisine never induced slow depolarizations, nAChRs mediating this response also do not contain β₄-subunits. Therefore, somatodendritic nAChRs on AH neurons and nAChRs on nerve terminals releasing slow excitatory neurotransmitter(s) probably do not contain β₄-subunits. This conclusion is consistent with the finding that the pharmacological properties of TTX-resistant, nAChR-mediated noncholinergic transmission to the longitudinal smooth muscle of guinea pig ileum are characteristic of nAChRs containing β₂-subunits (9).

Postsynaptic nAChRs. The most consistent response to nicotine was a fast depolarization that was TTX resistant and occurred in the presence of scopolamine. The receptors that mediate these fast depolarizations are nAChRs localized to the somatodendritic region of postsynaptic neurons (20, 31). fEPSPs within the myenteric plexus are mediated partly by nAChRs (22). In the present study, fEPSPs were recorded from S but not AH neurons. It is surprising, then, that a fast depolarization was induced by nicotine in most AH neurons. Using the amplitude of single channel and whole cell currents activated by ACh, it has been determined that myenteric AH neurons have fewer somatodendritic nAChRs than S neurons (40), and this could account for the smaller nicotine-induced fast depolarizations in AH neurons compared with responses in S neurons. The smaller response to nicotine in AH neurons is consistent with immunofluorescence studies demonstrating that nAChR immunoactivity in S neurons is very dense and is present in clusters on
the somatodendritic region, contrasting with nAChR immunoreactivity in AH neurons, which is less intense and more diffuse (20).

Summary and conclusions. TTX-resistant, nicotine-induced slow depolarizations of myenteric neurons are mediated by stimulation of nAChRs localized to tachykinin peptide-containing nerve terminals. Myenteric neurons contain and release substance P (SP) and neurokinin A (NKA) (16), and one or both of these peptides are the likely mediators of the nicotine-induced slow response. The data presented here indicate that SP and NKA act at postsynaptic NK2 receptors on S and AH neurons. AH neurons are intrinsic primary afferent neurons and provide synaptic inputs to other AH neurons, interneurons, and motoneurons (7). AH neurons connect with other AH neurons via slow excitatory synapses (21). AH neurons express NK3 receptors (18) and contain choline acetyltransferase and tachykinin peptides (6), but ACh released from the nerve terminals of AH neurons does not appear to play a postsynaptic role at connections between AH neurons. The data presented here suggest that ACh released at synaptic connections between AH neurons could act presynaptically, at cytisine-insensitive nAChRs, in a positive feedback mechanism to release SP and NKA (Fig. 8A). Somatodendritic nAChRs are present on myenteric neurons. Because AH neurons do not receive fast cholinergic synaptic input, somatodendritic nAChRs on AH neurons must be extrasynaptic; these nAChRs are cytosine insensitive. Because cytosine did not elicit slow responses in any neuron or fast responses in AH neurons, nerve terminal nAChRs and those present at the somatodendritic region of AH neurons may differ in subunit composition from those on the somatodendritic region of S neurons. S neurons in the myenteric plexus are interneurons and motoneurons, and there are several classes of each of these functional groups (6). The specific subset of S neurons receiving slow excitatory input activated by nerve terminal nAChRs is unknown, but AH neurons do make synaptic connections with both interneurons and motoneurons (18). ACh released by AH neurons at synaptic connections with S neurons acts postsynaptically to mediate fEPSPs and sEPSPs (29) and may also act presynaptically to facilitate SP and NKA release (Fig. 8B).

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