Pharmacological Properties of Nicotinic Acetylcholine Receptors Expressed by Guinea Pig Small Intestinal Myenteric Neurons

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ABSTRACT

The electrophysiological and pharmacological properties of nicotinic acetylcholine receptors (nAChRs) were studied in guinea pig small intestinal myenteric neurons maintained in culture or in acutely isolated preparations. Acetylcholine and nicotine caused inward currents that desensitized in ~4 s. The current-voltage (I-V) relationship rectified inwardly with a reversal potential near 0 mV. The agonist rank order potency was 1,1-dimethyl-4-phenyl-piperazinium > acetylcholine = nicotine > cytisine. Agonist-induced currents were blocked by nAChR antagonists with a rank order potency of hexamethonium > dihydro-β-erythroidine (DHβE); mecamylamine and DHβE exhibit high potency at β4 and β2 subunit-containing nAChRs, respectively. α-Bungarotoxin (0.1 μM) or α-methyllycaconitine (0.1 μM), antagonists that block nAChRs containing α7 subunits, did not affect acetylcholine-induced responses. Immunohistochemical studies revealed that nearly every neuron in culture was labeled by an antibody (mAb35) that recognizes nAChr α3 and α5 subunits. Antibodies selective for α3, α5, or β2 subunits also stained most neurons, whereas an α7 subunit antibody revealed very few neurons. In neurons in the intact myenteric plexus from newborn and adult guinea pigs, local application of acetylcholine (1 mM) and cytisine (1 mM) caused similar amplitude depolarizations, and these responses were blocked by nAChR antagonists with a rank order potency of mecamylamine > hexamethonium > DHβE. These data indicate that myenteric neurons maintained in culture predominately express nAChRs composed of α3, α5, β2, and β4 subunits. These subunits may be in a homogenous population of receptors with unique pharmacological properties, or multiple receptors of different subunit composition may be expressed by individual neurons.

In the nervous system, nAChRs are ligand-gated cation channels composed of pentameric combinations of 11 subunits (α2–α9, β2–β4) (Sargent, 1993; Lukas et al., 1999). The specific subunit composition yields receptors with pharmacological and electrophysiological properties that are unique to that subunit combination (Luetje and Patrick, 1991; Gerzanich et al., 1998). For example, antagonists can discriminate among nAChRs with different subunit compositions. α-Methyllycaconitine and α-bungarotoxin (α-BGT) are potent and selective antagonists of α7 subunit-containing nAChRs (Couturier et al., 1990; Ward et al., 1990). Receptors composed of the α4β2 or α3β2 subunit combinations are more sensitive to block by dihydro-β-erythroidine (DHβE) than receptors composed of other subunit pairs. Alternatively, mecamylamine has high affinity for nAChRs composed of α3β4 subunits (Albuquerque et al., 1997).

There are two main classes of neuron in the enteric nervous system: S neurons and AH neurons. S neurons are enteric interneurons or motor neurons (Kunze and Furness, 1999). S neurons receive fast excitatory synaptic input mediated partly by acetylcholine acting at nAChRs (Galligan and Bertrand, 1994). Studies of the pharmacological properties of nAChRs in the enteric nervous system would provide information about the mechanisms of synaptic excitation of enteric motor neurons and interneurons. AH neurons are enteric sensory neurons (Furness et al., 1998). The action potential in AH neurons is followed by a long-lasting afterhyperpolarization, and AH neurons contain the calcium binding protein calbindin (Furness et al., 1998). Although most AH neurons do not receive fast excitatory synaptic input, they express functional nAChRs because exogenously applied nAChR agonists depolarize AH neurons (Schneider and Galligan, 2000). Detailed studies of the properties of enteric neurons...
nAChRs may help to identify the role of these receptors on enteric sensory neurons.

Recent immunohistochemical studies using antibodies that recognize α3, α5, α7, and β2 subunits have localized nAChR subunit immunoreactivity (ir) to nerve cell bodies, dendrites, and nerve terminals in enteric ganglia in acutely isolated myenteric plexus preparations (Kirchgessner and Liu, 1998). It is not known which of these subunits coassemble to form the functional receptors. The purpose of the present study was to characterize the pharmacological and electrophysiological properties of nAChRs in myenteric neurons in an effort to identify the nAChR subunit composition. Determining the subunit composition of nAChRs will help to understand the physiological role played by these receptors and to develop new drugs that may target particular nAChRs in the gut.

**Methods and Materials**

**Tissue Culture.** Myenteric neurons were cultured as described previously (Zhou and Galligan, 1996). Two newborn guinea pigs (1–2 days old) were sacrificed by severing the major neck blood vessels after halothane anesthesia. These procedures were approved by the All University Committee on Animal Use and Care at Michigan State University. The small intestine was placed in cold (4°C) sterile-filtered Krebs’ solution of the following composition 117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose. The longitudinal muscle with attached myenteric plexus was stripped free using a cotton swab and cut into 5-mm-long segments. Tissues were divided into four aliquots, and each aliquot was transferred to 1 ml of Krebs’ solution containing 1600 U of trypsin for 25 to 30 min at 37°C. The tissues were triturated and then centrifuged at 900 g for 5 min using a benchtop centrifuge. The supernatant was discarded, and the pellet was resuspended and incubated (30 min at 37°C) in Krebs’ solution containing 2000 U of crab hepatopancreas collagenase. The suspension was triturated and then centrifuged for 5 min. The pellet was resuspended in Eagle’s minimum essential medium containing 10% fetal bovine serum, gentamicin (10 μg/ml), and streptomycin (50 μg/ml). Cells were plated on plastic dishes coated with (poly)lysine and maintained in an incubator at 37°C with an atmosphere containing 5% CO₂ for up to 2 weeks. After 2 days in culture, 10 μM cytosine arabinoside was added to the Eagle’s minimum essential medium to limit smooth muscle and fibroblast proliferation. Medium was changed twice weekly.

**Whole-Cell Recording Technique.** Patch-clamp recordings were carried out at room temperature with 3- to 5-MΩ patch pipettes; seal resistances were greater than 5 GΩ. The composition of the patch pipette solution was 160 mM CsCl, 2 mM MgCl₂, 1 mM EGTA, 10 mM HEPES, 1 mM ATP, and 0.25 mM GTP; the pH and osmolality were adjusted to 7.4 (using CsOH) and 320 mOsm/l. EGTA, 10 mM HEPES, 1 mM ATP, and 0.25 mM GTP; the pH and osmolality were adjusted to 7.4 (using CsOH) and 320 mOsm/l. The concentration near the neurons is a critical factor affecting the responses to drugs. The concentration of drug application, computer-controlled solenoid valves (General Valve, Fairfield, NJ) were used to gate solution flow through the tubes. The concentration of drug in the pipette was 1 mM and was ejected from the pipette using a brief nitrogen pulse (10 psi) controlled by a Picospripter (General Valve).

**Immunohistochemistry.** The medium in culture dishes was replaced with 3 ml of Zamboni’s fixative (2% (v/v) formaldehyde and 0.2% (v/v) picric acid in 0.1 M sodium phosphate buffer, pH 7.0), and cells were fixed overnight at 4°C. The fixative was cleared using three washes of dimethyl sulfoxide at 10-min intervals. Cells were then washed three times with phosphate-buffered saline (PBS) (0.01 M; pH 7.2) at 10-min intervals and subsequently incubated overnight with primary antibody at room temperature. The primary antibodies, target antigens, host species, and working dilutions are listed in Table 1. In some experiments, antibodies raised against the α3 and α5 subunits were preincubated with the antigen peptide provided by the commercial supplier (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For these experiments, diluted antibody was incubated with 10 μg of the antigen peptide for 2 h at room temper-

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ature. The antigen peptide preincubated antibody was then applied to preparations as described above. After primary antibody incubation, cells were washed three times at 10-min intervals with PBS. Cells were then incubated (1 h at 23°C) with sheep anti-mouse IgG (1:40 dilution in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA) conjugated to fluorescein isothiocyanate to reveal calbindin-d1b, donkey anti-goat IgG (1:200 dilution in PBS; Jackson ImmunoResearch Laboratories) conjugated to Cy3 to reveal α3 or α5 subunit-ir, goat anti-rat IgG (1:200 dilution in PBS; Jackson ImmunoResearch Laboratories) conjugated to Cy3 to reveal mAb35-ir (α3 and α5 subunits) and mAb270-ir (β2 subunit) or goat anti-rabbit IgG (1:200 dilution in PBS; Jackson ImmunoResearch Laboratories) conjugated to Cy3 to reveal α7 subunit-ir. Cells were then washed with three times with PBS and mounted in buffered glycerol for fluorescence microscopy using a LabroluxS upright microscope and a PL Fluotar 40 × 0.7 numerical aperture objective (Leica, Wetzlar, Germany). When using this microscope, digital images were obtained using a SPOT-2 cooled charge-coupled device color camera (Diagnostic Instruments, Sterling Heights, MI). Some images were obtained using Leica TCS-SL confocal scanning system (Leica Microsystems, Heidelberg, Germany) and a DMLFA upright microscope with a HCX PL APO 63 × 1.32 numerical aperture oil immersion objective (Leica). Confocal image depth was 1.1 μm, and four images were averaged to obtain a single image for presentation. All images were then processed using Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA) and Powerpoint 7.0 (Microsoft, Redmond, WA) software.

Drugs. Crab hepatopancreas collagenase was obtained from Calbiochem-Novabiochem (La Jolla, CA). All other drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Statistics. Data are expressed as the mean ± S.E.M. Agonist concentration-response curves obtained from individual neurons were fit using the Hill equation as follows.

\[ y = E_{\text{max}} \left( \frac{x^n}{EC_{50}^n + x^n} \right) \]

where \( E_{\text{max}} \) is the maximum response, \( EC_{50} \) is the half-maximal effective concentration, \( n \) is the slope factor, and \( x \) is the ligand concentration. Mean values for each parameter obtained from a number of neurons in different treatment groups were compared using Student’s t test. For electrophysiological studies, \( n \) values refer to the number of neurons from which data were obtained. For data from the immunohistochemical studies, \( n \) values refer to the number of separate batches of neuronal cultures on which observations were made; two guinea pigs provided neurons for each batch of cultures.

Results

Agonist Concentration-Response Relationships. ACh (1 mM) caused an inward current in every neuron tested (holding potential of −60 mV). The amplitude of currents activated by ACh remained stable during repeated ACh application for up to 60 min after whole-cell formation when recordings were obtained using an ATP/GTP-free pipette solution. At 2 min after establishing whole-cell recording conditions, the mean ACh current amplitude was 2.5 ± 0.6 nA, whereas at 60 min the mean current amplitude was 2.4 ± 0.6 nA (\( n = 4; P > 0.05 \)).

Concentration-response curves were obtained for inward currents caused by ACh, nicotine, DMPP, and cytisine (Fig. 1). All responses were normalized to the amplitude of the inward current caused by 1 mM ACh in each neuron. The \( EC_{50}, E_{\text{max}} \), and Hill coefficients derived from these concentration-response curves are presented in Table 2. The rank order potency based on agonist \( EC_{50} \) values was DMPP > nicotine = ACh > cytisine. The mean \( E_{\text{max}} \) for ACh was significantly greater than that for each of the other agonists, whereas the \( E_{\text{max}} \) values for nicotine and DMPP were greater than that for cytisine. The Hill coefficient for DMPP was significantly lower than that for ACh and cytisine, whereas there were no differences between Hill coefficients for ACh, nicotine, and cytisine.

The shallow slope for the DMPP concentration-response curve and the smaller \( E_{\text{max}} \) suggested that DMPP may have been acting as a partial agonist. This possibility was tested by using a subthreshold concentration of DMPP (10 μM) to block responses caused by ACh. DMPP pretreatment caused a rightward shift in the ACh concentration-response curve (Fig. 2). Under control conditions the ACh \( EC_{50} \) value was 242 ± 20 μM, whereas in the presence of DMPP the ACh \( EC_{50} \) value was 1481 ± 459 μM (\( P < 0.0002 \)). The control ACh Hill coefficient was 1.9 ± 0.15, whereas in the presence of DMPP this value was 0.8 ± 0.1 (\( t = 3.98; P < 0.01 \)). DMPP did not decrease the ACh \( E_{\text{max}} \) (Fig. 2).

Effects of Antagonists. Because nAChR subtypes can be differentiated on the basis of their sensitivity to block by nAChR antagonists, inhibition curves for hexamethonium, mecamylamine, and DHβE against responses caused by ACh (1 mM) were obtained. In these experiments, neurons were pretreated with each antagonist for 5 min, and responses to repeated applications of ACh in the presence of increasing
The mean age of the response caused by 1 mM ACh in each neuron tested. Points are the mean ± S.E.M. of the data obtained from the indicated number of neurons.

concentration of each antagonist were obtained. The data summarized in Fig. 3 show antagonist inhibition curves where responses were normalized to the peak current induced by ACh alone. Mecamylamine was the most potent antagonist; the mecamylamine IC_{50} value (micromolar) was 0.1 ± 0.04 (n = 6). The IC_{50} (micromolar) value for hexamethonium was 1.6 ± 0.4 (n = 6). Full concentration-response curves for DHβE were not obtained because concentrations higher than 300 µM were not tested. However, at 30 µM, DHβE reduced the ACh response by 50 ± 4% (n = 5).

α-BGT blocks nAChRs that contain the α7 subunit (Zhang et al., 1994). To determine whether α7 subunit-containing nAChRs contributed to the responses caused by ACh, neurons were preincubated with α-BGT (0.1 µM) for 1 to 3 h. It was found that the concentration-response curves for ACh and nicotine were not altered by α-BGT preincubation compared with concentration-response curves obtained from untreated cells (Fig. 4, A and B). Similarly, acute treatment of individual neurons with MLA (0.1 µM), an antagonist of α7 subunit-containing nAChRs (Ward et al., 1990), had no effect on ACh concentration-response curves in myenteric neurons (Fig. 4, C and D).

Current-Voltage Relationship of ACh-Induced Current. Fig. 5 shows the inwardly rectifying I-V relationship for responses caused by ACh (300 µM). The slope conductances measured at −50 and 50 mV were 21 ± 3 and 0.7 ± 0.3 nS, respectively (G_{−50}/G_{+50} = 66 ± 19; n = 10). The reversal potential of ACh-induced current was approximately 0 mV in the normal Krebs' solution (n = 18), and this was shifted by −20 mV when the external Na⁺ concentration was reduced by 50% (n = 3).

Kinetic Properties of Currents Induced by Nicotinic Agonists. At −60 mV, the 10 to 90% rise times for ACh (1 mM)- and nicotine (1 mM)-induced currents were 79 ± 6 and 76 ± 7 ms, respectively. Currents caused by nicotine decayed more rapidly than those caused by ACh but more slowly than those activated by DMPP. The increase in the rate of current decay was accompanied by a rebound inward current after nicotine and DMPP washout (Fig. 6, arrow). To analyze the contributions of open-channel block, the decay rate of currents caused by ACh, nicotine, and DMPP were compared at different membrane potentials (Fig. 6). The 10 to 90% decay time for ACh-induced currents was voltage-independent (4 ± 0.4 s at −100 mV, 4 ± 0.3 s at −50 mV; n = 16; P > 0.05), whereas those for nicotine and DMPP were voltage-dependant (Fig. 6). The ratio of 10 to 90% decay times measured at −50 and −110 mV was 1.6 for nicotine (3.6/2.2 s; n = 10; P < 0.05) and 2.8 for DMPP (1.5/0.54 s; n = 11; P < 0.05).

Immunohistochemical Localization of nAChR Subunits in Myenteric Neurons. The monoclonal antibody mAb35 recognizes neuronal α3 and α5 nAChR subunits. mAb35-ir was found in nearly every myenteric neuron maintained in primary culture (n = 5) (Fig. 7A). Calbindin-ir was also localized to a subset of mAb35-ir neurons (n = 2) (Fig. 7B). Because mAb35 recognizes α3 and α5 subunits, antibodies that recognize α3 or α5 subunits were used to determine whether one or both of these subunits was expressed by myenteric neurons. These antibodies revealed that most neurons were immunoreactive for the α3 (n = 5) and α5 (n = 4) subunits (Fig. 7, B and C). Labeling revealed by these two antibodies was blocked when the antibodies were preincubated with the antigen peptide (n = 3) (data not shown). These data indicate that myenteric neurons express nAChRs that contain α3 and α5 subunits. A monoclonal antibody (mAb270) that recognizes the β2 subunit revealed that myenteric neurons also expressed nAChRs containing this subunit (n = 3) (Fig. 7D). Finally, an antibody that recognizes the α7 subunit revealed only a few faintly stained neurons and nerve fibers (n = 2) (Fig. 7E).

Studies in Acutely Isolated Myenteric Plexus. The data described above for agonist effects on myenteric neurons maintained in primary culture show that cytisine was a weak agonist at nAChRs expressed by these neurons. However, previous work in the acutely isolated myenteric plexus from adult guinea pigs showed that cytisine was equieffective with...
nicotine at causing depolarizations of a subset of myenteric neurons (Schneider and Galligan, 2000). This apparent difference in results could be due to the use of tissue culture or whole-cell recording conditions or to developmental differences because the neurons used for primary culture studies were obtained from 1- to 2-day-old guinea pigs. This issue was addressed by directly comparing responses to cytisine and nicotine in neurons in acutely isolated myenteric plexus preparations from adult and newborn guinea pigs. Nicotine and cytisine (each at 1 mM) were applied by pressure ejection from a pipette positioned near the impaled neuron. Both agonists caused a rapid depolarization associated with a fall in input resistance in neurons from the adult and newborn myenteric plexus (Fig. 8A). Furthermore, response amplitudes were also similar for nicotine and cytisine in adult and newborn neurons (Fig. 8B). These data indicate that nAChRs expressed by adult and newborn guinea pig myenteric neurons are similar in their sensitivity to cytisine.

To further characterize the pharmacological properties of nAChRs, sensitivity to blockade by mecamylamine, hexamethonium, and DHβE was tested by using ionophoretically applied ACh to activate nAChRs in the myenteric plexus from adult ileum. In these experiments, the initial membrane potential was held near −90 mV to minimize the occurrence of action potentials during ACh responses. Under these conditions, the amplitude of the ACh response was 19 ± 2 mV (n = 23). IC50 values (μM) for inhibition of ACh responses by mecamylamine, hexamethonium, and DHβE were 0.2 ± 0.1 (n = 6), 10 ± 5 (n = 8), and 14 ± 4 (n = 7), respectively (Fig. 9). Therefore, the antagonist IC50 values and rank order potency for inhibition of ACh responses recorded from neurons in the myenteric plexus were similar to
those for neurons in culture. MLA (30 nM) did not change the amplitude of fast excitatory postsynaptic potentials or the amplitude of depolarizations caused by pressure application of nicotine (1 mM) directly onto neurons (control = 26 ± 7 mV, MLA = 29 ± 9 mV; P > 0.05; n = 3).

Discussion

Immunohistochemical Studies. Previous studies of nAChR subunits in whole mounts of guinea pig gut showed that most enteric neurons were labeled by mAb35 (Kirchgessner and Liu, 1998; Obaid et al., 1999), an antibody that recognizes α3 and α5 subunits (Conroy et al., 1992; Conroy and Berg, 1995). Similarly, most myenteric neurons maintained in culture were labeled by mAb35 and some of these neurons contained calbindin. Calbindin is a marker for intrinsic sensory/AH type neurons (Furness et al., 1998) so most mAb35-labeled neurons were likely to be S-type neurons.

It is not clear whether mAb35 is labeling α3, α5, or both subunits in myenteric neurons. This issue was addressed by showing that antibodies raised against α3 and α5 subunits labeled myenteric neurons, indicating that nAChRs expressed by myenteric neurons contain both subunits. Studies using mAb270, an antibody that recognizes β2 subunits (Conroy and Berg, 1995), showed that this subunit is expressed by myenteric neurons. Previous studies of nAChR subunits in guinea pig submucosal plexus revealed that these neurons also express β2 subunits (Obaid et al., 1999). Finally, an antibody raised against the α7 subunit labeled few myenteric neurons maintained in primary culture. α7 subunit staining is present in many nerve fibers in the myenteric plexus (Kirchgessner and Liu, 1998), but in the present study few fibers were stained by the α7 subunit antibody, indicat-

Fig. 6. Voltage dependence of decay of nAChR-mediated inward currents. A, representative traces of inward currents caused by ACh, nicotine, and DMPP recorded at holding potentials of -110 mV (traces labeled 1) and at -50 mV (traces labeled 2). Recordings obtained at -50 mV were scaled to the same size as the traces obtained at -110 mV to compare decay rates. B, histograms show the mean 10 to 90% decay times for currents caused by ACh, nicotine, and DMPP at the indicated membrane potentials. The decay rates for nicotine and DMPP-induced currents significantly faster at -110 mV compared with -50 mV.

Electrophysiological Properties of Myenteric nAChRs. Whole-cell currents evoked by ACh were associated with a conductance increase, a reversal potential of 0 mV, and inward rectification. These properties are similar to those of nAChRs in other autonomic neurons (Mathie et al., 1990; Aibara and Akaike, 1991; Fieber and Adams, 1991). Although currents caused by nicotinic agonists desensitized, ACh produced stable responses if the period of agonist application was ≤2 s and the application interval was ≥2 min. Therefore, decay of whole-cell currents during agonist application is not time-dependent rundown. In addition, desensitization is the main mechanism for decay of ACh-induced currents, whereas desensitization and channel blockade contribute to the decay of currents activated by nicotine and DMPP. This latter conclusion is based on the voltage independence of the decay of ACh currents, whereas decay of nicotine and DMPP currents was faster at hyperpolarized potentials. Nicotine and DMPP are open channel blockers of nAChRs in myenteric neurons as in other neurons (Mathie et al., 1991; Maconochie and Knight, 1992).

Heterologously expressed and native nAChRs composed of α7 subunits desensitize rapidly and are blocked by low concentrations of α-BGT (Couturier et al., 1990; Seguela et al., 1993; Zhang et al., 1994). In the present study, it was found that whole-cell currents activated by nicotinic agonists desensitize more slowly (~4 s) than homomeric α7 subunit-containing nAChRs (<100 ms; Zhang et al., 1994). These data indicate that most myenteric neurons do not express homomeric α7 subunit-containing nAChRs on the cell body. α7 subunits coassemble with β2 subunits to form functional nAChRs, and α7β2 heteromeric receptors desensitize more slowly than homomeric α7 nAChRs (Khiroug et al., 2002). Immunohistochemical data indicate that some myenteric neurons express α7 subunits that could combine with β2 or β4 subunits to form a subset of nAChRs with unique properties.

Agonist Responses. The agonist rank order potency for myenteric neuronal nAChRs is DMPP > ACh = nicotine > cytisine. Because the agonist rank order potency for activation of homomeric α7 subunit nAChRs is nicotine = DMPP > cytisine > ACh (Couturier et al., 1990; Zhang et al., 1994), it is unlikely that myenteric neurons express these receptors on the cell body. Although DMPP was the most potent agonist, the Hill coefficient and E_{max} for DMPP were lower than those for ACh and nicotine. However, DMPP is a partial agonist at myenteric neuronal nAChRs and blockade of nAChRs is due partly to voltage-dependent channel block by DMPP. At high DMPP concentrations, the channel blocking effect would be most prominent accounting for the shallow slope of the concentration-response curve and decreased E_{max}. Therefore, the potency and efficacy of DMPP as an agonist at myenteric neuronal nAChRs was underestimated. Because DMPP is the most potent agonist at nAChRs composed of α3β2 or α3β4 subunits (Chavez-Noriega et al., 1997; Gerzanich et al., 1998), the data presented herein indicate that myenteric neurons expressed α7 subunits and that these receptors may play a significant role in submucosal plexus function.
neurons express nAChRs composed of one or both of these subunit compositions.

Cytisine discriminates between nAChRs containing β2 and β4 subunits (Luetje and Patrick, 1991). For nAChRs containing β2 subunits, cytisine is a partial agonist (Papke and Heinemann, 1991), and cytisine produced little effect in myenteric neurons in culture. This result is consistent with the immunohistochemical data showing that myenteric neurons contain β2 subunits. However, previous electrophysiological studies showed that myenteric neurons are depolarized by cytisine (Schneider and Galligan, 2000). This result was also confirmed in the present study when intracellular electrophysiological methods were used to record from neurons in the acutely isolated myenteric plexus from 1- to 2-day-old

Fig. 7. Immunohistochemical localization of ir for nAChR subunits and calbindin in myenteric neurons maintained primary culture. A and B, photomicrographs showing colocalization of mAb35-ir (A) and calbindin-ir (B). Most mAb35-ir neurons did not contain calbindin-ir. mAb35 localizes α3 and α5 nAChR subunits. Arrows indicate a single neuron labeled by both antibodies. C, localization of α5 subunit-ir in myenteric neurons. D, confocal image showing localization of α3 subunit-ir in myenteric neurons. E, photomicrograph showing localization of mAb270-ir in myenteric neurons. mAb270 localizes β2 subunits. F, confocal image showing faint labeling of two myenteric neurons with α7 subunit-ir. Scale bars, 35 μm.
were obtained. Values are the number of neurons from which data were obtained.

Nicotine and cytisine were equieffective at depolarizing myenteric neurons. Although cytisine and adult guinea pigs. Nicotine and cytisine were equieffective at depolarizing myenteric neurons. Although cytisine

Fig. 8. Cytisine and nicotine depolarize myenteric neurons in the acutely isolated myenteric plexus from newborn (1–2 day-old) and adult guinea pig small intestine. A, representative traces of depolarizations caused by nicotine or cytisine applied from a pipette positioned near the impaled neuron. Recordings were obtained using conventional intracellular microelectrodes. The pipette drug concentration was 1 mM. Downward deflections are voltage responses to 200-pA, 20-ms current pulses passed through the intracellular recording electrode. B, mean data from experiments illustrated in A. Data are mean ± S.E.M. depolarization amplitude caused by nicotine or cytisine in myenteric neurons from newborn or adult small intestine; n is the number of neurons from which the data were obtained.

Fig. 9. Antagonist concentration-response curves for inhibition of depolarizations caused by ionophoretically applied ACh (1 M) onto myenteric neurons in the myenteric plexus preparation from adult guinea pig ileum. Points are the mean ± S.E.M. inhibition of the ACh response caused by each concentration of antagonist. Data are percentage of inhibition of ACh responses obtained in each neuron before antagonist application; n values are the number of neurons from which data were obtained.

and adult guinea pigs. Nicotine and cytisine were equieffective at depolarizing myenteric neurons. Although cytisine activates nAChRs containing β4 subunits, it is a low potency/low efficacy agonist at these receptors (Chavez-Noriega et al., 1997; Gerzanich et al., 1998). Cytisine may not be a reliable tool for discriminating nAChRs containing β2 versus β4 subunits if nAChRs are present in low concentrations. Myenteric neurons maintained in culture may contain fewer nAChRs than neurons in the intact plexus, and therefore cytisine would produce little effect in cultured neurons.

The α5 subunit does not contribute to agonist binding but alters the pharmacological properties of nAChRs (Gerzanich et al., 1998). α3β2 subunit-containing nAChRs have low micromolar (<30 μM) EC50 values for ACh and nicotine, and addition of α5 subunits reduces the EC50 value to <3 μM (Wang et al., 1996). α3β4 subunit-containing nAChRs have high EC50 values (>100 μM) for ACh and nicotine, and α5 subunits do not alter EC50 values (Wang et al., 1996). nAChRs expressed by myenteric neurons have EC50 values (~200 μM) for ACh and nicotine similar to those for α3β4- or α3α5β4-containing nAChRs.

Hill coefficients for agonists acting at α3β2 nAChRs are close to 1 but α3β4 subunit-containing nAChRs have Hill coefficients near 2 (Cohen et al., 1995; Chavez-Noreiga et al., 1997). In myenteric neurons, the Hill coefficients for ACh, nicotine, and cytisine were not different from 2. The Hill coefficient for DMPP was less than 1, but the channel blocking properties of this agonist would reduce its Hill coefficient. The immunohistochemical data showed that myenteric neurons express α5 subunits, and taken together these data are consistent with a myenteric neuronal nAChRs subunit composition of α3, α5, and β4 subunits.

Studies with Antagonists. α-BGT and MLA block α7 subunit-containing AChRs (Couturier et al., 1990; Zhang et al., 1994), but nAChR-mediated responses in myenteric neurons were insensitive to these antagonists. Therefore, it is unlikely that guinea pig myenteric neurons express homomeric α7 subunit-containing nAChRs on the soma. Similar conclusions about the absence of somatodendritic α7 subunit-containing nAChRs in myenteric neurons have been reached by others (Töröcsik et al., 1991; Barajas-Lopez et al., 2001).

Mecamylamine, hexamethonium, and DHβE block ACh responses in myenteric neurons with a rank order potency of mecamylamine > hexamethonium > DHβE. DHβE is the most potent antagonist of nAChR-containing β2 subunits, whereas mecamylamine is the most potent antagonist of nAChRs-containing β4 subunits (Harvey and Luetje, 1996). IC50 values obtained for mecamylamine (0.1 μM) and DHβE (15–30 μM) in the present study are similar to those for inhibition of α3β4 subunit-containing nAChRs (Harvey and Luetje, 1996). In addition, nAChRs containing α4β2 or α3β4 subunits have DHβE IC50 values of 0.2 and 23 μM, respectively (Harvey et al., 1996). The IC50 values for DHβE and mecamylamine inhibition of nAChRs in myenteric neurons indicate that functional nAChRs contain α3 and β4 subunits.

Summary and Conclusions. These studies used immunohistochemical and electrophysiological approaches to identify the subunit composition of myenteric neuronal nAChRs. Immunohistochemical studies revealed the predominant presence of α3, α5, and β2 subunits. Although cytisine was a weak agonist at nAChRs expressed by myenteric neurons in culture, it was equieffective with nicotine at depolarizing neurons in the myenteric plexus. Furthermore, EC50 values for ACh, nicotine, and DMPP obtained in neurons maintained in culture are similar to
those for heterologously expressed α3β4 subunit-containing receptors. In addition, necamylamine was the most potent antagonist of myenteric neuronal nAChRs. These data indicate that myenteric neurons predominantly express nAChRs composed of α3, α5, β2, and β4 subunits as occurs in a subset of nAChRs in chick ciliary ganglia (Conroy and Berg, 1995). These subunits may be in a homogeneous population of receptors with unique pharmacological properties, or multiple receptors of different subunit composition may be expressed by individual neurons.

References

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