Dorsal Root Ganglion Neurons Express Multiple Nicotinic Acetylcholine Receptor Subtypes

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Genzen, Jonathan R., William Van Cleve, and Daniel S. McGehee. Dorsal root ganglion neurons express multiple nicotinic acetylcholine receptor subtypes. J Neurophysiol 86: 1773–1782, 2001. Although nicotinic agonists can modulate sensory transmission, particularly nociceptive signaling, remarkably little is known about the functional expression of nicotinic acetylcholine receptors (nAChRs) on primary sensory neurons. We have utilized molecular and electrophysiological techniques to characterize the functional diversity of nAChR expression on mammalian dorsal root ganglion (DRG) neurons. RT-PCR analysis of subunit mRNA in DRG tissue revealed the presence of nAChR subunits α2-7 and β2–β4. Using whole cell patch-clamp recording and rapid application of nicotinic agonists, four pharmacologically distinct categories of nicotinic responses were identified in cultured DRG neurons. Capacitance measurements were used to divide neurons into populations of large and small cells, and the prevalence of nicotinic responses was compared between groups. Category I (α7-like) responses were seen in 77% of large neurons and 32% of small neurons and were antagonized by 10 nM methyllycaconitine citrate (MLA) or 50 nM α-bungarotoxin (α-BTX). Category II (α3β4-like) responses were seen in 16% of large neurons and 9% of small neurons and were antagonized by 20 μM mecamylamine but not 10 nM MLA or 1 μM DHβE. Category II responses had a higher sensitivity to cytisine than nicotine. Two other types of responses were identified in a much smaller percentage of neurons and were classified as either category III (α4β2-like) or category IV (subtype unknown) responses. Both the α7-like and α3β4-like responses could be desensitized by prolonged applications of the analgesic epibatidine.

INTRODUCTION

Dorsal root ganglion (DRG) neurons convey somatic and visceral sensory information from peripheral tissues to the spinal cord. DRG neurons are a diverse population, and different size classes can correlate with modality (Devor 1999). Neurons with small diameter axons and cell bodies are likely to convey information about pain and temperature, whereas neurons with larger diameter axons and cell bodies are more likely to convey mechanoreceptive information. DRG neurons express a diverse array of neurotransmitter receptors, and this expression can also correlate with cell size and modality. For example, small-diameter nociceptive neurons express the vanilloid receptor (VR-1), which is sensitive to capsaicin, low pH, and high temperature (Caterina et al. 1997; Tominaga et al. 1998). Interestingly, capsaicin can have both irritative and analgesic effects, presumably due to mechanisms of receptor excitation and desensitization (Fitzgerald 1983).

In behavioral studies, nicotinic agonists can also induce analgesic and irritative effects. Receptor knockout experiments have demonstrated that nicotine-induced analgesia is significantly reduced in animals that lack the high-affinity α4 and β2-containing nicotinic acetylcholine receptors (Marubio and Changeux 2000; Marubio et al. 1999). Although most reports focus on central sites of action (Damaj et al. 1998; Davis et al. 1932; Khan et al. 1994; Masner 1972; Sahley and Berntson 1979), cholinergic agonists excite peripheral sensory nerve endings (Stein and Reeh 1993), and there is evidence for nicotinic acetylcholine receptor (nAChR) expression on both DRG and trigeminal ganglion (TG) neurons (Boyd et al. 1991; Hu and Li 1997; Liu and Simon 1997; Liu et al. 1993; Morita and Katayama 1989; Roberts et al. 1995; Sucher et al. 1990). There has not been an extensive functional characterization of the nAChR diversity on mammalian DRG neurons.

Neuronal nAChRs are pentameric ligand-gated ion channels. Nine α and three β subunits have been identified by molecular cloning, and these subunits assemble in numerous combinations to form functional nAChR subtypes (Elgoyhen et al. 2001; Lindstrom 1996; Lukas et al. 1999; McGehee 1999; McGehee and Role 1995; Sargent 1993). Receptor subunit composition is the principal determinant of nAChR properties, including agonist and antagonist selectivity profiles, as well as the kinetics of activation and desensitization. Pharmacological characteristics of nAChR-mediated responses reflect the presence of specific receptor subunits. For example, the α7 nAChR is activated by millimolar concentrations of choline and is selectively antagonized by nanomolar concentrations of methyllycaconitine citrate (MLA) or α-bungarotoxin (α-BTX) (Alkondon et al. 1992, 1997; Coutieret al. 1990).

In this study, we utilize a pharmacological approach to assess the prevalence of distinct nAChR subtypes on cultured DRG neurons, focusing on the comparative distribution of nicotinic responses between small and large neurons. Desensitization properties of these responses were also examined.

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Methods

Cell culture

Tissue was isolated from postnatal day 1–3 Sprague-Dawley rats. Briefly, animals were anesthetized with urethan (2 mg/kg ip). DRGs were removed, washed in Hank’s balanced salt solution (HBSS; Life Technologies, Rockville, MD), and dissociated by 60-min trypsin treatment (0.25% in Ca2+-free HBSS) at room temperature followed by mild trituration in modified neuronal MEM (nMEM), which included 10% fetal calf serum (HyClone, Logan, UT), 50 ng/ml 75 NGF (Alomone Labs, Jerusalem, Israel), and 1% Penicillin/Streptomycin (Life Technologies) in Dulbecco’s MEM (Life Technologies). nMEM also served as the culture medium. Cells were plated on collagen-coated glass coverslips and maintained at 37°C in a humidified incubator with 5% CO2. To limit the problems associated with uncontrolled voltage changes in long neurites, nAChR characterization experiments were carried out using DRG neurons during the first 3 days in culture.

Electrophysiology

Neurons were visualized using an Axiovert 25 inverted microscope (Zeiss, Göttingen, Germany) and visualized with a ×32 objective with Varel contrast enhancement. Standard external solution (SES) was constantly perfused at 0.6 ml/min and contained (in mM) 140 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 glucose, pH 7.4. All recordings were done at room temperature. The pipette solution contained (in mM) 145 K-gluconate, 10 KCl, 1 EGTA, 10 HEPES, and 10 glucose, and 5 K-ATP, pH 7.4. Guanosine triphosphate (100 µM) was added for receptor desensitization experiments. An EPC7 Patch Clamp (HEKA, Lambrecht, Germany) and a Digidata 1200 interface (Axon Instruments, Foster City, CA) were used to digitally acquire data to a Pentium computer running pCLAMP8 software (Axon Instruments). Recording electrodes were manufactured using a P-97 electrode puller (Sutter Instruments, Novato, CA) and borosilicate micropipettes (Warner Instruments, Hamden, CT). Electrode resistance was between 2 and 5 MΩ. Recordings were sampled at 10 kHz and filtered at 3 kHz. Neurons were identified by their phase-bright appearance, spherical cell bodies, and the presence of voltage-activated sodium currents as measured in response to depolarizing voltage steps. Recordings were not included in the analysis if the series resistance (Rs) exceeded 25 MΩ. Rs error correction was accomplished by postrecording calculation of corrected peak current values.

Drug applications

A piezo-controlled solution exchange system (Piezo Systems; Cambridge, MA) was used for rapid application of nicotinic agonists onto DRG neurons in culture. This system used a three-barreled glass perfusion head (<20 ms exchange at the cell), and cells were exposed to a constant flow of bath solution between drug applications.

Reagents

Nicotine tartrate (Nic), choline chloride (Chol), cytisine (Cyt), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), acetylcholine chloride (ACh), methyllycaconitine citrate (MLA), mecamylamine (Mec), epibatidine (Epi), and dihydro-β-erythroidine hydrobromide (DHβE) were all obtained from Sigma/RBI (St. Louis, MO). Capsaicin (Cap) was obtained from ICN Biochemicals (Aurora, OH). α-Bungarotoxin (α-BTX) was obtained from Biotoxins (St. Cloud, FL). All drugs were dissolved in SES. Capsaicin was initially dissolved in 50% ethanol/H2O aliquots at 1,000 times final concentration, and DMPP was dissolved using a small volume of DMSO at >1,000 times final concentration, and pH and osmolality were measured for each drug solution to prevent activation of either proton-sensitive or mechanoreceptive currents.

RT-PCR

RT-PCR was used to detect mRNA expression in acutely dissected DRGs. DRGs were obtained using the dissection procedures outlined above. Three DRG equivalents were used per PCR reaction tube. DRGs were washed in HBSS, which was then subsequently replaced with 1 ml Trizol reagent (Life Technologies). Tissue was homogenized and chloroform was added to each tube followed by RNA extraction and precipitation. Oligo(dT) primers were used to generate cDNA template by reverse transcription using the GibcoBRL SuperScript Preamplification System (50 min at 42°C). RNase H (Life Technologies) was then used to cleave the remaining mRNA strand. PCR amplification of specific products was carried out with the PCR Core System II (Promega, Madison, WI). Thirty-five PCR cycles were completed with the protocol 95°C 1 min, 55°C 1 min, 73°C 2 min. Products were separated on 2% agarose gel and visualized with ethidium bromide fluorescence. PCR products were purified using the QiAquick PCR Purification Kit (Qiagen, Valencia, CA) and were subsequently cleaved with appropriate restriction enzymes (New England Biolabs, Beverly, MA) to verify product length and identity. Reverse transcriptase–negative controls were run in parallel for each nAChR subunit assayed to ensure that primers were not amplifying contaminant DNA. Primer design was conducted using Primer3 Software (Rozen and Skaltsky 1997), sequence alignments were conducted using the Wisconsin Package (GGC) with sequences obtained from GenBank, and restriction maps were generated by DNA Strider (Marck 1988). Primers were designed to cross exon boundaries to distinguish amplified product from mRNA versus genomic DNA. Primer melting temperatures were determined using the following equation from Operon, modified for our salt concentrations: Tm = 81.5 + 16.6 × log [Na+] + 41 × (G+C) length − 500/length, where [Na+] = 0.075 M. Primer sequences, melting temperature, predicted product sizes, and restriction enzyme information are included in Table 1.

Data analysis and statistics

For whole cell currents, Clampfit 8.0 (Axon Instruments) was used to determine current amplitude and rise/decay values. These values were exported to SigmaPlot 5.0 (SPSS, Chicago, IL) for statistical analysis and display. For individual agonist applications, peak amplitudes values that were >10 times the baseline RMS noise measured by the Mini Analysis Program (Synaptosoft) were characterized as “responsive.”

Results

nAChR mRNA expression in the dorsal root ganglia

We have examined nAChR mRNA expression in the rat DRG using RT-PCR analysis. Ganglia were removed from neonatal pups of the same age used for our primary cultures (1–3 days postnatal), and mRNA was isolated from a combined pool of excised DRGs from both male and female pups. PCR primer information is located in Table 1. As Fig. 1A indicates, amplification products of the predicted sizes were detected for each of the alpha (α2, α3, α4, α5, α6, and α7) and beta subunits (β2, β3, and β4) examined. The chick α8 (Anand and Lindstrom 1992) and vestibulocochlear α9 (Elgoyhen et al. 1994) were not examined. α2 expression was faint but visually detectable. In all cases, reverse transcriptase–negative controls yielded no product, ensuring that amplified template was originally from mRNA sources. Figure 1B illustrates the results of restriction enzyme treatment of the PCR products, which yielded fragments of the predicted sizes as indicated in Table 1.
Electrophysiological recordings were made from neonatal DRG neurons in culture. Membrane capacitance measurements were recorded for every neuron in our experiments and were used to divide cells into distinct size categories. Cell diameter measurements were obtained for a subpopulation of neurons using a calibrated reticle and were calculated as an average of the shortest and longest axes of each soma. A plot comparing capacitance and diameter measurements for a representative sample of DRG neurons is shown in Fig. 2. The solid line represents the relationship between membrane capacitance and diameter of a spherical cell, based on the specific membrane capacitance of 1 μF/cm². Divergence of data points from this line is likely the result of increased membrane area of the neuronal processes. A threshold of 18.7 pF was used as the cutoff between large and small neurons (dashed line). The mean ± SD of capacitance measurements from 86 visually identified small neurons is 18.7 pF (average diameter, 16.0 ± 2.7 μm, mean ± SD).

TABLE 1. Primers, products, and restriction enzymes used in RT-PCR analysis of DRG

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Primer</th>
<th>T(m), °C</th>
<th>Size, bp</th>
<th>Accession No.</th>
<th>Restriction Enzyme (size products, bp)</th>
</tr>
</thead>
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<tr>
<td>α2</td>
<td>Sense</td>
<td>5’ AACTGCAAGATGAAGTTTG 3’</td>
<td>54</td>
<td>944</td>
<td>L10077.1</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5’ AGGAATATCCCGGTCTACCAC 3’</td>
<td>56</td>
<td></td>
<td>Dra III (305, 639)</td>
</tr>
<tr>
<td>α3</td>
<td>Sense</td>
<td>5’ CGACGTGACCTACTTCCCAT 3’</td>
<td>60</td>
<td>1,066</td>
<td>L31621.1</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5’ ACAGTGAGCTCTGGAAC 3’</td>
<td>60</td>
<td></td>
<td>MspA1 I (238, 828)</td>
</tr>
<tr>
<td>α4</td>
<td>Sense</td>
<td>5’ CTCTTCTCGGGTTACAAAC 3’</td>
<td>56</td>
<td>1,069</td>
<td>L31620.1</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5’ TGCAAGTGACCTGAAGATG 3’</td>
<td>54</td>
<td></td>
<td>Dra III (695, 374)</td>
</tr>
<tr>
<td>α5</td>
<td>Sense</td>
<td>5’ GTGGCTGAACATTCTCTG 3’</td>
<td>58</td>
<td>1,039</td>
<td>J05231.1</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5’ ACTCCTGACTTCTCTCT 3’</td>
<td>58</td>
<td></td>
<td>Msp I (579, 460)</td>
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<tr>
<td>α6</td>
<td>Sense</td>
<td>5’ TGGATGAAATGACACGATT 3’</td>
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<td></td>
<td>Antisense</td>
<td>5’ CTGGGTCTGTGTAGTGTAG 3’</td>
<td>60</td>
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<td>Sense</td>
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<td>56</td>
<td>668</td>
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<td></td>
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<td>50</td>
<td></td>
<td>Eco0109 I (425, 243)</td>
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<td>β2</td>
<td>Sense</td>
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<td>5’ GACCTTCACCGGAAGAAAA 3’</td>
<td>56</td>
<td></td>
<td>Msp I (282, 830)</td>
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<td>β3</td>
<td>Sense</td>
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<td>1,106</td>
<td>J04636.1</td>
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<td>Antisense</td>
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<td>Eco0109 I (715, 391)</td>
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<tr>
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<td>56</td>
<td></td>
<td>Dra III (283, 427)</td>
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**DRG neuron size categorization**

Electrophysiological recordings were made from neonatal DRG neurons in culture. Membrane capacitance measurements were recorded for every neuron in our experiments and were used to divide cells into distinct size categories. Cell diameter measurements were obtained for a subpopulation of neurons using a calibrated reticle and were calculated as an average of the shortest and longest axes of each soma. A plot comparing capacitance and diameter measurements of a representative sample of DRG neurons is shown in Fig. 2. The solid line represents the relationship between membrane capacitance and diameter of a spherical cell, based on the specific membrane capacitance of 1 μF/cm². Divergence of data points from this line is likely the result of increased membrane area of the neuronal processes. A threshold of 18.7 pF was used as the cutoff between large and small neurons (dashed line). The mean ± SD of capacitance measurements from 86 visually identified small neurons is 18.7 pF (average diameter, 16.0 ± 2.7 μm, mean ± SD).
DRG neurons respond to nicotinic agonists

A rapid, piezo-controlled solution exchange system was used for nicotinic agonist applications onto DRG neurons in culture (Fig. 3A). Nicotinic agonists evoked rapid excitatory currents in a concentration-dependent manner in some DRG neurons (Fig. 3B). Initial experiments determined appropriate agonist concentration ranges (between 50 and 80% of the maximal response), which were then utilized for the more extensive pharmacological characterization below. Currents were observed following application of several nicotinic agonists including epibatidine, DMPP, cytisine, nicotine, acetylcholine, and choline.

Category I response: functional evidence for \( \alpha 7 \) receptor expression by DRG neurons

The initial experiments indicated that a subpopulation of DRG neurons responded to choline. As choline has been reported to act as an \( \alpha 7 \)-selective agonist (Alkondon et al. 1997), we tested the nicotinic responses in choline-sensitive neurons pharmacologically to determine whether DRG neurons express functional \( \alpha 7 \) receptors. Figure 4A shows a DRG neuron that responds to both choline (10 mM) and acetylcholine (1 mM). Both the choline and acetylcholine-induced currents in this cell were reversibly blocked by a 3-min preincubation with the \( \alpha 7 \)-selective antagonist MLA (10 nM; Fig. 4B). After a 12-min wash, both agonists evoked rapidly activating currents again. The choline and acetylcholine-induced currents were then completely blocked by an irreversible \( \alpha 7 \) antagonist, \( \alpha \)-BTX (50 nM; Fig. 4C). No current could be activated by either choline or acetylcholine even after a 40-min wash, which is consistent with the \( \alpha 7 \) subtype nAChR. In five of five cells tested with both antagonists, choline-induced currents were completely blocked by 10 nM MLA and 50 nM \( \alpha \)-BTX.

Prevalence, amplitude, rise time, and decay information for each of the nicotinic response categories is presented in Table 2. A total of 220 neurons was surveyed for the presence of functional \( \alpha 7 \) receptors. The \( \alpha 7 \) receptors were the most common nAChR in DRG neurons. Seventy-seven percent of large neurons and 32% of small neurons had these category I responses, which had rapid activation and desensitization kinetics. There were no statistical differences in current density, rise time, or decay time for category I currents between the populations of large and small DRG neurons.

We compared agonist potency on \( \alpha 7 \)-expressing DRG neurons by testing responses to 100-\( \mu \)M test doses (\( n = 3 \) cells per agonist) and then normalizing to the 100-\( \mu \)M nicotine response in each cell.
experiments if the responses to both choline and ACh were completely blocked by 10 nM MLA (as in Fig. 4, A and B). Although choline is the least potent agonist at α7 receptors, it is also selective, even at high millimolar concentrations. The rank order potency for nicotinic agonists at α7-expressing neurons was as follows: DMPP ≈ cytisine > nicotine ≈ acetylcholine > choline.

Category II response: functional evidence for α3β4 receptor expression by DRG neurons

While conducting the pharmacological characterization of α7 responses in DRG neurons, MLA-insensitive ACh currents were also observed in some cells. These MLA-insensitive currents could also be activated by cytisine, which is a full agonist at β4-containing receptors yet only a partial agonist at receptors containing the β2 subunit (Papke and Heinemann 1994). Figure 5A shows an example of a cytisine-induced current that was not blocked by 10 nM MLA. To examine the properties of these non-α7 currents, 10 nM MLA was included in perfusion solutions.

A total of 266 DRG neurons was surveyed for the expression of MLA-insensitive nAChRs. Under these conditions, neurons that responded to cytisine always displayed a greater sensitivity to cytisine than nicotine, regardless of the order of application. For example, Fig. 5B shows a cytisine-induced current in the presence of 10 nM MLA compared with a nicotine-induced current in the same cell. These category II responses were the second-most common nicotinic response in DRG neurons, seen in 16% of large cells and 9% of small cells (see Table 2). Category II responses also had slower activation and desensitization kinetics than were observed for α7. There were no statistical differences in current density, rise time, or decay time for category II currents between the populations of large and small DRG neurons.

The rank order potency of nicotinic agonists on category II responses [using 50–200 μM test doses for all drugs except epibatidine (1 μM); n = 3 cells per agonist] was as follows: epibatidine ≫ acetylcholine ≈ cytisine ≈ DMPP > nicotine ≫ choline (Fig. 5C). These MLA-insensitive currents could be blocked by 20 μM mecamylamine (Fig. 5D; n = 6) but not 1 μM DHβE (n = 3). The cell shown in Fig. 5D was also sensitive to 10 μM capsaicin (data not shown). Category II responses were therefore characterized by insensitivity to 10 nM MLA or 1 μM DHβE, activation by cytisine with a greater potency than nicotine, and antagonism by 20 μM mecamylamine. These observations point to the expression of α3β4 nAChRs in a subpopulation of DRG neurons (see discussion for additional evidence).

Category III and IV responses: functional evidence for two other categories of nicotinic responses in DRG neurons

While investigating the properties of category II responses on DRG neurons, two other categories of responses were also observed. These responses were identified in a much smaller population of neurons, which precluded extensive pharmacological characterization.

Of the neurons used for the characterization of category II responses, a category III (α4β2-like) response was seen in 4% of the large DRG neurons and in <1% of the small DRG neurons (see Table 2). This category III response was insensitive to cytisine (50 μM; n = 6), but activated by nicotine (n = 2), DMPP (n = 1), or epibatidine (n = 3). These currents could be antagonized by DHβE (100 nM, n = 1; 1 μM, n = 2), a potent selective antagonist of α4β2-receptors (Alkondon and Albuquerque 1993; Luejt et al. 1990). Figure 6A shows representative currents from a small DRG neuron that was insensitive to 50 μM cytisine but activated by 50 μM nicotine. This current could be reversibly antagonized by 100 nM DHβE. The profound insensitivity to cytisine indicates that this receptor subtype is not likely to contain the β4 subunit, and antagonism by DHβE in nanomolar concentrations serves as evidence of an α4β2-like nAChR subtype in a small fraction of DRG neurons in culture.

One final category of response was observed in DRG neurons. These category IV responses were relatively large compared with those described above, were insensitive to cytisine, and had much slower activation and desensitization kinetics (see Table 2). Figure 6B shows one large DRG neuron in which 25 μM nicotine induced a response that lasted over 100 s with a persistent current of >1 nA. When we reviewed all of the recordings from these experiments (including preliminary analyses and receptor categorizations I–III), category IV responses were evident in 3% of large neurons and in 1% of small neurons. These slower responses have been observed with nicotine (n = 6), epibatidine (n = 4), and choline (n = 4). Category IV responses could also be discriminated from categories I–III by their insensitivity to 10 nM MLA (n = 5), 100 nM α-BTX (n = 3), 1 μM DHβE (n = 2), and 100 μM mecamylamine (n = 1).

Functional consequences of nAChR expression on sensory neurons

This pharmacological investigation illustrates the diversity of nAChR subtypes expressed by DRG neurons. As nAChR

<table>
<thead>
<tr>
<th>Category</th>
<th>Prevalence, %</th>
<th>Average Capacitance, pF</th>
<th>Amplitude, pA</th>
<th>10–90 Rise</th>
<th>Decay at 400 ms, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Large Cells</td>
<td>Small Cells</td>
<td>Large Cells</td>
<td>Small Cells</td>
<td>Large Cells</td>
</tr>
<tr>
<td></td>
<td>57 (11/192)</td>
<td>39 (11/288)</td>
<td>35.7 ± 1.4</td>
<td>12.1 ± 0.8</td>
<td>545.2 ± 70.1</td>
</tr>
<tr>
<td>II</td>
<td>16 (4/137)</td>
<td>9 (11/129)</td>
<td>27.6 ± 3.2</td>
<td>13.2 ± 0.7</td>
<td>421.5 ± 92.7</td>
</tr>
<tr>
<td>III</td>
<td>4 (5/137)</td>
<td>&lt;1 (1/129)</td>
<td>35 ± 8.5</td>
<td>11 ± 0.0</td>
<td>273.7 ± 121d</td>
</tr>
<tr>
<td>IV</td>
<td>3 (1/355)</td>
<td>1 (3/248)</td>
<td>24.4 ± 1.0</td>
<td>9.7 ± 4.1</td>
<td>1,626.0 ± 531</td>
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</table>
desensitization might interfere with any endogenous cholinergic transmission, we have examined the desensitization properties of these nicotinic responses in DRG neurons. As is shown in Fig. 7A, a DRG neuron that initially responds to a 100-μM ACh application with an inward current no longer responds after the receptors are desensitized by a prolonged epibatidine application. The kinetics of recovery from desensitization were investigated for both category I and II responses in DRG neurons and are shown in Fig. 7B. Epibatidine applications (1 μM, 30 s) were used to desensitize responses to 100 μM ACh, and recovery from desensitization was measured using subsequent ACh applications every 40–60 s. The data were normalized to an initial 100-μM ACh response before desensitization and was corrected for measured rundown observed in the absence of epibatidine desensitization. Recovery times from desensitization were remarkably similar for category I and II responses (τ of 243 and 234 s, respectively), although the initial rate of desensitization for these categories is different (see Table 2). If endogenous cholinergic transmission plays a role in sensory transduction or transmission, desensitization of nAChRs by exogenous nicotinic agonists could inhibit this endogenous activity.

**DISCUSSION**

The results of this study show that DRG neurons express multiple subtypes of nAChRs, and that the distribution of receptor expression varies according to cell size. RT-PCR analysis of mRNA expression in DRG tissue indicated the presence of nearly every known nAChR subunit (α2–7, β2–4).
A pharmacological approach was chosen to detect and characterize the nAChRs functionally expressed on DRG neurons in culture, and four response categories were observed.

Category I (α7-like) responses were seen in 77% of large neurons and in 32% of small neurons. These findings demonstrate that the α7 nAChR subtype is the most prevalent nAChR expressed by neonatal DRG neurons in culture. The exact role of α7 receptors on these neurons is unclear, although α7 receptors are known to play a role in presynaptic modulation of glutamatergic transmission in other systems (McGehee et al. 1995), and we have preliminary evidence indicating that this also occurs in the dorsal horn of the spinal cord, the central projection site of DRG neurons (Genzen and McGehee 1999).

The α7 receptor is unique in that the channel is extremely permeable to Ca²⁺ (Seguela et al. 1993), which makes it an ideal channel for presynaptic modulation, or as an activator of Ca²⁺-mediated second-messenger cascades. This report demonstrates functional α7 receptors on primary somatosensory neurons, and it provides an explanation for the autoradiographic evidence for α-BTX binding sites on sensory neurons demonstrated two decades ago (Ninkovic and Hunt 1983; Polz-Tejera et al. 1980). Additionally, intense α7 immunolabeling has been detected in the spinal mesencephalic nucleus of the trigeminal nerve, a region known to contain jaw muscle afferents (Dominguez del Toro et al. 1994).

Category II (α3β4-like) responses were the second most common response observed in DRG neurons, seen in approximately 16% of large neurons and in 9% of small neurons. The α3β4 designation of category II responses is based on the similarity between the pharmacological profile we observed and that of nAChRs generated by pair-wise expression in *Xenopus* oocytes. This idea is also supported by immunoprecipitation and binding experiments in sensory neurons. The relative potency of cytisine versus nicotine can be used to implicate the presence of the β4 subunit (Lena et al. 1999), which is the case for our category II response. While we observed a higher sensitivity to ACh and DMPP than was seen in oocyte expression studies, the similar potency of these two agonists is consistent with α3β4 expression (Luetje and Patrick 1991; Papke and Heinemann 1994). Evidence for α3β4 receptor expression has also been demonstrated in trigeminal ganglion tissue (Flores et al. 1996). The prevalence of category II responses in our experiments matched the distribution of α3 mRNA transcripts seen using in situ hybridization in both chick DRG and rat trigeminal ganglion (Boyd et al. 1991; Flores et al. 1996).

The less common category III response (α4β2-like) was seen in approximately 4% of large neurons and <1% of small neurons. These responses were remarkably insensitive to cytisine and were antagonized by DHβE. Our results therefore suggest that functional α4β2 nAChRs are expressed in only a small percentage of DRG neurons, although it is unknown whether this expression is rare in all classes of DRG neurons, or possibly restricted to a modality-specific subpopulation. The low prevalence of category III responses in our culture preparation closely matched the distribution of α4 mRNA transcripts in chick DRGs (Boyd et al. 1991), although less so with the distribution in adult rat trigeminal ganglion (Flores et al. 1996), where a more diffuse labeling was seen in up to 60–80% of neurons.

Category IV responses remain unclassified due to rare expression in our culture conditions, although the strikingly different time course, amplitude, and pharmacology are worthy of note. Category IV responses were seen in 3% of large neurons and 1% of small neurons and could be activated by nicotine, epibatidine, or choline but not cytisine.

It should be noted that other nAChR subunits are likely to be included in each of the receptor categorizations above. α5, α6, and β3 subunit mRNA expression was also detected in DRG tissue by our RT-PCR analysis, and these subunits have been shown to co-assemble with other nAChRs to create more diverse receptor profiles. For example, α5 addition to α3β4 or α3β2 receptors expressed in *Xenopus* oocytes enhances receptor desensitization and increases Ca²⁺ permeability (Gerzanich et al. 1998). α6 and β3 might also contribute to functional nAChRs. These subunits are largely colocialized in the CNS (Le Novere et al. 1996), and mRNAs for both subunits are coexpressed in a subpopulation of nicotine-responsive locus.
coeruleus neurons (Lena et al. 1999). We do not know whether these subunits contribute to the category IV response, or whether this response is due to an unidentified receptor protein complex. The suggested receptor subunit compositions outlined above are likely to represent minimal receptor complexes that may in fact contain additional nAChR subunits (Lukas et al. 1999).

While the present results are derived from agonist applications onto the somata of cultured DRG neurons, it is reasonable to question whether nAChRs are also expressed on the terminals of DRG afferents. Evidence for peripheral nAChR expression comes from single fiber nerve recordings by Steen and Reeh (1993), who demonstrated that nicotinic activation of peripheral nerve terminals can result in excitation. Single-unit spinal cord microelectrode recordings have also demonstrated that most wide-dynamic range dorsal horn neurons respond to intracutaneous nicotine application (Jinks and Carstens 1999), and nicotinic agonists have also been shown to excite afferents in the rabbit cornea (Tanelian 1991). Furthermore, Ninkovic and Hunt (1983) observed evidence for both peripheral and central and transport of α-BTX binding sites on sensory neurons, and dorsal horn α-BTX binding is largely eliminated after dorsal rhizotomy.

The spinal cord contains a subpopulation of inhibitory interneurons that possess the synthesizing enzyme for acetylcholine (ChAT, choline acetyltransferase), and ChAT-positive varicosities can make presynaptic contact onto the terminals of primary afferents in the dorsal horn (Barber et al. 1984; Ribeiro-da-Silva and Cuello 1990; Todd 1991). Acetylcholine in the spinal cord might therefore activate nAChRs expressed on the central terminals of DRG afferents. It is also possible that choline, a product of ACh degradation by acetylcholinesterase, may also activate these receptors depending on concentrations at the synapse. The sources of acetylcholine that could activate nAChRs on peripheral nerve terminals are much more speculative, although there is evidence that many nonneuronal cells either contain or can manufacture acetylcholine (Wessler et al. 1999).

While peripheral applications of nicotinic agonists can cause the excitatory effects described above, behavioral experiments have demonstrated that nicotinic agonists can also have analgesic properties when delivered via multiple routes of administration (Davis et al. 1932; Masner 1972; Sahley and Berntson 1993). Epibatidine is a nicotinic agonist with an analgesic potency greater than morphine (Spande et al. 1992), and ABT-594 (a novel nicotinic agonist synthesized by Abbott Laboratories) can produce analgesia when administered systemically, intradermally, or centrally in the nucleus raphe magnus, a site involved in the descending modulatory system of analgesia (Bannon et al. 1998; Bitner et al. 1998; Boyce et al. 2000; Kesingland et al. 2000). While some reports have identified dose-dependent analgesia after both intrathecal and subcutaneous applications of nicotinic agonists (Damaj et al. 1998), others demonstrate irritable and autonomic responses (Khan et al. 1994) or hyperalgesia at subanalgiesic doses (Masner 1972).

It is likely that the variation in response to nicotinic agonist administration in vivo may involve a number of factors, and identification of nAChR properties on sensory neurons in vitro will help reveal the underlying mechanisms.

Excitatory effects of nicotinic agonists are primarily due to nAChR activation, and subsequent neuronal depolarization leads to Na⁺ or Ca²⁺-channel activation. Inhibitory effects may be due to receptor desensitization, Na⁺ or Ca²⁺-channel inactivation, Ca²⁺-mediated second-messenger pathways, or simply current shunting. Of the inhibitory effects, receptor desensitization occurs at much lower agonist concentrations than those needed for activation (Fenster et al. 1997) and may also play an important role in modulating sensory activity.

Discrepancies between analgesic, hyperalgesic, or irritative actions of nicotinic ligands might therefore be due to a complex balance between receptor expression patterns and basic drug pharmacokinetics and pharmacodynamics. The fact that a majority of large DRG neurons express functional nAChRs implies that modulation of nonnociceptive modalities could contribute to the behavioral effects of these compounds. For example, subpopulations of visceral sensory afferents in the nodose ganglia respond to cholinergic agonists (Baccaglini and Cooper 1982) and are labeled by α-BTX autoradiography (Ashworth-Preece et al. 1998), supporting the idea that nAChRs may play a role in the modulation of visceral as well as somatic sensory transmission. Understanding the sensory effects of nicotinic agonists will necessarily involve identifying the specific nAChRs expressed at each location along these sensory pathways.

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