Characterization of Neuronal Nicotinic Acetylcholine Receptors in the Membrane of Unmyelinated Human C-Fiber Axons by In Vitro Studies

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Lang, P. M., R. Burgstahler, W. Sippel, D. Irnich, B. Schlotter-Weigel, and P. Grafe. Characterization of neuronal nicotinic acetylcholine receptors in the membrane of unmyelinated human C-fiber axons by in vitro studies. J Neurophysiol 90: 3295–3303, 2003. First published July 23, 2003; 10.1152/jn.00512.2003. Application of acetylcholine to peripheral nerve terminals in the skin is a widely used test in studies of human small-fiber functions. However, a detailed pharmacological profile and the subunit composition of nicotinic acetylcholine receptors in human C-fiber axons are not known. In the present study, we recorded acetylcholine-induced changes of the excitability and of the intracellular Ca2+ concentration in C-fiber axons of isolated human nerve segments. In addition, using immunohistochemistry, an antibody of a subtype of nicotinic acetylcholine receptor was tested. Acetylcholine and agonists reduced the current necessary for the generation of action potentials in C fibers by ≤30%. This increase in axonal excitability was accompanied by a rise in the free intracellular Ca2+ concentration. The following rank order of potency for agonists was found: epibatidine > 5-Iodo-A-85380 > 1,1-dimethyl-4-phenylpiperazinium iodide > nicotine > cytisine > acetylcholine; choline had no effect. The epibatidine-induced increase in axonal excitability was blocked by mecamylamine and, less efficiently, by methyllycacontine and dihydro-β-erythroidine. Many C-fiber axons were labeled by an antibody that recognizes the 5 subunit of nicotinic acetylcholine receptors. In summary, electrophysiological and immunohistochemical data indicate the functional expression of nicotinic acetylcholine receptors composed of α3, α5, and β4 but not of α4/β2 or of α7 subunits in the axonal membrane of unmyelinated human C fibers. In addition, the observations suggest that the axonal membrane of C fibers in isolated segments of human sural nerve can be used as a model for presumed cholinergic chemosensitivity of axonal terminals.

INTRODUCTION

The evaluation of unmyelinated C-fiber function in the peripheral human nervous system is not possible with conventional electrophysiological techniques. As an alternative, unmyelinated sympathetic efferents and nociceptive afferents can be tested by transdermal iontophoresis of acetylcholine (ACh) to nerve endings in human skin. In such experiments, ACh induces sweat production by activation of sympathetic sudomotor axons (Low et al. 1983) and flare by release of vasoactive neuropeptides from terminals of nociceptive afferents (Benarroch and Low 1991; Parkhouse and Le Quesne 1988; Walmsley and Wiles 1990). Many investigators have used this technique for the evaluation of C-fiber function in diabetic and other neuropathies (Caselli et al. 2003; Kilo et al. 2000; Low 1993; Walmsley and Wiles 1991). Important components of the ACh-induced axon reflexes have been demonstrated already. First, a contribution of nicotinic receptors in the membrane of C-fiber axons is revealed by the antagonism of hexamethonium (Benarroch and Low 1991). Second, the conduction of action potentials via axon collaterals to a region outside the area of ACh application has been confirmed by dermal anesthesia (Caselli et al. 2003). However, to our knowledge, a detailed analysis of the subtype of nicotinic receptor involved in the generation of ACh-induced axon reflexes has not been performed.

Neuronal nicotinic ACh receptors (nAChRs) are composed of various pentameric combinations of ≥11 subunits (α2–α7, α9, α10, β2–β4). Major nAChR subtypes present in the central and/or the peripheral nervous system are α4β2, α3α5β4, and a homomeric α7 subtype (Lukas et al. 1999; Paterson and Nordberg 2000). The various nAChR subtypes show distinct pharmacological profiles and can be separated by differences in the rank order of potency for receptor agonists and antagonists. Recently, we have shown that functional studies of ligand-gated ion channels in human C-fiber axons can be performed using threshold tracking (Bostock et al. 1998) of compound action potentials in isolated segments of human nerve fascicles (Irnich et al. 2002; Lang et al. 2002). In the present study, we demonstrate that agonists at nAChRs increase the excitability of C-fiber axons in the trunk of the human sural nerve. The possible subunit composition of the underlying receptor(s) is discussed according to the pharmacological profile and observations obtained by using immunohistochemistry.

METHODS

Human sural nerves

The experiments on isolated segments of human sural nerves were carried out on 81 fascicles from 18 patients. Approval for this procedure was obtained from the Ethics Committee of the University of Munich, and the patients gave written informed consent. The patients (male, n = 14; female, n = 4) had a median age of 68 yr (47–86) at the day of surgery. Seven of the patients underwent a biopsy of the sural nerve for histological examination, and in 11 patients, an amputation of the lower limb below the knee was performed (9 patients due to peripheral vascular diseases and 2 for other reasons). The isolated sural nerves were cut in segments of 15–25 mm in length and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
single nerve fascicles were dissected under a microscope (Quasthoff et al. 1995).

**Experimental setup**

The experimental setup used in the present study has been described before (Lang et al. 2002). In brief, isolated human nerve fascicles were held at each end by suction electrodes in an organ bath. One suction electrode was used to elicit action potentials, while the other was used as a recording electrode. The distance between stimulating and recording electrodes was ~4 mm. The organ bath (volume: 1 ml) was continuously perfused with solution at a flow rate of 6–8 ml/min, at a temperature of 32°C. The perfusion solution contained (in mM) 118 NaCl, 3.0 KCl, 1.5 CaCl$_2$, 1.0 MgCl$_2$, 5.0 NaHCO$_3$, and 1.2 NaH$_2$PO$_4$. A small decrease of the baseline. Antagonists at nAChRs were usually given 10 min prior to the application of the agonists.

**Threshold tracking**

Axonal excitability was measured using the threshold tracking technique, making use of the QTRAC program (Institute of Neurology, London, UK). QTRAC is a flexible, stimulus-response data-acquisition program, originally written for studies of human nerves in vivo (Bostock et al. 1998; Burke et al. 2001) but also suitable for electrophysiological recordings from isolated human peripheral nerve (Grafe et al. 1997; Irnich et al. 2002; Lang et al. 2002). In the present study, QTRAC was used to record compound action potentials from peripheral C fibers, to generate stimuli, and to display the results. Isolated fascicles were stimulated with a linear stimulus isolator (A395, WPL Sarasota, FL) with a maximal output of 500 μA. The stimulus was controlled by a computer via a data-acquisition board (Data Translation DT2812, Marlboro, MA). Nerve excitability was tested with 1-ms current pulses, automatically adjusted to maintain the constant supramaximal stimulus-induced compound action potential (CAP) at a constant amplitude (40% of the maximum, defined as “threshold”). This test pulse was preceded for 50 ms by a hyperpolarizing prepulse (amplitude: 20% of the test pulse). This prepulse was used to compensate for a possible axonal membrane depolarization due to the dissection procedure.

**Intracellular Ca$^{2+}$ concentration**

Nerve fascicles were loaded with membrane-permeable esters of the fluorescent dyes Calcium Green-1 and Fura Red (Molecular Probes, Leiden, The Netherlands) by bath application (Mayer et al. 1998). The organ bath containing the nerve fascicles was mounted on an inverted fluorescence microscope (Axiovert 35, Zeiss Jena, Germany) with a custom-made photometric attachment, and illuminated at 0.33 Hz with 10-ms light pulses at 485 nm. Intensity of the emitted fluorescent light was measured after filtering by two photodiodes at 530 nm (Calcium Green-1) and 660 nm (Fura Red). Emission intensities were recorded using the QTRAC program. The ratio of the two emission intensities was calculated off-line to give a measure of intracellular Ca$^{2+}$ concentration.

**Immunohistochemistry**

Single-nerve fascicles were cut in small pieces of 2–3 mm in length and treated with collagenase (type XI, 0.3%, 30 min at room temperature). After washing with PBS, the nerve fragments were teased and placed onto gelatin-coated microscope slides. The teased preparations were allowed to air dry overnight before they were fixed in 4% paraformaldehyde (40 min at room temperature) and incubated in 0.4% Tween 20 (40 min at room temperature). Specimens were stained in duplicate overnight at 4°C with the following primary antibodies: goat anti-nAChR α5 (1:200, and corresponding blocking peptide Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-peripherin (1:200, Chemicon, Temecula, CA). Highly cross-adsorbed secondary antibodies Alexa Fluor 488 donkey anti-goat (A-11055), and Alexa Fluor 594 goat anti-mouse (A-11032) were purchased from Molecular Probes and were used at 4 μg/ml (2 h at room temperature). To exclude unwanted cross-reactivity of the secondary antibodies, staining was performed sequentially with Alexa Fluor 488 donkey anti-goat first, followed, after four washing steps, by incubation with Alexa Fluor 594 goat anti-mouse. Images were digitized using a Bio-Rad MRC-1024 confocal microscope (PMT1: Ex 594 nm, Em: 660DF50; PMT2: Ex 488 nm, Em: 530DF30).

**Chemicals**

Acetylcholine (ACh), ATP, 1–1-dimethyl-4-phenyl-piperazinium iodide (DMP), choline chloride, collagenase type XI, dihydro-beta-erythroidine hydrobromide (DHßE), mecamylamine hydrochloride, nicotine, phosphate buffered saline (PBS), and TWEEN 20 were purchased from Sigma (Taukirchen, Germany). (−)-Cytisine, (±)-epibatidine, 5-iodo-A-85380, and methyllycaconitine (MLA) were purchased from Biotrend (Köln, Germany), and capsaicin from Roth (Karlsruhe, Germany). Agonists at nAChRs were administered for 1–3 min with application free intervals of ≥10 min after recovery to baseline. Antagonists at nAChRs were usually given 10 min prior to the application of the agonists.

**RESULTS**

**C-fiber CAPs of isolated human sural nerve**

Recordings of compound action potentials from isolated fascicles of human sural nerves have been described in detail (Lambert and Dyck 1993; Quasthoff et al. 1995). A representative example of compound action potentials obtained in the present study is illustrated in Fig. 1. Fast-conducting A fibers and slowly conducting C fibers were separated by adjusting the stimulus strength (5–500 μA) and the stimulus duration (0.1–1 ms). Quantitatively, the amplitude of the C-fiber compound potential (Fig. 1C) varied between 0.1 and 1.5 mV [0.56 ± 0.31 (SD) mV; n = 81]. Compound C-fiber action potentials could be recorded for ≥48 h after the excision of the nerves when stored at 4°C.

**Agonists at nAChRs enhance the excitability of human C-fiber axons**

Changes in the excitability of human C axons induced by ACh are revealed by threshold tracking. Three stimulus conditions were tested in succession, using three channels of the program QTRAC (Fig. 2). When tested using a constant supramaximal stimulus, a small or no decrease in the amplitude of the C-fiber CAP was observed during bath application of 100 μM ACh (Fig. 2, channel 1). However ACh induced an increase in the amplitude of the submaximal C-fiber compound potential. This change in the peak amplitude resulted in a reduction of the stimulus current (threshold current) necessary to maintain the submaximal peak at 40% of the maximum (Fig. 2, channels 2 and 3). A decrease in the threshold current in combination with a constant supramaximal stimulus-induced CAP clearly indicates an increase in the excitability of C-fiber axons.

In a series of experiments, concentration-response curves for agonists at nAChRs in unmyelinated human C-fiber axons were determined. A typical example for these experiments is illustrated in Fig. 3. Nicotine was applied in ascending concentrations via the bathing solution. A small decrease of the
threshold current was seen at the least effective concentration (3 μM). At higher concentrations, the tracking speed was not fast enough to maintain the submaximal CAP at a constant amplitude. Furthermore, at the highest concentration tested (30 μM), nicotine produced a decrease in the amplitude of the supramaximal response. As a consequence of these multiple effects (on threshold current, peak amplitude of submaximal supramaximal response), concentration-response curves were plotted for the minimal effective concentration, i.e., the lowest concentration at which agonists at nAChRs produce changes in threshold current only. The data are plotted in Fig. 4 and reveal a pharmacological profile with the following order of potency: epibatidine ≫ 5-Iodo-A-85380 > DMPP > nicotine > cytisine > acetylcholine. In addition, effects of choline (10 mM, an agonist at the α7 subtype of nAChRs) was tested on the excitability of C-fiber axons in four fascicles from three different human sural nerves. Application of choline to the bathing solution did not induce an increase in axonal excitability (Fig. 6A).

In few experiments, the application time for agonists at nAChRs was prolonged for more than the usual 1–3 min. Under these conditions, receptor desensitization well known for nAChRs (e.g., Tan et al. 1998 in sympathetic ganglion neurons) was revealed. A typical experiment is illustrated in Fig. 5. DMPP (30 μM) was applied to the bathing solution for 11 min. The effects of this agonist on the supramaximal stimulus-induced CAP, on the peak of the CAP produced by submaximal stimulation, and on the threshold current faded completely within this time period.

**Pharmacological profile of nAChR antagonists**

The various subunit compositions of nAChRs can be separated by pharmacological antagonists. Therefore the effects of DHβE, mecamylamine, and MLA (widely used antagonist for discrimination of α4/β2, α3/β4, and of α7 subunits) were tested on the increase in C-fiber excitability produced by epibatidine (10 nM). Representative experiments are illustrated in Fig. 6, B and C, and a summary of the data is plotted in Fig. 7. It was found that mecamylamine strongly reduced the epibatidine-induced increase in excitability in concentrations >3 nM. In contrast, DHβE had a blocking effect when applied in concentrations ~1,000 times higher as compared with mecamylamine. MLA antagonized the epibatidine-induced increase in axonal excitability only in concentrations higher than those effective at α7 subunits (Zhao et al. 2003).

**Effects on intracellular Ca²⁺ concentration**

Epibatidine was tested for effects on the free intracellular Ca²⁺ concentration in isolated segments of human sural nerve. In these experiments, possible Ca²⁺ transients in axons, Schwann cells, and/or other cell types within the endoneurium were registered by photometry. A representative example is illustrated in Fig. 8. Application of either epibatidine (1 μM), capsaicin (1 μM), or ATP (100 μM) to the bathing solution led to a rise in [Ca²⁺], and an increase in axonal excitability. The magnitude of the effects on [Ca²⁺] differed considerably. ATP had the most prominent effect (P2Y receptors in Schwann cells, see Mayer et al. 1998), capsaicin produced a smaller rise in [Ca²⁺], and epibatidine had an effect of [Ca²⁺], close to the detection limit of the method only. However, the epibatidine-induced changes in the emissions at the two wavelengths used (530 nm for Calcium Green and 660 nm for Fura Red) were in opposite directions (see Fig. 8, inset), and such a ratiometric behavior excludes possible artifacts such as a movement of the preparation. Effects of epibatidine on [Ca²⁺], were tested in different concentrations and a transient rise in [Ca²⁺], was seen at 50 nM, 0.1 μM, and 1 μM (n = 5 in 3 different preparations). Effects of epibatidine on [Ca²⁺], were blocked by mecamylamine (1 μM; n = 2; not illustrated).

**Immunohistochemistry**

The pharmacological profile described in the preceding text indicates a contribution of α3 subunits to the effects of ACh on the excitability of human C-fiber axons (see Discussion). α3-subunits can be present in two subtypes of nAChRs. These subtypes are composed either of α3 plus β subunits or of α3 in combination with α5 and β subunits. A separation of these two subunit combinations by a pharmacological profile is not clear cut (see Discussion). Therefore in immunohistochemical studies, we analyzed unmyelinated axons in six different nerve fascicles for the presence of the α5 subunit of nAChRs. Unmyelinated axons were identified by immunostaining of peripherin, a neurofilament present in sensory and sympathetic C-fibers (Derer et al. 1989; Goldstein et al. 1991). Double staining of six different teased nerve fiber preparations with anti-α5 and anti-peripherin antibodies revealed co-localization of both proteins in many of the unmyelinated axons. A separation of these two subtypes of nAChRs was not possible.
myelinated axons (Fig. 9, A and B). In contrast, myelinated nerve fibers (identified in images using light transmission) were not immuno-positive for α5-subunits (Fig. 9, C and D).

**DISCUSSION**

**Axonal localization of nAChRs**

Excitatory effects of ACh on unmyelinated fibers in the peripheral mammalian nervous system are known for many decades (Douglas and Ritchie 1960), and a depolarizing effect of ACh has been recorded in single mammalian ganglion cells (Genzen et al. 2001; Liu et al. 1993; Wood and Docherty 1997). Electrophysiological studies on single human or rat C-fiber axons have demonstrated that sensitivity to ACh can be found in unmyelinated sympathetic efferents (Schmidt et al. 1999) and in unmyelinated sensory afferents (Bernardini et al. 2001; Schmelz et al. 2003; Steen and Reeh 1993). However, there is less agreement whether receptors for ACh...
are restricted to nerve endings or whether they are also functionally active in the trunk of an unmyelinated peripheral axon. For example, Diamond (1959) has injected ACh intra-arterially into the rabbit’s sural nerve in vivo, and he has found that it failed to excite fibers of which the axons but not the endings were exposed to the drug (citation in Douglas and Ritchie 1960). In contrast, direct depolarizing effects of ACh on unmyelinated axons were seen in the isolated rabbit vagus nerve (Armett and Ritchie 1963) and in human C axons by the method of threshold tracking used in the present study. A possible explanation for these findings is that activation of axonal ACh receptors produces membrane depolarization and an increase in membrane excitability which is not sufficient for generation of action potentials. Alternatively, it has been discussed that damage of axons due to nerve transection produces abnormal chemosensitivity of the axonal membrane e.g., by insertion of receptors into the axonal membrane (Michaelis et al. 1997). We cannot disprove this hypothesis, however, effects of ACh on human C-axons were seen already one hour after transection of the sural nerves and an increase in the sensitivity to ACh was not observed in the following 48 h. We assume that such a time-dependent effect should be seen if more and more channels are translocated from the cytoplasm to the axonal membrane.

Activation of nAChRs on nonneuronal cells in the surrounding of axons might contribute to an increase in axonal excitability, e.g., by release of K⁺ or other chemical mediators. We cannot completely exclude this possibility, however, clear evidence for the presence of axonal nAChRs was found in our immunohistological studies (see Fig. 9). Furthermore, in a previous study (Lang et al. 2002), changes in extracellular K⁺ activity due to opening of ionotropic axonal receptors (P2X) in an isolated peripheral nerve preparation were measured by

![Graph showing minimal effective concentrations of agonists at nicotinic ACh receptors (nAChRs) in human C-fiber axons.](https://example.com/graph.png)

**FIG. 4.** Minimal effective concentrations of agonists at nicotinic ACh receptors (nAChRs) in human C-fiber axons. All 6 agonists were tested for the lowest concentration necessary for a decrease in threshold current (i.e., an increase in axonal excitability). The complete concentration-response curve has not been determined because, at higher concentrations of the agonists, effects on the peak amplitude of the C-fiber CAP interfered with the determination of excitability (i.e., changes in threshold current at constant supramaximal peak amplitude). All agonists were applied for 3 min to the bathing solution (data in the plot show means ± SE; number of observations for each agonist are given at right).

![Graph showing desensitization observed during long-lasting application of agonists at nAChRs.](https://example.com/graph.png)

**FIG. 5.** Desensitization observed during long-lasting application of agonists at nAChRs. Illustrated are the effects of 1-1-dimethyl-4-phenyl-piperazinium iodide (DMPP) in high concentration (30 μM) on the peak amplitude of the supramaximal stimulus-induced CAP (top) and on the peak amplitude and the stimulus strength used to maintain the action potential at 40% (threshold current) of the supramaximal stimulus-induced CAP (middle and bottom). Note, that all parameters returned to baseline values in spite of the continuous presence of this agonist at nAChRs in the bathing solution.

![Graph showing components in the pharmacological profile of nAChRs in human C-fiber axons.](https://example.com/graph.png)

**FIG. 6.** Components in the pharmacological profile of nAChRs in human C-fiber axons. A: epibatidine (10 nM) enhances the excitability (measured as reduction in threshold current) of unmyelinated human axons. No such effect was observed by bath application of choline (10 mM). B and C: differential sensitivity to mecamylamine and dihydro-β-erythroidine hydrobromide (DHβE) shown in representative recordings. The increase in excitability caused by epibatidine (10 nM) is reduced in the presence of nanomolar concentrations of mecamylamine. In contrast, the antagonistic effect of DHβE was observed in micromolar concentrations only. The experimental protocol is similar to Fig. 3; a summary of data is illustrated in Fig. 7.
K⁺-sensitive microelectrodes. It was observed that the extra- cellular K⁺ concentration increases by ~250 µM in the initial phase of the membrane depolarization only. The time course of this effect does not correlate with the changes in excitability and/or peak amplitude. The possible release of a chemical mediator by agonists at nAChRs cannot be excluded. However, the characteristics of the electrophysiological changes (including desensitization) resemble observations made when agonists at nAChRs are applied directly to single peripheral neurons (e.g., Tan et al. 1998).

**Subunit composition derived from the pharmacological profile and immunohistochemistry**

The isolated human nerve fascicles used in the present study enable a detailed pharmacological profile of ACh-induced changes in membrane excitability. The possible subtype composition of nAChRs present in unmyelinated axons in a peripheral human nerve can be derived from this profile. An

**FIG. 7.** Concentration-response curves for antagonists at nAChRs in human C-fiber axons. Antagonists at nAChRs were tested for an inhibitory effect against an increase in C-fiber excitability produced by epibatidine (10 nM, normalized to 100%). All compounds were applied via the bathing solution for ≥10 min before the effects of epibatidine were tested (data in the plot show means ± SE; number of observations for each agonist are given at right).

**FIG. 8.** Epibatidine induces an increase of [Ca^{2+}] in human sural nerve. Simultaneous registration of changes in [Ca^{2+}], and C-fiber excitability (threshold current necessary to maintain the peak amplitude at 40% of the maximum) from an isolated fascicle of human sural nerve. Epibatidine, capsaicin, and ATP produced both, a transient rise in [Ca^{2+}], and an increase in axonal excitability. *Inset:* the opposite changes in the emission of the Ca²⁺-sensitive dyes (Calcium Green, 530 nm; Fura Red, 660 nm; observation in a different nerve fascicle).

**FIG. 9.** Immunohistochemical evidence for the α5 subunit of nAChRs in human C-fiber axons. A and B: laser scanning confocal images of teased nerve fiber preparation show colocalization of nAChR-α5 (A) and peripherin (B). C and D: confocal images from the same area within the preparation reveals binding of anti-nAChR-α5 antibody to unmyelinated (C) but not to myelinated (D) axons. E: lower magnification shows the labeling of many unmyelinated C-fibers with the nAChR-α5 antibody. F: a control image was obtained from a teased nerve fiber preparation by treatment with anti-nAChR-α5 antibody that was preincubated with blocking peptide and subsequently stained with Alexa Fluor 488-labeled anti-goat antibody. A–D and F: objective, ×60; E: objective, ×40; all scale bars: 20 µm.
important contribution of α2 or α4 subunits can be excluded because 5-Iodo-A-85380, an agonist about equipotent to epibatidine at the α3β2 subtype (Mukhin et al. 2000), was ~100 times less potent than epibatidine on human C-fiber axons (Fig. 4). Furthermore, in cells that have been transfected with combinations of human α2β4 or α4β4, cytisine had much stronger effects as compared with DMPP (Stauderman et al. 1998). The opposite rank order of potency (DMPP > cytisine) was found in human C-fiber axons (Fig. 4). Such a high sensitivity to DMPP has been observed in cells expressing a composition of human α3 and β2 or β4 subunits (Chavez-Noriega et al. 2000; Stauderman et al. 1998).

Further clues for the functional importance of the α3 and β4 subunits comes from the rank order of potency for antagonists. The observation that mecamylamine is more potent than DHβE in blocking epibatidine-induced responses (see Figs. 6 and 7) is compatible with the presence of α3 but not of α4 subunits (Chavez-Noriega et al. 2000; Harvey et al. 1996). Also, DHβE is less potent at combinations of α3β4 as compared with α3β2 subunits (Harvey and Luetje 1996).

The immunohistochemical data indicate that the α5 subunit is also expressed by human C-fiber axons. Binding of the antibody against the α5 subunit was found in many of the unmyelinated axons in the human sural nerve fascicles (see Fig. 9). Whether α5 subunits contribute to the pharmacological profile is difficult to answer. Transfection of α3β4 and α3β2 cell lines with the α5 subunit had little effect on their agonist sensitivities (Gerzanich et al. 1998; Nelson et al. 2001). On the other hand, a functional contribution of α5 subunits not only to agonist but also to antagonist sensitivity of natively expressed nAChR channels has been described for chick sympathetic ganglion neurons (Yu and Role 1998).

Our findings on human axons are in general accordance with observations made in the peripheral nervous system of other mammalian species. The importance of α3, α5, β2, and β4 subunits for peripheral autonomic neurons has been revealed in electrophysiological and/or immunohistochemical studies in rat superior cervical ganglia (Skok et al. 1999), in guinea pig myenteric neurons (Zhou et al. 2002), and in the autonomic nervous system of mice (Wang et al. 2002). Furthermore, high levels of antibodies against the α3 subunit have been found in autoimmune autonomic neuropathies (Lennon et al. 2003; Vernoino et al. 2000). Also in afferent sensory neurons, expression of α3 and β4 has been found (Flores et al. 1996).

A study on cultured DRG neurons from postnatal rats came to the conclusion that nicotinic responses in 77% of the large neurons and in 32% of the small neurons are caused by the α7 subunit of nAChRs (Genzen et al. 2001). We did not find functional evidence for activation of α7 subunits in unmyelinated human axons because two characteristics of this subunit, i.e., activation by choline and blockade by low concentrations of MLA, could not be observed. It is known that ACh-induced membrane currents induced by binding to the α7 subunit are rapidly desensitizing, and the bath application used in the present study might be too slow for such an activation. In addition, developmental changes in the expression of nAChR subunits have been described (Zoli et al. 1995) that may explain differences between cultured postnatal rat DRGs and adult human axons. Sensory neurons in DRGs also express the α9 and α10 subunits of nAChRs (Lips et al. 2002). A contribution of these subunits to the effect of ACh on human C-fiber axons is unlikely because the pharmacological profile (Rothlin et al. 2003) is very different from the one observed in the present study.

**Function of axonal nAChRs**

Although ACh is clearly able to excite a variety of sensory receptors, the involvement of ACh in normal or pathological activation of unmyelinated nerve fibers remains to be established (Carr and Prosko 1996). The presence of nAChRs in the axonal membrane of human peripheral C axons is another example for nonsynaptic nicotinic receptors in neurons (Vizi and Lendvai 1999). However, the function of axonal receptors is unclear. One reason is that the possible source(s) for release of ACh in the trunk of a peripheral nerve have not been identified (yet). Such cells may be Schwann cells (Dennis and Miledi 1974; Evans et al. 1999), keratinocytes (Sharma and Vijayaraghavan 2002), endothelial cells (Macklin et al. 1998), and cholinergic sudomotor axons (Low 1993). It is possible that release of ACh from such cells in the vicinity of unmyelinated axons in the trunk of a peripheral nerve may contribute to ectopic axonal excitation in inflammatory or neuropathic pain (Scholz and Woolf 2002).

Interestingly, systemically administered nicotine results in an antinociceptive effect. This phenomenon has been attributed mainly to the activity of α4 subunits in the CNS because it is reduced in α4 subunit knockout mice (Marubio et al. 1999; Picciotto et al. 2001). However, in models of neuropathic pain states, local administration of agonists at nAChRs into the hindpaw (Bannon et al. 1998) or onto dorsal root ganglia (Ruetter et al. 2003) also produced antinociceptive effects. The mechanism underlying this peripheral site of action is unclear. A finding in the present study referring to this is the decrease in the amplitude of the C-fiber CAP seen at higher concentrations of agonists at nAChRs (see Figs. 2, 3, and 5). This effect may be caused either by fewer fibers contributing to the CAP or by a decrease in the amplitude of individual action potentials (due to an increase in membrane conductance or inactivation of sodium channels during membrane depolarization). The data suggest that agonists at nAChRs should be tested for their potential ability to prevent the generation of ectopic action potentials at sites of peripheral nerve injury.

In conclusion, studies on isolated human nerve fascicles indicate that chemosensitivity to ACh expected at nerve terminals can be studied at the axonal membrane. The use of isolated human nerve fascicles in combination with threshold tracking enables the recording of a pharmacological profile of nAChRs in unmyelinated human axons. Based on these data, the possible subunit combination (α3, α5, and β4) can be derived. It is likely, that isolated human C-fiber axons can be useful for studies of abnormal sensitivity to ACh in neuropathies and may be a helpful supplement in quantitative sensory tests of small fiber functions.

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**DISCLOSURES**

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