ACCELERATED COMMUNICATION

Inhibition of Tetrodotoxin (TTX)-Resistant and TTX-Sensitive Neuronal Na⁺ Channels by the Secretolytic Ambroxol

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ABSTRACT

Ambroxol has a long history for the treatment of airway diseases because of its beneficial effects on surfactant synthesis and mucus-modifying properties. Some findings, however, point to an additional effect on neuronal signal transduction: ambroxol can suppress reflexes such as the cough or the corneal reflex. The airways and the cornea are innervated by C-fibers, which express voltage-gated Na⁺ channels with and without sensitivity to tetrodotoxin (TTX). In this study, we performed voltage-clamp experiments to investigate whether ambroxol affects these channel types. In rat dorsal root ganglia, TTX-resistant Na⁺ currents were suppressed in a concentration-dependent manner with IC₅₀ values of 35.2 and 22.5 µM for tonic and phasic block, respectively. Depolarizing prepulses increased the potency of ambroxol, and steady-state inhibition curves were shifted to more negative values. The inhibition was not frequency-dependent. TTX-sensitive currents were inhibited with lower potency (~50% inhibition with 100 µM). Recombinant rat brain IIA channels in Chinese hamster ovary cells were blocked with IC₅₀ values of 111.5 and 57.6 µM for tonic and phasic block, respectively; in contrast to TTX-resistant channels the block was frequency-dependent. Thus, ambroxol indeed blocks neuronal voltage-gated Na⁺ channels, and TTX-resistant channels in sensory neurons were more sensitive than TTX-sensitive. Compared with known local anesthetics (e.g., lidocaine or benzocaine), the potency for Na⁺ channel block was relatively high. A recent clinical trial has further confirmed that ambroxol relieved pain and was beneficial in patients who suffered from sore throat.

Ambroxol is a well-known medication for the treatment of disorders of the respiratory system. It is an effective expectorant, has been shown to normalize the structure of surfactant, to prevent the infant respiratory distress syndrome, and to be effective for the treatment of bronchitis (Renovanz, 1975; Laoag-Fernandez et al., 2000; Matthys et al., 2000). Interestingly, it has been reported that ambroxol has pharmacological effects that cannot be explained by its already described properties on airway epithelia: this compound has been shown to suppress the cough reflex. Moreover, instillation of ambroxol into the eye inhibited the corneal reflex (Klier and Papendick, 1977; Karlsson, 1996; Nemcekova et al., 1998). A recent study showed that ambroxol suppressed the pain associated with sore throat (Fischer et al., 2002). These findings support the hypothesis that ambroxol can affect neuronal excitation and/or signal transduction in sensory neurons.

In the airways, as well as in the cornea, irritant stimuli are detected and encoded by C-fiber neurons (Lalloo et al., 1996; Brock et al., 1998), and one key mechanism for the suppression of neuronal signal transduction is the blockade of voltage-gated Na⁺ channels. In this study, therefore, we investigated whether ambroxol interacts with voltage-gated Na⁺ channels. We performed voltage-clamp experiments on small (C-fiber) neurons of rat dorsal root ganglia, as well as on cells transfected with rat brain type IIA α subunits to investigate whether ambroxol inhibited Na⁺ channel block was relatively high. A recent clinical trial has further confirmed that ambroxol relieved pain and was beneficial in patients who suffered from sore throat.

Materials and Methods

Cell Culture. Adult Wistar rats, weighing 150 to 300 g, were anesthetized with Ethane (Abbott GmbH, Wiesbaden, Germany) and decapitated. The spinal column was removed and immersed in Hibernate A (Invitrogen, Carlsbad, CA), supplemented with 10%
fetal calf serum (Roche Applied Science, Mannheim, Germany) and 1.5% penicillin/streptomycin (Seromed, Berlin, Germany). Approximately 10 to 15 dorsal root ganglia (DRG) were extracted from the full length of the column. Axonal and connective tissue was cut away. The remaining tissue was incubated for 1 h in a solution of trypsin (2.5 µg/ml; Sigma-Aldrich, St. Louis, MO) and collagenase (2.5 µg/ml; Sigma) dissolved in Hibernate A at 37°C. Subsequent passage through soft glass Pasteur pipettes of narrowing diameter produced a homogenous cell suspension. The cells were plated on glass cover slips coated with poly(L-lysine) and laminin and stored under atmospheric conditions at a room temperature of approximately 25°C. Electrophysiological experiments were carried out on cells 1 to 4 days after culture.

Cells stably transfected with rat brain type IIA channel α subunits were cultured as described by West et al. (1992) in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum (Roche Applied Science), 200–400 µg/ml G418 (Geneticin; Invitrogen), and 5.75 mg/ml l-proline (Calbiochem, UK). Cells were plated at low density on untreated coverslips and stored at 37°C in 10% CO₂ and at a relative humidity of 100%. Electrophysiological experiments were carried out 2 to 7 days after seeding.

**Electrophysiology.** Na⁺ currents were recorded in the whole-cell, voltage-clamp configuration at room temperature (Hamill et al., 1981). The extracellular solution contained 140.0 mM NaCl, 5.3 mM KCl, 27.0 mM glucose, 10.0 mM HEPES, 0.8 mM MgCl₂, and 1.8 mM CaCl₂, pH 7.4. In most of the experiments on DRG neurons, 300 nM

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**Fig. 1.** Block of TTX-resistant Na⁺ currents in DRG neurons by ambroxol. A, chemical structure of ambroxol. B, inhibition of Na⁺ currents by 30 µM ambroxol. The cell was stimulated with a pulse train of 100 depolarizations (from −100 to 10 mV, 5 Hz frequency). Responses to the first pulse in the absence and to the first and the last pulse with ambroxol are shown. C, concentration-response curve of ambroxol effects on TTX-r currents. IC₅₀ values for tonic (●) and phasic block (○) were 35.2 ± 2.5 and 22.5 ± 3.8 µM, respectively. Channels in cells that had been depolarized before the application of the test pulses were blocked with an IC₅₀ value of 10.5 ± 1.1 µM (○). The inset shows the pulse-dependent current decay for 30 µM ambroxol. All experiments were performed in the presence of 300 nM TTX.

**Fig. 2.** Effects of stimulation frequency on ambroxol block of TTX-r Na⁺ currents. A, original recordings performed in the absence (left) and presence (right) of 30 µM ambroxol. The cell was stimulated with pulses at the indicated frequencies from a holding potential of −100 mV (test potential, 0 mV). Even in the absence of drug, the currents were reduced by the stimulus trains. The 20th response for each frequency is shown. B, responses in the presence of 3 (●), 10 (○), and 30 µM (△) ambroxol plotted against the pulse number. Cells were stimulated with 20 pulses at each of the frequencies given in the graph, and the responses were normalized to the corresponding controls. C, quantitative representation of the data. Means of the responses in the presence of the drug were normalized to the corresponding control values. Ambroxol did not induce significant frequency-dependent block (one-way analysis of variance with Dunnett test; n = 6 per concentration). Experiments were performed in the presence of 300 nM TTX.
tetrodotoxin (TTX) was added. The intracellular medium consisted of 50.0 mM CaCl₂, 90.0 mM CsF, 10.0 mM NaF, 10.0 mM HEPES, 10.0 mM EGTA, and 2.0 mM MgCl₂, pH 7.4. Recording pipettes had resistances of 0.8 to 1.8 MΩ when filled with intracellular solution. Ambroxol was synthesized at Boehringer Ingelheim (Ingelheim, Germany), and stock solutions were prepared in extracellular medium or in DMSO (Carl Roth GmbH, Karlsruhe, Germany).

Currents were recorded using an EPC-9 amplifier (HEKA, Lambrecht/Pfalz, Germany). Voltage-clamp commands were delivered and currents recorded using the TIDA system (HEKA). Whole-cell capacitance was compensated for using the internal voltage-clamp circuitry; usually, 70% of series resistance was compensated. Data analysis and curve fitting was performed using Prism version 3.02 (GraphPad Software, San Diego CA). If not otherwise stated, data points are the means of at least five experiments, and grouped data are reported ± S.E.M.

**Results**

The principal characteristics of TTX-r Na⁺ channel block by ambroxol are illustrated by the current traces in Fig. 1. DRG neurons were voltage-clamped at a holding potential of −100 mV in the presence of 300 nM TTX, and control current traces were recorded in response to a pulse train consisting of 100 depolarizations to 0 mV (of 10-ms duration and 5-Hz frequency). The cell was then exposed to 30 μM ambroxol, and the pulse protocol was repeated. Approximately 50% of the current was tonically blocked in the first pulse at this concentration, and an additional 20% block was observed at

![Fig. 3](Image)

**Fig. 3.** Effects of ambroxol on TTX-r and TTX-s Na⁺ currents assessed in the same cells. A, steady-state inhibition curve of Na⁺ currents in a DRG neuron in the presence of 300 nM TTX (●) and 300 nM TTX with 30 μM ambroxol (○). Data were normalized to the responses at −120 mV and were fitted with Boltzmann functions. Ambroxol shifted the curves by −8.7 ± 0.3 mV (n = 8). B, steady-state inhibition curves of Na⁺ currents in a DRG neuron. From a holding potential of −120 mV, 1-s depolarizing prepulses to various potentials were applied to the cell before the test pulse to 0 mV. The data points were normalized to the response at −120 mV in the absence of the drug. Double Boltzmann functions were fitted to the data, and the differences between their minimum and maximum were used to calculate the relative responses. The more depolarized part was attributed to TTX-r channels (dotted lines) and the more hyperpolarized part to TTX-s channels (solid lines). The contribution of the two channel populations was calculated in the control (●), as well as in the presence of 30 μM ambroxol (○). C, relative TTX-r currents in the presence of various concentrations of ambroxol. With 30 μM drug, approximately 50% block was observed. D, relative TTX-s currents in dependence of the ambroxol concentrations. A nonsignificant apparent increase of the amplitudes could be observed. Ambroxol at 100 μM induced approximately 50% block. Each point in C and D represents data from a single cell. The vehicle values (“veh”) in C and D were obtained from cells that were superfused with drug-free solution between recording the inactivation curves.
cell. A decrease in peak current was observed in control experiments with higher stimulation frequencies. Upon the application of ambroxol there was a concentration-dependent increase in block, but inhibition was almost unaffected by stimulus frequency (Fig. 2, B and C). At the highest concentration tested (30 μM), increasing the pulse frequency to 25 Hz induced only an additional 17% of block.

The fact that depolarization increased the potency of ambroxol (see Fig. 1C) suggested that the compound might preferentially bind to inactivated channels. We therefore tested whether steady-state inactivation curves after increasingly depolarizing pulses (from −110 to −10 mV, 1-s duration, holding potential −120 mV) were shifted by ambroxol in the presence of 300 nM of TTX. Indeed, 30 μM of the drug induced a shift of −8.7 ± 0.3 mV (Fig. 3A).

Sensory neurons have been shown to possess TTX-s as well as TTX-r Na⁺ channels, and we were interested whether ambroxol also affects the former. To assess the effects on both types of channels in the same neuron, we made use of their different voltage-dependencies of inactivation (with V₅₀ values of −45.8 ± 0.9 mV for TTX-r, and −80.3 ± 1.7 mV for TTX-s currents; n = 36, Fig. 3A). A DRG neuron was held at a holding potential of −120 mV, and an inactivation curve was generated by applying increasingly depolarizing pulses (from −110 to −10 mV, 1-s duration) before the test pulse to 0 mV. The data points recorded in the absence and in the presence of ambroxol were fitted by double Boltzmann curves. V₅₀ values were used to identify the part of the curve contributed by each of the channel types (TTX-r was more depolarized and TTX-s was more hyperpolarized). The differences between minimum and maximum of the Boltzmann functions were used to calculate the relative amplitudes of TTX-r and TTX-s current fractions. A typical graph is shown in Fig. 3B. The unblocked fractions of TTX-r and TTX-s are plotted in Fig. 3, C and D, respectively.

These scatterplots show a general decrease in the currents mediated by TTX-r channels. Ambroxol significantly reduced...
the TTX-r currents to ~50% at a concentration of 30 μM, correlating with the IC₅₀ value calculated from the concentration response curve for tonic block (Fig. 1C). TTX-s currents seemed to be half-maximally inhibited with 100 μM of the drug (Fig. 3D). These data points showed a considerably higher scatter compared with those obtained for TTX-r Na⁺ channels. The current means at the concentrations of 3, 10, and 30 μM, however, were not significantly different from those in the controls. This suggested that ambroxol inhibited TTX-s channels with lower potency, compared with their TTX-r counterparts.

In our DRG preparation, we observed only very rarely neurons that had only TTX-s Na⁺ currents. It was therefore not possible to perform a more detailed analysis of ambroxol effects on these channels. Thus, we used a recombinant cell line expressing rat brain type IIA α subunits as a surrogate. This channel type can be assumed to represent the prototype of TTX-s neuronal Na⁺ channels and in terms of ambroxol block should be comparable with TTX-s channels in sensory neurons.

To confirm this assumption, and to characterize the block of rat brain IIA α channels by ambroxol, we recorded concentration-response curves for tonic and phasic block similar to those described for Fig. 1. The only difference in the protocol was that we omitted TTX from the extracellular solution.

In this 100-pulse protocol, 100 μM ambroxol tonically inhibited Na⁺ currents in CNaIIA cells by 50%, and block increased to 70% with pulse 100 (Fig. 4A). In contrast to the block of TTX-r currents, there was a larger difference between tonic and phasic block with IC₅₀ values for tonic and phasic block of 111.5 ± 6.0 and 57.6 ± 4.1 μM, respectively. Na⁺ channels in depolarized cells were half-maximally inhibited with 20.6 ± 2.8 μM. Moreover, pulse-dependent block developed faster, compared with TTX-r currents in DRG neurons. In CNaIIA cells, e-fold increase in block could be observed at 1.06 depolarizing pulses (with 100 μM ambroxol), whereas with 30 μM of the compound 4.5 pulses were required for TTX-r currents in DRG neurons (insets in Figs. 1B and 4B). Steady-state availability curves were shifted −11.8 + 1.2 mV by 100 μM ambroxol (data not shown).

Ambroxol did not significantly alter the kinetics of Na⁺ channel activation and inactivation in CNaIIA cells: when cells were depolarized from −100 to 0 mV, time-to-peak (0.16 ± 0.006 ms for the grouped controls) and time constants of inactivation (0.44 ± 0.02 ms, n = 46) were not changed by ambroxol (Fig. 5, A-C).

The higher discrimination between the IC₅₀ values for tonic and phasic block in CNaIIA cells (ratio of IC₅₀ tonic/phasic block in CNaIIA cells was about 2, compared with about 1.5 for TTX-r currents) suggested that the frequency-dependence of ambroxol block might also be different for TTX-s currents. This was indeed the case; when we applied the experimental protocol described for Fig. 2 to CNaIIA cells, increasing stimulation frequencies did augment the inhibition of rat brain IIA channels, and the application of 25-Hz stimulus trains induced an additional 63% of block, compared with 1 Hz frequency (Fig. 6).

Fig. 6. Effects of stimulation frequency on ambroxol block of Na⁺ currents in CNaIIA cells. A, responses in the presence of 10 (□), 30 (○), and 100 μM (△) ambroxol plotted against the pulse number. Cells were stimulated with 20 pulses at each of the frequencies given in the graph. Data from one typical cell for each concentration are shown. B, quantitative representation of the data. Ambroxol showed a pronounced frequency-dependent block of rbIIA Na⁺ channel α subunits. Same conditions as for the experiments shown in Fig. 2. Statistical significance was tested using one-way analysis of variance with Dunnett test; n = 5 to 9 per concentration. **, p < 0.01.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Summary of the effects of ambroxol on TTX-r and TTX-s Na⁺ channels</th>
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<td></td>
<td>TTX-r</td>
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<tr>
<td>IC₅₀</td>
<td>μM</td>
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<tr>
<td>Tonic³</td>
<td>~30</td>
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<tr>
<td>Tonic²</td>
<td>35.2 ± 2.5</td>
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<tr>
<td>Phasic³</td>
<td>22.5 ± 3.8</td>
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<tr>
<td>Depolarized³</td>
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<tr>
<td>Shift of inactivation</td>
<td>−8.7 + 0.3 mV(@30μM)</td>
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N.T., not tested.

² From analysis of double Boltzmann fits in DRG neurons (Figs. 3).
³ From 100 pulse protocols (Figs. 1 and 4).
⁴ After 5-s depolarizing prepulses to −40 mV (Figs. 1 and 4).
Thus, ambroxol inhibits TTX-r Na⁺ currents in sensory neurons with higher potency, compared with TTX-s channels, but the inhibition is less use-dependent. The main findings of this study are summarized in Table 1.

**Discussion**

In this study we showed that ambroxol inhibits Na⁺ channels in sensory neurons. The potency for tonic block of TTX-r channels is relatively high; in this respect, ambroxol can be compared with, for example, the local anesthetic bupivacaine, which has a reported IC₅₀ value of 32 μM for the tonic inhibition of TTX-r Na⁺ currents in small DRG neurons (in a thin-preparation; Scholz et al., 1998b). In the same study, TTX-s channels were found to be tonically blocked with an IC₅₀ value of 13 μM. This rank order of potency was also reported for lidocaine, as well as the newer Na⁺ channel blockers riluzole and 4030W92 (Song et al., 1997; Scholz et al., 1998b; Trezise et al., 1998; Gold and Thut, 2001). In contrast, ambroxol inhibited TTX-r currents with higher potency compared with their TTX-s counterparts, which sets this compound apart from known Na⁺ channel blockers. To our knowledge, only the volatile anesthetic halothane showed a preference for TTX-r channels (with IC₅₀ values of about 6 mM for TTX-r and 12 mM for TTX-s channels; Scholz et al., 1998a).

Interestingly, ambroxol affected the Na⁺ current kinetics of TTX-r and TTX-s channels differently. In CNaIIA cells, the compound behaved like a charged local anesthetic: the block was dependent on stimulus number and increased with higher frequencies in a train of depolarizing stimuli. Time course of activation, as well as inactivation, was not significantly affected. TTX-r channels behaved differently, showing a low use- and frequency-dependence. In both channel types, however, depolarization increased the block, and steady-state inactivation curves were shifted to more negative values. In this respect, block of TTX-r channels by ambroxol shares similarities with the effects of benzocaine on Na⁺ channels (DeLuca et al., 1991; Baker, 2000). Nevertheless, ambroxol has a much higher (about 10-fold) potency compared with benzocaine. The low use dependence, however, should not affect the ambroxol's analgetic effects. C-fibers have relatively low action potential frequencies; therefore, frequency dependence of a Na⁺ channel blocker can be anticipated to be of limited importance for analgesia (Raymond et al., 1990; Schmelz et al., 1995).

Ambroxol also showed some remarkable properties with respect to differential effects on resting and inactivated channels: In CNaIIA cells, the drug inhibited inactivated channels 5.5-fold more potently than resting channels (Fig. 5C). The corresponding factor for TTX-r channels was only 3.3 (Fig. 1C). Taken together, the mechanism of action on TTX-r and TTX-s channels seems to be different. Future experiments [e.g., using channels with mutated binding sites for local anesthetics (Ragdale et al., 1994; Weiser et al., 1999)] would help to further clarify this issue.

One can speculate that the preferential blockade of TTX-r Na⁺ channels in C-fiber neurons can be synergistic with the already described beneficial effect of ambroxol on respiratory function. A recent clinical study confirmed that ambroxol lozenges relieved pain associated with sore throat (Fischer et al., 2002). The identification of ambroxol’s effects on neuronal signal transduction should open up new avenues for future clinical applications.

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**References**


