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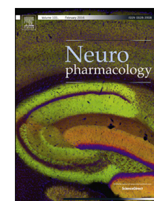

Benzonatate inhibition of voltage-gated sodium currents

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ABSTRACT

Benzonatate was FDA-approved in 1958 as an antitussive. Its mechanism of action is thought to be anesthesia of vagal sensory nerve fibers that mediate cough. Vagal sensory neurons highly express the Nav1.7 subtype of voltage-gated sodium channels, and inhibition of this channel inhibits the cough reflex. Local anesthetics inhibit voltage-gated sodium channels, but there are no reports of whether benzonatate affects these channels. Our hypothesis is that benzonatate inhibits Nav1.7 voltage-gated sodium channels.

We used whole cell voltage clamp recording to test the effects of benzonatate on voltage-gated sodium (Na^+) currents in two murine cell lines, catecholamine A differentiated (CAD) cells, which express primarily Nav1.7, and N1E-115, which express primarily Nav1.3.

We found that, like local anesthetics, benzonatate strongly and reversibly inhibits voltage-gated Na^+ channels. Benzonatate causes both tonic and phasic inhibition. It has greater effects on channel inactivation than on activation, and its potency is much greater at depolarized potentials, indicating inactivated-state-specific effects. Na^+ currents in CAD cells and N1E-115 cells are similarly affected, indicating that benzonatate is not Na^+ channel subtype-specific. Benzonatate is a mixture of polyethoxy esters of 4-(butylamino) benzoic acid having varying degrees of hydrophobicity. We found that Na^+ currents are inhibited most potently by a benzonatate fraction containing the 9-ethoxy component. Detectable effects of benzonatate occur at concentrations as low as 0.3 μM, which has been reported in humans.

We conclude that benzonatate has local anesthetic-like effects on voltage-gated sodium channels, including Nav1.7, which is a possible mechanism for cough suppression by the drug.

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1. Introduction

Benzonatate (Tessalon®) is a non-narcotic cough suppressant approved for human use by the United States Food and Drug Administration (FDA) in 1958 for symptomatic relief of cough in patients over 10 years of age. Benzonatate is classified as an ester-type local anesthetic chemically related to tetracaine, procaine and cocaine. The mechanism of cough suppression by benzonatate is reported to be through inhibition of pulmonary stretch receptors (Pfizer Laboratories, 2014; Michelson and Schiller, 1957; Tomokazu, 1967; Wilson et al., 1958), whose fibers travel in the vagus nerves to the brain. Benzonatate has a numbing effect when applied directly to the oral and pharyngeal mucosa. Direct mucosal application has been used as a method for rapid oral anesthesia for awake intubation (Mongan and Culling, 1992), but for cough relief it is given orally, with drug reaching its site of action through the bloodstream after gastrointestinal absorption. Although benzonatate is reported to inhibit pulmonary stretch receptors, the mechanism of that inhibition is unknown.

The primary mechanism of action of local anesthetics is inhibition of voltage-gated sodium channels. Benzonatate was patented (Matter, 1955) and approved for human use before the importance and ubiquity of these channels was fully understood, and before the first description of local anesthetic effects on voltage-gated sodium channels (TAYLOR, 1959). Whether benzonatate, any of its components, or its primary metabolite 4(butylamino)benzoic acid (BABA), affect voltage-gated sodium channels

Abbreviations: CAD, catecholamine A differentiated; Na+, sodium ion; BABA, 4(butylamino)benzoic acid; TTX, tetrodotoxin; PABA, para aminobenzoic acid.

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has not yet been reported.

There are nine subtypes of mammalian voltage-gated sodium channels, denoted Nav1.1 through 1.9. The tetrodotoxin (TTX)-sensitive sodium channel Nav1.7 is the main sodium channel present in vagal sensory neurons, with a large contribution from the TTX-insensitive subtypes Nav1.8 and Nav1.9 (Kwong et al., 2008). Muroi et al. (Muroi et al., 2013) have shown that short hairpin RNA knockdown of Nav1.7 in the nodose ganglion of the vagus inhibits the cough reflex, suggesting that benzonatate inhibition of Nav1.7 in pulmonary sensory nerve fibers could relieve cough.

Our study was designed to determine whether benzonatate has local anesthetic effects on voltage-gated sodium channels, specifically on Nav1.7. We tested this using a murine CNS cell line that highly expresses Nav1.7 (CAD cells) and compared the results to a murine neuroblastoma cell line that expresses primarily Nav1.3 (N1E-115 cells).

2. Material and methods

2.1. Cell culture

Catecholamine A differentiated (CAD) cells were a gift of Raj Khanna, University of Arizona. CAD cells were grown at 37 °C in 21% O_2, 5% CO_2, in Ham's DMEM/F12 medium (Gibco®; Life Technologies, Grand Island, NY) supplemented with with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.2% Glutamax (all from Life Technologies, Grand Island, NY). Cells were grown in 35 mm plastic culture dishes and passaged at a 1:4 dilution every 3–5 days after reaching 90% confluency. CAD cells can grow in a "differentiated" form when cultured without serum (Wang and Olsen, 2000). Our studies used the "undifferentiated" form.

N1E-115 cells were obtained from American Type Culture Collection (Manassas, VA). N1E-115 cells were grown at 37 °C in 21% O_2, 5% CO_2, in DMEM-high glucose with glutamine medium (Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were grown in 35 mm plastic culture dishes and passaged at a 1:2 dilution every 2–3 days after reaching 90% confluency.

For both lines, cells used for electrophysiological study were grown in 35 mm dishes containing 12 mm round glass coverslips.

2.2. Materials

Benzonatate was obtained from Toronto Research Chemicals (Toronto, Canada), Santa Cruz Biotechnology (Dallas, Tx) and Zydus Pharmaceuticals (Pennington, NJ). Tetrodotoxin was obtained from Tocris Bioscience (Bristol, United Kingdom). 4-(butylamino)benzoic acid (BABA), para aminobenzoic acid (PABA), tetracaine and all electrolytes were obtained from Sigma (St. Louis, MO). Benzonatate concentrations of 10 μM or less were dissolved directly in the particular extracellular solution used for recording. For higher concentrations a stock solution was dissolved in 70% ethanol and added to extracellular solutions. Final ethanol concentrations were always less than 1%. Control and wash solutions always contained the same amount of ethanol as benzonatate-containing solutions and the concentrations of ethanol used did not affect Na^+ currents.

2.3. HPLC-MS

To examine benzonatate composition, 1.0 ng of diluted benzonatate was injected into a Waters 2795 Alliance HT High Performance Liquid Chromatograph (HPLC; Waters Corporation, Milford, MA) equipped with a combination Phenomenex Aeris™ 3.6 μm PEPTIDE XB-C18 100 Å guard cartridge coupled with Phenomenex Aeris™ 3.6 μm PEPTIDE XB-C18 100 Å column 150 × 2.1 mm 3.6 μm (Phenomenex Inc., Torrance, CA) at a flow rate of 0.150 mL/min; a 2.00 min hold at 90:10 A:B (Mobil Phase A = 0.01% Formic Acid; Mobil Phase B = acetonitrile:methanol 1:1) followed by a binary mobile phase gradient to 10:90 A:B in 30.00 min. The effluent stream from the chromatographic separation was coupled to a Waters Quattro Ultima Triple Stage Quadrupole (TSQ) mass spectrometer (MS) with atmospheric pressure ionization and electrospray (ESI) probe, linear hexapole assembly collision cell, post acceleration conversion dynode and photomultiplier detection system (MassLynx 4.0 & QuanLynx). Full scan collection in a mass range from 150 to 1000 m/z through a quadrupole filter (Q1) was obtained with a capillary temperature of 275 °C maintained at 3.5 kV. All mass spectra show the major molecular $[M+H]^+$ ion.

2.4. Separation of benzonatate fractions

To fractionate benzonatate, 1.0 mg of diluted benzonatate was injected into a BioRad BioLogic DuoFlow High Performance Liquid Chromatograph (Bio-Rad Laboratories Inc., Hercules, CA) equipped with an Agilent 5.0 μM Zorbax SB-C18 250 × 7.6 mm column (Agilent Technologies, Santa Clara, CA) at a flow rate of 3.0 mL/min; a 2.00 min hold at 90:10 A:B (Mobil Phase A = 0.01% Formic Acid; Mobil Phase B = acetonitrile:methanol 1:1) followed by a binary mobile phase gradient to 10:90 A:B in 30.00 min. The effluent stream from the chromatographic separation was coupled to a Büchi C-630 UV Monitor (254 nm) (Flawil, Switzerland) and a BioRad BioLogic Fraction Collector. The fraction collection was adjusted to collect the first one third of eluting n-ethoxy compounds, the middle one third and the latter one third; each one third represented a different overall hydrophobicity and n-ethoxy chain length group.

2.5. Whole cell recording

Whole cell voltage-clamp recordings were done at room temperature using an Axopatch 200b amplifier (Molecular Devices, Sunnyvale CA). Electrodes were pulled from thin-walled borosilicate glass capillaries (1.5 OD, 1.0 ID; King Precision Glass, Inc., Claremont, CA) with a P-97 electrode puller (Sutter Instrument Co., Novato, CA) with electrode resistances of 1–2 MΩ when filled with internal solution. The internal solution for recording Na^+ currents contained (in mM): 110 CsCl, 5 $MgSO_4$, 10 EGTA (in CsOH), 4 ATP Na_2-ATP, 25 HEPES (pH 7.2). The external solution contained (in mM): 100 NaCl, 10 tetraethylammonium chloride (TEA-Cl), 1 $CaCl_2$, 1 $CdCl_2$, 1 $MgCl_2$, 10 D-glucose, 1 3,4 diaminopyridine, 0.1 $NiCl_2$, 10 HEPES (pH 7.3). To allow complete dialysis of the pipette solution with the intracellular contents and stabilize Na^+ currents, which tended to increase gradually after establishing whole cell mode, recording began after 5–10 min. Whole-cell capacitance and series resistance were compensated with the amplifier. Series resistance in whole cell mode was 2–3 MΩ and compensated by 50–80%. Linear leak currents were digitally subtracted using a P/4 protocol. Currents were filtered at 5 kHz using the low pass Bessel filter of the amplifier and sampled at 10–20 kHz using a Digidata 1322A interface and PClamp 10 software (Molecular Devices). Cells used for study had stable seal resistances of at least 1 GΩ, input resistances of at least 500 MΩ, and Na^+ currents of at least 500 pA.

The holding potential in all experiments was −80 mV unless stated otherwise. Currents were elicited at a rate of not greater than every 20 s, which eliminated use-dependent ("phasic") effects of benzonatate on Na^+ currents. In some experiments we specifically studied phasic inhibition by benzonatate, and currents were elicited at a rate of 20 Hz from a holding potential of −100 mV.

For current clamp experiments the internal solution was K aspartate 130, KCl 20, MgCl 1, D-glucose 10, EGTA 1 (in KOH), and

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