Ranolazine inhibits voltage-gated mechanosensitive sodium channels in human colon circular smooth muscle cells

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Neshatian L, Strege PR, Rhee P, Kraichely RE, Mazzone A, Bernard CE, Cima RR, Larson DW, Dozois EJ, Kline CF, Mohler PJ, Beyder A, Farrugia G. Ranolazine inhibits voltage-gated mechanosensitive sodium channels in human colon circular smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 309: G506–G512, 2015. First published July 16, 2015; doi:10.1152/ajpgi.00051.2015.—Human jejunal smooth muscle cells (SMCs) and interstitial cells of Cajal (ICCs) express the SCN5A-encoded voltage-gated, mechanosensitive sodium channel Nav1.5. Nav1.5 contributes to small bowel excitability, and Nav1.5 inhibitor ranolazine produces constipation by an unknown mechanism. We aimed to determine the presence and molecular identity of Na+ current in the human colon smooth muscle and to examine the effects of ranolazine on Na+ current, mechanosensitivity, and smooth muscle contractility. Inward currents were recorded by whole cell voltage clamp from freshly dissociated human colon SMCs at rest and with shear stress. SCN5A mRNA and Nav1.5 protein were examined by RT-PCR and Western blots, respectively. Ascending human colon strip contractility was examined in a muscle bath preparation. SCN5A mRNA and Nav1.5 protein were identified in human colon circular muscle. Freshly dissociated human colon SMCs had Na+ currents (~1.36 ± 0.36 pA/pF), shear stress increased Na+ peaks by 17.8 ± 1.8% and accelerated the time to peak activation by 0.7 ± 0.3 ms. Ranolazine (50 μM) blocked peak Na+ current by 43.2 ± 9.3% and inhibited shear sensitivity by 25.2 ± 3.2%. In human ascending colon strips, ranolazine decreased resting tension (31%), reduced the frequency of spontaneous events (68%), and decreased the response to smooth muscle electrical field stimulation (61%). In conclusion, SCN5A-encoded Nav1.5 is found in human colonic circular smooth muscle. Ranolazine blocks both peak amplitude and mechanosensitivity of Na+ current in human colon SMCs and decreases contractility of human colon muscle strips. Our data provide a likely mechanistic explanation for constipation induced by ranolazine.

colon; smooth muscle; sodium channel; SCN5A; ranolazine

Sodium Channel Nav1.5 Is Functionally Significant in Human GI Smooth Muscle

ION CHANNELS are directly involved in the regulation of electrical properties and excitability of electrically active tissues such as smooth muscle and nerves. GI smooth muscle excitability relies on multiple types of ion channels (3). Intracellular recordings from human small intestine smooth muscle strips have shown that voltage-gated sodium channels (NavV) are involved in the regulation of resting membrane potential and slow wave frequency and morphology (10, 22). We have previously shown that SCN5A-encoded NavV1.5, a voltage-gated sodium selective ion channel, is responsible for the sodium (Na+) current in human jejunal circular smooth muscle cells (SMC) and interstitial cells of Cajal (ICC) (10, 21, 22). Sodium current is also present in human colon SMC, but the molecular nature of this current is unclear (23, 24, 25).

Smooth Muscle Voltage-Gated Sodium Channels Are Mechanosensitive

Mechanical stimuli are important in the GI tract and known to affect the electrophysiology of smooth muscle (11). Voltage-gated sodium channels, including NavV1.5 in the GI tract, are mechanosensitive (10, 15, 20). Mechanical stimulation with shear stress increases peak Na+ currents in the human jejunal circular SMC and ICC (17, 21). In heterologous expression systems, mechanical stimuli significantly increase peak Nav1.5 currents, accelerate channel activation and inactivation, and increase single channel activity at resting potentials (5, 7, 17). Stretch increases slow wave frequency in the human jejunal (22). It is currently unknown whether Na+ current in the human colon is also mechanosensitive.

SCN5A Mutations in IBS Patients Lead to Abnormal NavV1.5 Function

Studies suggest that NavV1.5 is clinically relevant in GI functional disorders (12) such as irritable bowel syndrome (IBS) (4, 12, 17). A proportion of IBS patients have SCN5A mutations that lead to abnormal NavV1.5 function (4, 17). A majority of these SCN5A missense mutations cause a loss-of-function NavV1.5 phenotype in vitro, and the loss of NavV1.5 activity is associated with a constipation-predominant IBS in the majority of these IBS patients. Importantly, restoration of NavV1.5 function in a patient leads to improvement of constipation (4).
Ranolazine Is a NaV1.5 Inhibitor Associated with Constipation

Ranolazine is a piperazine derivative NaV1.5 inhibitor that blocks Nav1.5 voltage-dependent peak current and mechanosensitivity (7). Ranolazine is clinically available as an antianginal therapy with a particular advantage over other antianginal therapies in that it does not decrease heart rate and blood pressure (14). Ranolazine blocks NaV1.5 at an IC50 \( \sim 135 \) \( \mu \)M (9) and has a low antagonist activity against L-type voltage-gated calcium channels (\( \text{CaV}_{1.2} \)) [IC50 \( \sim 300 \) \( \mu \)M (1)], consistent with its clinical lack of effect on blood pressure (8). Intriguingly, multiple large-scale clinical and postmarketing trials showed that constipation is one of the most commonly reported side effects of ranolazine, with a severalfold higher incidence for ranolazine than for placebo (14). It is unclear whether blockade of these currents contributes to constipation related to ranolazine use.

The goals of this study were to examine the molecular identity of the Na\(^+\) current and examine the effect of ranolazine on Na\(^+\) current, mechanosensitivity, and contractility in human colonic circular smooth muscle cells and muscle strips.

METHODS

The Institutional Review Boards of the respective institutions approved the use of normal human colonic tissue resected as part of the surgical procedure for nonobstructing colon cancer.

Tissue Dissection

Colon specimens not involved by cancer (at least 10 cm away) were harvested directly into chilled F12 buffer solution (F-12: Gibco, Invitrogen, Grand Island, NY; 10 mM antibiotic antimycotic A5955; Sigma, St. Louis, MO; 14 mM NaHCO\(_3\); pH 7.35) and transported to the laboratory within 20 min. Colonic tissue was transferred from F12, cut along the mesentery, and pinned mucosa side down onto a Sylgard (Dow Corning, Midland, MI)-coated dish filled with ice-cold Krebs solution (in mM, 137.4 Na\(^+\), 5.9 K\(^+\), 2.5 Ca\(^{2+}\), 1.2 Mg\(^{2+}\), 134 Cl\(^-\), 15.5 NaHCO\(_3\), 1.2 HPO\(_4^{2-}\), 11.5 glucose, pH 7.4). The mucosa was cut away with a sharp scissors and discarded. The remaining tissue was repinned, serosa side up. The longitudinal muscle layer was peeled away from the circular layer with forceps. Circular muscle strips were shaved off of the connective tissue with a scalpel.

Reverse Transcription PCR

RT-PCR was carried out on RNA isolated from colonic smooth muscle strips using specific primers designed against the human SCN5A sequence as described before (21, 22).

Western Blots

**Tissue preparation.** Flash-frozen samples of human heart and colon circular muscle were homogenized in 1 ml of homogenization buffer [0.025 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4, with protease inhibitors (Sigma)] using a handheld homogenizer. The homogenates were centrifuged at high speed for 30 min at 4°C and the supernatant quantitated for protein concentration (ThermoScientific BCA Kit).

**Immunoblotting.** Forty micrograms of human colon circular muscle lysates and 10 \( \mu \)g of human heart lysate (not boiled) were loaded for each sample and electrophoresed on a 4–15% Tris-glycine gel. Proteins were transferred to nitrocellulose (0.4 \( \mu \)m; BioRad) and blocked for 1 h in 5% NFDM at 4°C. Blots were incubated in anti-Na,1.5 Ig (Covance) or anti-GAPDH (Fitzgerald) overnight at 4°C. Blots were washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Laboratories) for 2 h at 4°C. Blots were washed and developed using the BioRad Clarity ECL kit and the BioRad Gel Documentation System.

Human Colon Smooth Muscle Cell Dissociation

Single smooth muscle cells from the human colonic circular layer were obtained by a method adapted from that previously described (10, 19). Circular smooth muscle strips were dissected out as described above in Tissue Dissection. Circular muscle layer strips were placed temporarily into a separate Krebs bath during dissection, then minced in 35 \( \times \) 10 mm cell culture dishes filled with a Ca\(^{2+}\)-free Hanks solution (Cellgro, Herndon, VA) supplemented with 1.5–2.0 mg/ml papain and 0.5 mg/ml \( \beta \)-dithiothreitol (DTT; Sigma). The tissue was transferred to a test tube and stirred for 3 min at 800 rpm at 32°C on an isometric magnetic stir plate (Fisher Scientific, Pittsburgh, PA). Next, cells were spun (1,000 rpm or 170 g for 90 s), and the supernatant was replaced with a collagenase solution (Hanks; 1.0 mg/ml collagenase type II; 2.0 mg/ml BSA; 5.0 mg/ml DTT). Colon cells in collagenase were returned to the hotplate and stirred at 600 rpm in the test tube at 32°C. After cells began to appear in the top fraction of the supernatant, the cells were spun as described above and rinsed twice with fresh enzyme-free Hanks. The cells were returned to the stir plate (450–500 rpm), and in 5-min intervals, a 7-ml cell suspension was transferred into tubes containing 2 ml modified minimal essential medium [10.4 g/l MEM (Sigma, St. Louis, MO) containing (in mM) 4.17 Na\(^+\), 0.5 Ca\(^{2+}\), 0.5 Mg\(^{2+}\), 2 Cl\(^-\), 4.17 HCO\(_3^{-}\), 10 HEPES, pH 7.2, 276 mOsm].

**HEK293 Cell Transfection**

L-type Ca\(^{2+}\) channel subunits \( \alpha_{1C} \) and \( \beta_2 \) were cotransfected with green fluorescent protein into HEK293 cells and voltage-clamp recordings were performed and recorded as previously described (13).

**Whole Cell Electrophysiology**

A Sutter Instruments P-97 puller (Sutter Instrument, Novato, CA) was used to pull electrodes from Kimble KG-12 glass. Electrodes were coated with heat-cured R6101 compound (Dow Corning, Midland, MI). Axon 200A amplifier, Digidata 1322A, and Clampex 10 (Molecular Devices, Union City, CA) software were used for voltage-clamp and data acquisition.

**Electrophysiology protocols.** Whole cell inward currents were measured by standard patch-clamp technique as described previously (21). Cells were held at –100 mV and stepped to –80 through 35 mV in 5-mV steps. Start-to-start time was 1 s. Mechanical stimulation was induced by bath flow of solution through a 0.7-ml elliptical bath chamber, calibrated at 10 ml/min.

**Human Ascending Colon Muscle Strips Contractility**

Human colon strips were collected as above. Muscle strips (2 \( \times \) 10 mm) were oriented in circular smooth muscle axis on the organ chamber and equilibrated over 60 min. Ranolazine (300 \( \mu \)M) and TTX (1 \( \mu \)M) were added to the bath solution. In specific experiments, electrical field stimulation applied using 150 V, 3 ms, 20 Hz, and 20 s duration. Data were acquired using LabChart software (ADInstruments, Colorado Springs, CO).

**Drugs and Solutions**

**Electrophysiology.** The intracellular solution contained (in mM) 130 Cs\(^+\), 125 methanesulfonate, 20 Cl\(^-\), 5 Na\(^+\), 5 Mg\(^{2+}\), 5 HEPES, 2 EGTA, 2.5 ATP, 0.1 GTP, pH 7.0, and osmolality was 300 mmol/kgH\(_2\)O. Extracellular NaCl Ringer solution contained (in mM) 149.2 Na\(^+\), 159 Cl\(^-\), 4.74 K\(^+\), 2.54 Ca\(^{2+}\), 5 glucose, and 5 HEPES. For ion substitution experiments, Na\(^+\) was replaced with 149.2 mM N-methyl-D-glucamine (NMDG\(^+\)), or Ca\(^{2+}\) was replaced with 2.54...
The voltage dependence of activation and inactivation is voltage-gated sodium channels (NaV). exchanging Ca2+ constants of 2.2/0.43/H11006, B0.8 (Fig. 2). The current-voltage data were fit by using a two-state Boltzmann function. The calculation of the voltage-dependent currents with a two-state Boltzmann curve (half-point of the voltage dependence of activation at 20 mV and remained after peak). These currents activated with a time constant of 0.5 ms and 21.1 ms, while the extracellular solution containing 150 mM NaCl and no added Ca2+, while the extracellular solution containing 150 mM Na+ and 2.5 mM Ca2+ was exchanged. A: total inward current. B: isolation of the fast component of inward current from the same cell as A by replacing extracellular Ca2+ with Mn2+. C: isolation of slow component of inward current from the same cell as A and B by replacing extracellular Na+ with NMDG+. D: average current-voltage relationships of peak inward currents, as shown in Fig. A, fast (●, B) and slow (○, C) components of inward current, normalized to the conductance of each of cell, n = 6 cells.

Fig. 1. Fast Na+ and slow Ca2+ components contribute to total inward current in human colon myocytes. A–C: representative whole cell inward currents recorded from a human colonic circular smooth muscle cell evoked by stepping for 50 ms to −80 through +35 mV in 5-mV intervals from the −100 mV holding potential. Intracellular solution contained 5 mM NaCl and no added Ca2+, while the extracellular solution containing 150 mM Na+ and 2.5 mM Ca2+ was exchanged. A: total inward current. B: isolation of the fast component of inward current from the same cell as A by replacing extracellular Ca2+ with Mn2+. C: isolation of slow component of inward current from the same cell as A and B by replacing extracellular Na+ with NMDG+. D: average current-voltage relationships of peak inward currents, as shown in A, fast (●, B) and slow (○, C) components of inward current, normalized to the conductance of each cell, n = 6 cells.

SCN5A-Encoded Na\textsubscript{v}1.5 Is Present in the Circular Smooth Muscle of Human Colon

We next aimed to determine whether SCN5A, which encodes Na\textsubscript{v}1.5, is present in circular smooth muscle cells. Using RT-PCR on the smooth muscle layer with previously validated primers we found evidence for SCN5A mRNA in human colonic smooth muscle (Fig. 3A). We also performed Western blots on human heart and human colon circular smooth muscle and demonstrate the presence of the Na\textsubscript{v}1.5 protein in human colon circular smooth muscle (Fig. 3B).

Na+ Channel in Circular Smooth Muscle of Human Colon is Mechanosensitive

We next aimed to determine whether the human colon smooth muscle cell Na+ current is mechanosensitive using shear stress as a model of mechanostimulation. Voltage-clamped colonic smooth muscle cells were superfused with NaCl Ringer (bath) solution at a rate of 10 ml/min. In response to shear stress, peak Na+ current increased 18 ± 2%, from −1.73 ± 0.45 pA/pF at rest to −20.3 ± 0.54 pA/pF with shear stress (n = 6, P < 0.05, Fig. 4, A and B). Shear stress also

### RESULTS

**Voltage-Gated Sodium Current Is Present in Human Colon Smooth Muscle Cells**

We carried out voltage-clamp experiments in whole cell configuration to characterize the inward currents from human colon circular layer smooth muscle cells (SMCs) (Fig. 1A). A robust inward current had a fast component in 35/44 (80%) cells, a slow component in 30/44 (68%) cells, and both components in 25/44 (57%) cells (Fig. 1A).

We next aimed to determine which ion species were involved in carrying these inward currents. The slower component peaked around 0 mV and was eliminated by the replacement of Ca2+ by Mn2+ (Fig. 1, C and D), suggesting that this current was due to voltage-gated calcium channels (Ca\textsubscript{v}). The slower component peaked near −20 mV and remained after exchanging Ca2+ with Mn2+ (Fig. 1, B and D), but it was eliminated by the replacement of Na+ by NMDG+ (Fig. 1C). These results suggest that the faster current was carried by voltage-gated sodium channels (Na\textsubscript{v}).

We then analyzed the voltage-dependence and kinetics of the Na\textsubscript{v} currents with the slower Ca2+ currents eliminated using Mn2+ (Fig. 2A). We fit these voltage-dependent currents using three exponentials (one time constant for activation, the other two time constants for inactivation) and the peaks of the voltage-dependent currents with a two-state Boltzmann function. The current-voltage data were fit by using two-state Boltzmann curves with half-point of the voltage dependence of activation at −37.4 ± 1.7 mV, slope 7.9 ± 0.8 (Fig. 2B). At peak, these currents activated with a time constant of 0.43 ± 0.06 ms, and inactivated with time constants of 2.2 ± 0.5 ms and 21.1 ± 3.5 ms (Fig. 2, C–E). The voltage dependence of activation and inactivation is shown in Fig. 2B.

**Data Analysis**

Data were analyzed using Clampfit 10 software (Molecular Devices, Sunnyvale, CA), Microsoft Excel 2003 (Microsoft, Redmond, WA) and SigmaPlot 12 (SPSS, Chicago, IL).

**Electrophysiology.** Inward currents displaying fast components were measured within the first 10 ms of the voltage step. For current-voltage graphs, the maximum inward current of a single control trace was normalized to 1 using the equation: $I_{\text{NORMAL}} = (I_0/I_{\text{MAX}})$, where $I_{\text{NORMAL}}$ is normalized current, $I_0$ is measured current at a given voltage, and $I_{\text{MAX}}$ is maximum peak inward current of the control trace. Thus peaks of all other traces per cell were expressed as a fraction of 1. Statistical comparisons were performed on raw data using a paired two-tailed Student’s $t$-test. Statistical significance was accepted when $P < 0.05$. Data are expressed as mean values ± SE.

**Muscle strips.** Resting muscle tone was measured as baseline tension of two spans ≥10 min immediately before and 30 min after addition of ranolazine (300 μM). Contractile activity was measured as the contraction frequency and average contraction amplitude over two spans ≥30 min before and after addition of the drug. Response to electrical field stimulation (EFS) was measured as the difference between EFS peak and baseline tension prior to stimulus.
accelerated the activation time to peak at voltages between −30 and −10 mV (−30 mV, from 5.4 ± 0.6 ms at rest to 2.9 ± 0.4 ms with shear stress; n = 5, P < 0.05, Fig. 4C).

Ranolazine Decreases Nav Peak Current in Circular Smooth Muscle of the Human Colon

Ranolazine is a clinically employed NaV1.5-selective modulator which is known to inhibit NaV1.5 peak and late currents (1). We next examined the effect of ranolazine on inward currents from human colonic myocytes (Fig. 5). Addition of 50 μM ranolazine to the extracellular solution reversibly decreased peak Na⁺ currents by 43 ± 9%, from −1.73 ± 0.45 to −0.95 ± 0.28 pA/pF (n = 6, P < 0.05; Fig. 5, A and B). The kinetics of activation and inactivation remained unchanged (n = 6, P > 0.05; Fig. 5C). We tested the effects of ranolazine at this concentration on L-type Ca²⁺ channels in HEK cells, and ranolazine did not block L-type Ca²⁺ current (−3.6 ± 0.6 pA/pF before vs. −3.7 ± 0.5 pA/pF after ranolazine, +4.5 ± 9.3% change, n = 5 P > 0.05). Therefore, ranolazine decreases peak Na⁺ current in human colon smooth muscle cells.

Ranolazine Blocks the Mechanosensitivity of Nav Current in Human Colon Smooth Muscle Cells

To study whether ranolazine also affected mechanosensitivity of colonic Na⁺ current, we shear-stressed smooth muscle cells while in voltage-clamp in the presence or absence of 50 μM ranolazine. Ranolazine inhibited the increase in peak Na⁺ current induced by shear (−0.94 ± 0.43 pA/pF at rest with ranolazine, to −0.86 ± 0.38 pA/PF during shear with ranolazine; n = 4, P > 0.05; Fig. 6, A and B). Ranolazine also blocked the acceleration in activation caused by shear (3.6 ± 1.1 ms at rest with ranolazine, to 3.6 ± 1.2 ms during shear with ranolazine, n = 3, P > 0.05; Fig. 6C). Therefore, ranolazine not only decreased peak Na⁺ current (Fig. 5) but also separately inhibited the shear response (Fig. 6) in human colonic circular smooth muscle.
SCN5A-Encoded Nav1.5 Contributes to the Fast Voltage-Dependent Sodium Current in the Human Colon Smooth Muscle Cells

Our data show that there was a robust voltage-sensitive inward current ($-1.36 \pm 0.36 \text{ pA/pF}$) in human colon smooth muscle cells. We used ion substitution to determine that the faster activating and inactivating current was carried by sodium. We present several lines of evidence that Nav1.5 contributes significantly to inward Na$^+$ current in these smooth muscle cells. First, RT-PCR and Western blots of human colon circular smooth muscle identified SCN5A and Nav1.5, respectively (Fig. 3). Second, the voltage dependence and kinetic parameters of this Na$^+$ current are consistent with the published parameters of Nav1.5 (Figs. 1 and 2) (10). Third, these Na$^+$ currents were sensitive to shear to the same extent as the muscle cells (10) and interstitial cells of Cajal (22). In the human jejunum circular smooth muscle strips, inhibition of Na$^+$ currents by lidocaine (22) or ion substitution (2, 22) leads to a decrease in smooth muscle excitability. In patients with irritable bowel syndrome (IBS) loss of Nav1.5 function due to mutations is associated with constipation (4). Ranolazine, a novel clinically approved Nav1.5 antagonist, is also associated with constipation (14). In this study we found that in human colonic circular smooth muscle SCN5A-encoded Nav1.5 contributes to a voltage-dependent and mechanosensitive Na$^+$ current, and that ranolazine blocks both peak and mechanosensitivity of this Na$^+$ current and also decreases contractility of human colon muscle strips.

**DISCUSSION**

Voltage-sensitive sodium currents of unknown identity are present in rat and human colon smooth muscle cells (25). In the human jejunum, voltage-sensitive sodium currents are carried by SCN5A-encoded Nav1.5, which is expressed in smooth
Acute mechanical stimulation of NaV1.5 is excitatory, with both shear stress and pressure significantly increasing peak currents and accelerating kinetics (5, 7). Further, NaV1.5 mechanosensitivity may be important in GI motility, which is abnormal in some IBS-linked mutations (17). We have previously shown that ranolazine (7) and local anesthetics (6) inhibit NaV1.5 mechanosensitivity. These data in human colon myocytes are consistent, showing that 50 μM ranolazine blocks shear-induced increase in peak NaV currents to a similar degree previously reported for NaV1.5 channels (Fig. 6).

**Ranolazine Decreases Contractility of Human Colon Smooth Muscle Strips**

Previous studies in vitro and in vivo suggest that ranolazine decreases vascular and bladder smooth muscle excitability and tension (16, 18). In this study we extend these findings to the GI tract. We demonstrate that ranolazine decreases contractility of the human colon muscle strips (Fig. 7). Ranolazine decreased resting tension by 29% (Fig. 7, A and B), and the amplitude and frequency of spontaneous contractile activity by 73% and 68%, respectively (Fig. 7, A, C, and D). It also decreased evoked activity in response to electrical field stimulation (EFS) by 59% (Fig. 7, E and F). These results complement the electrophysiology data and together suggest that ranolazine significantly decreases excitability and contractility of the human colon smooth muscle. Future studies will have to determine if other targets also contribute to the contractility effect of ranolazine.

**Ranolazine Block of Human Colon Smooth Muscle Cell NaV Current May Contribute to the Constipation Phenotype Observed in Clinical Studies**

Since block of NaV currents in smooth muscle decreases excitability (2, 22), and NaV1.5 loss-of-function is associated with constipation-predominant IBS, while restoration of NaV1.5 current improves constipation (4), inhibition of NaV1.5 peak currents and NaV1.5 mechanosensitivity by ranolazine may provide a mechanism for ranolazine-induced constipation.

In summary, we demonstrate the presence of the voltage-gated mechanosensitive SCN5A-encoded NaV1.5 in the circular smooth muscle layer of human colon. The NaV1.5 peak currents and mechanosensitivity in human smooth muscle cells were inhibited by ranolazine which also decreased contractility. These findings suggest that the mechanism of constipation due to ranolazine may be due to block of NaV1.5 and its mechanosensitivity by this drug. These findings also open the door for novel diagnostic and therapeutic options in GI disorders targeting NaV1.5.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
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