UDC 591.185;616.8-009.7;616.-009.623

Differential effects of TRPV1 antagonist AMG-517 on activation of nociceptive skin endings

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The transient receptor potential vanilloid 1 (TRPV1) is a polymodal ion channel activated by capsaicin, protons, and heat. It is predominantly expressed in the peripheral endings of small-diameter nociceptive primary afferent neurons. The localization of TRPV1 receptors in nociceptors and their ability to integrate harmful physical and chemical irritants, as well as inflammation mediators, make them important pharmacological targets for pain therapy. This study aimed to assess the effect of the novel TRPV1 receptor antagonist, AMG-517, on the activation of sensitive nociceptive skin endings by capsaicin and heat. For this, we recorded the activity of single afferents in mouse n. saphenous-skin preparation. AMG-517 almost completely blocked responses of nerve fibers to the application of selective TRPV1 receptor agonist capsaicin (20 µmol/l), but did not affect the activation in response to temperature increase (50°C). The obtained results suggest significant pharmacological differences between cellular nociception models, such as spinal ganglion neurons and transfected cell lines, and native nerve endings. Thus, TRPV1 is not the only thermally activated sensor for heat responsiveness of cutaneous nociceptors.
Key words: skin-nerve preparation; TRPV1; nociception; heat; capsaicin; AMG-517.

INTRODUCTION

Mammalian skin is densely innervated by sensory neuron endings, forming a continuous receptive field for surface sensitivity. These nerve endings are widely recognized as a valuable model for electrophysiological and pharmacological studies of the molecular mechanisms underlying primary sensory perception. Sensory neurons play a crucial role in transmitting somatic sensations, including touch, pain, temperature, and proprioception. Most nociceptors are polymodal, meaning they respond to multiple harmful stimuli (e.g., heat, pressure, and chemical irritants such as acid) due to the expression of various receptor-channel complexes (TPRV, TPRM, P2X, P2Y, ASIC) [1]. TRPV1 is a polymodal ion channel that is activated by a pH drop below 6.0, an increase in temperature (> 43°C), and capsaicin [2]. The high expression level of TRPV1 in nociceptive neurons and its sensitivity to painful stimuli indicate the significant role of this receptor in nociceptive perception [3]. This also suggests that TRPV1 receptor are promising target for antinociceptive drugs. However, the use of TRPV1 receptor antagonists ruthenium red and AMG-517 has shown somewhat contradictory results in electrophysiological studies on cell cultures *in vitro* and on sensitive afferent endings of the skin *ex vivo* [4-6]. Our goal was to block the heat- and capsaicin-induced response directly at its origin - on the native sensitive endings of sensory nociceptive neurons - by applying the TRPV1 receptor antagonist AMG-517.

METHODS

The experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and Recommendations of the First National Congress on Bioethics Issues (Kyiv, Ukraine, September, 2001). The experimental

design and procedures were approved by the Bioethics Commission of Bogomoletz Institute of Physiology (protocol No. 28/5-KE from 12.04.2019).

Adult (6-7 weeks old, 22-30 g) BALB/c mice were used in this study. Animals were bred in the Institute vivarium, where they were housed on a 12-h light-dark cycle and given food and water *ad libitum*.

Murine skin-saphenous nerve preparation and single-fiber recording were used, as previously described [7]. Animals were anesthetized with an intraperitoneal injection of urethane (2 mg/kg; U2500, "Sigma", USA) dissolved in physiologic saline. The nerve saphenous was excised in continuity with the skin area on the dorsal side of the hind paw that it innervates. After dissection, animals were euthanized with an intraperitoneal injection of a lethal dose of urethane. The skin was mounted corium-side up in the organ bath. The end of the cut nerve was gently threaded through a small hole into a separate recording chamber for micromanipulation and single-unit recording. The organ bath and recording chamber were separately superfused with a modified Krebs–Henseleit solution composed of (in mmol/l): NaCl - 118, KCl - 5.4, NaH_2PO_4 - 1.0, $MgSO_4$ - 1.2, $CaCl_2$ - 1.9, $NaHCO_3$ - 25.0, and dextrose - 11.1, and gassed with 95% O_2-5% CO_2, pH 7.4, at a flow rate of 8 ml × min^{-1} and 3 ml × min^{-1}, respectively. The temperature was controlled to 30°C.

Single fiber activity was recorded with suction glass microelectrodes pulled using a Flaming-Brown micropipette puller (Model P-97, "Sutter Instrument Co.", USA). The recording signal was amplified (Model 3000, "A-M Systems, Inc.", USA) and filtered (low cut-off, 0.3 kHz; high cut-off, 1 kHz). Nerve fiber activities were recorded to a PC using WinEDR v 3.3.1 software via a Digidata 1200 analog-to-digital converter ("Axon Instruments", USA). Conduction latency of single nerve units was determined by electrical stimulation inside the receptive field with a fine steel electrode using an "A-M Systems" analog stimulus isolator model 2200, and the conduction distance was assessed to calculate conduction velocity. A cutoff of 1.0 m/s was used to distinguish between myelinated and unmyelinated fibers. Extracellular recordings were obtained only from single fibers that could be easily discriminated according to amplitude and shape.

For chemical or thermal stimulation of the receptive fields of nerve endings, a ring-shaped chamber enclosing a skin area was used to enhance the solution exchange rate. This chamber had an internal volume of 0.3 ml and was perfused separately at a rate of 4 ml/min. Capsaicin was added to the Krebs bicarbonate buffer solution at a concentration of 20 μmol/l for 5 min, this was sufficient for robust activation of capsaicin-sensitive nerve fibers. After a 5-minute pre-application, AMG-517 was applied together with capsaicin or a heated solution to the receptive field of nerve fibers. The first stimulation served as a control stimulus, while the last one tested recovery. Since capsaicin, depending on concentration and duration of exposure, induces temporary desensitization of nerve endings, each capsaicin stimulation was followed by a washout period of over 30 min, allowing nerve fibers to fully recover their response magnitude. For thermal stimulation, the perfusion solution was heated from 30 to 50°C over 60 s.

Our investigation focused specifically on the C mechano-heat-sensitive "polymodal" (CMH) subpopulation of nerve fibers, as these exhibit a significantly greater response to acidic pH. Other fiber types, including the A-fiber population with higher conduction velocities, were not examined. Single-fiber recordings were analyzed offline with a template-matching function of Spike 2 software ("CED", UK). Origin Pro 8.5 software ("Origin Lab. Corp.", USA) was used to conduct all statistical tests. Statistical analysis of the differences between nerve activities was assessed by one-way repeated measures ANOVA with the Tukey post-hoc test. All experimental data are presented as

mean ± SEM. All chemicals were purchased from "Sigma-Aldrich" (USA).

RESULTS

AMG-517 blocks capsaicin-induced nociceptor excitation. The effect of AMG-517 on the capsaicin response was tested in 16 C-units (Fig. 1). The application of capsaicin increased the frequency of action potentials from a basal activity of 0.031 ± 0.01 to 0.39 ± 0.048 imp/s (n = 16, $P = 3 \cdot 10^{-10}$). AMG-517 significantly reduced the overall response magnitude by 87.7% to 0.048 ± 0.015 ($P = 9 \cdot 10^{-9}$). The AMG-517 action was reversible: after washing out, the response to capsaicin was restored upon subsequent stimulation (0.31 ± 0.049; P = 0.21).

AMG-517 does not reduce the heat response of polymodal nociceptors. A population of CMH units (n = 13) was tested for heat in the presence of AMG-517 (Fig. 2, A). These fibers demonstrated spontaneous basal activity with the mean frequency of 0.048 ± 0.022 imp/s in control conditions (at 30°C). Increasing the superfusion solution temperature from 30 to 50°C led to an increase in the action potential frequency to 0.66 ± 0.14 imp/s (n = 13, $P = 2.5 \cdot 10^{-7}$). AMG-517 (5 µmol/l) did not affect the basal activity during pre-application and thermal excitation (0.7 ± 0.15; n = 13; P = 0.99). Fig. 2, B shows the averaged heat responses before and after AMG-517; no significant differences were found in the total number of spikes, the peak discharge rate, or the mean heat threshold.

DISCUSSION

We examined the effect of AMG-517 on capsaicin- and heat-evoked responses of nociceptive nerve endings in mouse skin-nerve preparation *ex vivo*. Our main conclusion was that AMG-517 selectively inhibits only the excitatory action of capsaicin, while not affecting heat-evoked responses in skin afferents.

Capsaicin causes burning pain by activating TRPV1 receptors on sensory nerve endings. The cloned capsaicin receptor integrates multiple pain-inducing stimuli: protons, harmful heat, and capsaicin. Each of these activators of TRPV1 mutually lowers the activation threshold for the others, enabling TRPV1 receptors to become activated at subthreshold levels under conditions of pH 5.9 and room temperature [8]. Capsaicin is a specific activator of TRPV1 receptor, and thus neuronal activation by capsaicin serves as a marker of TRPV1 expression. The localization of TRPV1 receptors in nociceptors and their ability to integrate harmful physical and chemical irritants, and inflammation me-

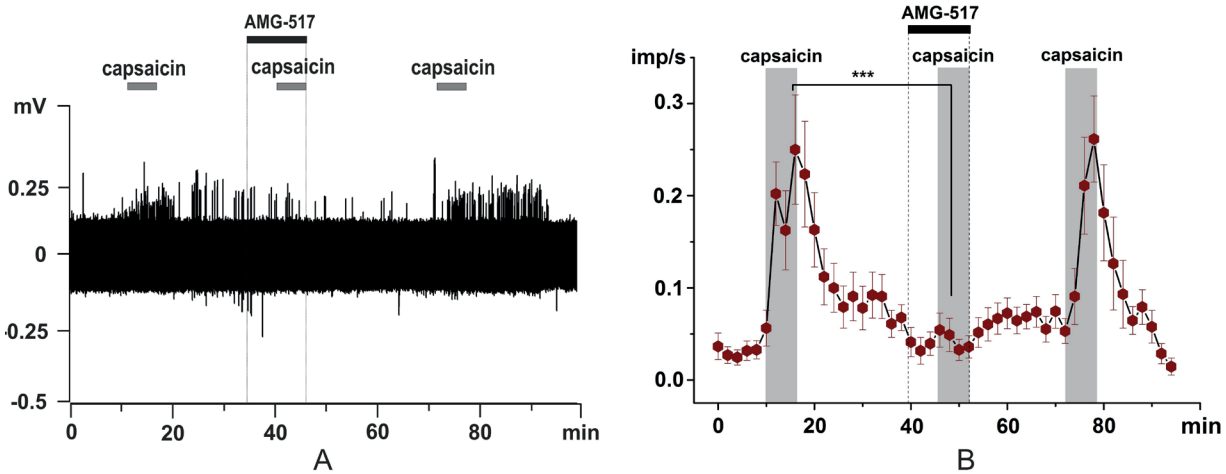

Fig. 1. A representative recording of CMH fibers activity under the action of capsaicin (20 µmol/l) and TRPV1 receptor antagonist AMG-517 (5 µmol/l) (A). Averaged (over 2 min intervals; n = 16) activity of CMH fibers (imp/s) (B). ***$P = 9 \cdot 10^{-9}$

diators make them important pharmacological targets for pain therapy. TRPV1 antagonists with distinct chemical structures − ruthenium red, capsazepine, and AMG-517 - suppress capsaicin-, proton-, and heat-induced currents in non-neuronal cells (CHO and HEK293 cells), which express human, rabbit, and rat VR1 receptors [8, 9]. However, in *in vitro* DRG neurons, they inhibit only capsaicin-activated currents, while not affecting proton-induced currents [10] or proton-induced excitation of sensory skin endings *ex vivo* [11]. Ruthenium red significantly reduced heat-induced currents in nociceptive neurons of rat DRG [12]. It was shown that ruthenium red inhibits only capsaicin-induced excitation and desensitization of rat skin nerve endings *ex vivo*. Conversely, ruthenium red did not affect proton-induced and heat-induced discharges [4]. AMG 517 inhibited transmembrane currents activated by capsaicin in rat DRG neurons and blocked capsaicin-induced flinching in rats [9]. We have now shown that AMG-517 suppressed only capsaicin-induced excitation of skin afferents and not affect thermal activation. In our previous study, AMG-517 alone did not alter the activity of CMH fibers in mouse skin *ex vivo* and did not cause any changes in discharge induced by low pH [5]. This can be explained by the fact that nociceptors can detect changes in pH using other acid-sensitive sensors, such as the acid-sensing ion channels (ASIC), proton-sensing G protein-coupled receptors, and several two-pore potassium channels, as well as proton-sodium exchanger NHE.

Receptors of the transient receptor potential (TRP) superfamily are expressed in primary sensory neurons and exhibit a wide range of thermal activation thresholds. TRPV1 is activated at temperatures above 43°C, TRPV2 at temperatures above 52°C, TRPV3 at 34-38°C, TRPV4 at 27-35°C, TRPM8 at 25-28°C, and TRPA1 is activated by temperatures dropping below 17°C [13]. This set of temperature sensors expressed on the sensitive endings of sensory neurons in the skin overlaps the range of temperature activation of the TRPV1 receptor. In a study comparing the properties of DRG neurons *in vitro*, sensitive skin endings in the "skin-nerve" preparation *ex vivo*, and pain behavior in VR1 knockout mice (TRPV1-/-) with the corresponding indicators in wild-type mice (TRPV1+/+), a complete lack of sensory response to vanilloids (capsaicin and resiniferatoxin) was observed in TRPV1-/- mice. At the same time, there was a reduction in response to proton-induced and noxious heat stimulation, although sensitivity to these stimuli was not entirely abolished. The sensory response to other stimuli (ATP, mechanical stimulation) did not differ significantly between TRPV1-/- and TRPV1+/+ mice [14, 15]. In mice with simultaneous genetic blockade of TRPV1-/- and TRPM2-/- receptors,

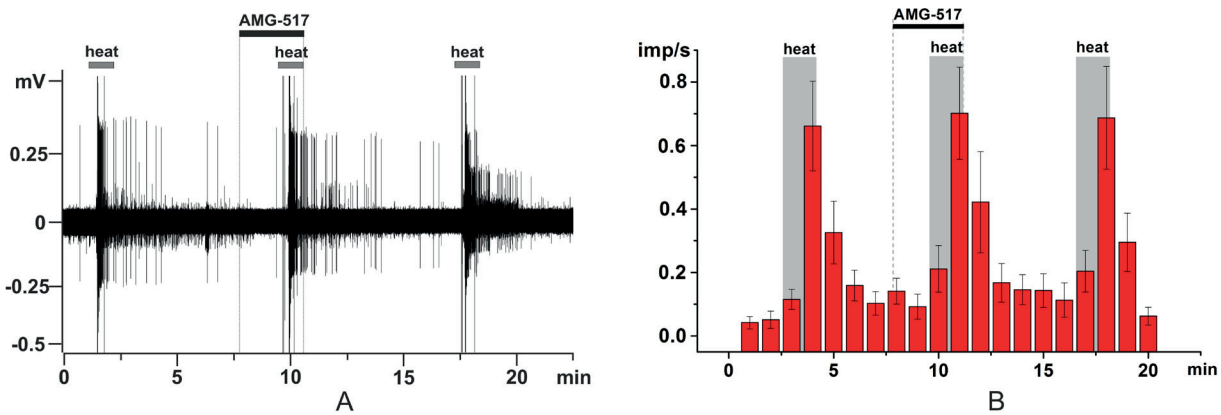

Fig. 2. A representative recording of CMH fibers activity under the influence of TRPV1 receptor antagonist AMG-517. AMG-517 (5 μmol/l) was applied before and during the activation of nerve fibers induced by increasing temperature from 30 to 50°C (A). Heat response of saphenous nerve. Averaged histogram of CMH-fibers activity (over 60 s intervals; n = 13) (B)

the response of sensitive nociceptive afferent endings in the skin *ex vivo* was reduced but not completely absent. In addition, mice with the TRPV1:TRPA1:TRPM3-/- triple knockout did not sense noxious heat but retained the ability to detect skin warming [16]. Thus, in non-neuronal cells (CHO, HEK293), where only the TRPV1 receptor-channel complex is expressed, TRPV1 antagonists block receptor activation by vanilloids, protons, and noxious heat. Meanwhile, in more complex systems, such as native sensory neurons of the skin and visceral organs, where TRPV1 is expressed alongside the natural set of other receptors, chemical TRPV1 antagonists block only capsaicin-induced excitation of sensory neurons and pain behavior in animals. Genetic blockade of TRPV1 also results in complete insensitivity of sensory neurons and test animals solely to capsaicin. Capsaicin is a potent and specific activator of TRPV1 receptors; therefore, the absence of a response to capsaicin or its analog resiniferatoxin during chemical and genetic blockade of the TRPV1 receptor is a marker of effective TRPV1 inhibition.

The selective TRPV1 receptor antagonist, AMG-517, effectively prevented the excitation of native nociceptive skin endings *ex vivo*, induced by capsaicin, but did not affect activation caused by harmful heat. The lack of effect of AMG-517 on the heat response is consistent with data from other studies that examined nociceptive responses using other TRPV1 antagonists, as well as in mice with a VR1 knockout. Thus, TRPV1 is not the only thermally activated sensor for heat responsiveness of cutaneous nociceptors.

This work was supported by a grant from the National Research Foundation of Ukraine No2023.05/0023.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ДИФЕРЕНЦІЙНА ДІЯ АНТАГОНІСТА TRPV1 РЕЦЕПТОРІВ AMG-517 НА АКТИВАЦІЮ НОЦИЦЕПТИВНИХ ЗАКІНЧЕНЬ ШКІРИ

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Ванілоїдний рецептор першого типу (TRPV1) — це полімодальний іонний канал, який активується капсаїцином, протонами та болісним нагріванням. Він переважно експресується в периферичних закінченнях ноцицептивних первинних аферентних нейронів малого діаметра. Локалізація рецепторів TRPV1 у ноцицепторах та їх здатність інтегрувати шкідливі фізичні та хімічні подразники, а також медіатори запалення, роблять їх важливою фармакологічною мішенню для терапії болю. Метою цього дослідження було оцінити дію новітнього антагоніста рецептора TRPV1, AMG-517, на активацію чутливих ноцицептивних закінчень шкіри капсаїцином та підвищенням температури. Для цього ми використали метод зовнішньо клітинної реєстрації активності окремих нервових волокон на ізольованому препараті n. *saphenus* миші. Показано, що AMG-517 майже повністю блокував відповідь на аплікацію селективного агоніста капсаїцину (20 мкмоль/л), проте не впливав на активацію чутливих нервових закінчень у відповідь на підвищення температури до 50°C. Отримані результати свідчать про значні відмінності між клітинними моделями ноцицепції, такими як нейрони спінальних гангліїв і трансфіковані клітинні лінії, та нативними аферентами. Таким чином, TRPV1 не є єдиним сенсором, який активується болісним нагріванням і забезпечує теплову чутливість ноцицепторів шкіри.
Ключові слова: ізольований препарат *n. saphenus*; ноцицепція; нагрівання; капсаїцин; TRPV1; AMG-517.

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Received 21.12.2024