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Substance MCS-18 Isolated from Helleborus Purpurascens Is a Potent Antagonist of the Capsaicin Receptor, TRPV1, in Rat Cultured Sensory Neurons

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Summary

Extracts of *Helleborus* roots were traditionally used in the Balkan area for their analgesic action. We report that the pure natural product MCS-18 isolated from this source is a potent, specific and reversible antagonist of the capsaicin receptor, TRPV1, expressed in rat dorsal root ganglion (DRG) neurons. TRPV1 is a non-selective cation channel expressed in a subset of cutaneous and visceral sensory nerve endings and activated by noxious heat, acidity and fatty acid metabolites of arachidonic acid, with a decisive role in inflammatory heat hyperalgesia. MCS-18 inhibited the increase in intracellular calcium concentration evoked in DRG neurons by capsaicin (300 nM) and low pH (5.5) but not by heat (43 ºC). The substance had no effect on the responses mediated by acid-sensing ion channels (ASICs) or the irritant receptor TRPA1. Whole-cell patch-clamp was used to confirm the inhibition of capsaicin-induced currents by MCS-18 which was dose-dependent. The mechanism of inhibition does not require an intact cell, as capsaicin-induced currents were also inhibited in the excised outside-out configuration. The antagonism of the capsaicin and proton action on native TRPV1 by MCS-18 may be of interest for pain therapy.

Key words

Pain • TRP channels • Plant extract • DRG • Capsaicin

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Introduction

Nociceptors are sensory neurons involved in the detection of harmful stimuli, which induce, or may induce, tissue damage. TRPV1 is a polymodal receptor expressed in nociceptive endings in cutaneous and visceral tissues, activated by a variety of stimuli such as noxious heat (>43 ºC), acid solutions, fatty acid derivatives of the arachidonic acid and exogenous compounds like capsaicin, the active ingredient in chili peppers (Caterina *et al.* 1997, for a review see Nagy *et al.* 2004). TRPV1 is a member of the TRP (transient receptor potential) superfamily of ion channels, a number of which are involved in sensory transduction (TRPV2-4, TRPA1, and TRPM8, for a review see Ramsey *et al.* 2006), with putative nociceptive roles. Although TRPV1 null-mutant mice still display sensitivity to noxious heat, the inflammatory heat hyperalgesia (an increased response to noxious heat in inflammatory states) is abolished in these animals, demonstrating a crucial role of TRPV1 in chronic inflammatory pain (Caterina *et al.* 2000, Davis *et al.* 2000). TRPV1 is expressed in nociceptive endings and its activation by acute noxious stimuli leads to depolarization, triggering action potential discharge and allowing Ca^{2+} entry. This in turn leads to neurogenic inflammation, through release of tachykinins like calcitonin-gene-related-peptide (CGRP), neurokinin A and substance P from nociceptive endings, inducing vasodilatation, edema and pain (Veronesi and Oortgiesen 2006). The biophysical properties and level of expression

of TRPV1 are subject to intense regulation in a variety of chronic pain states in humans, such as: inflammatory bowel disease, faecal urgency, irritable bowel syndrome, acute pancreatitis, cough, migraine headache, vulvodynia and mastalgia (Geppetti *et al.* 2006, Wick *et al.* 2006, Szallasi *et al.* 2007). Due to the prominent role of TRPV1 in inflammatory pain states, special attention has been focused on the development of TRPV1 antagonists for clinical use (Garcia-Martinez *et al.* 2006, McGaraughty *et al.* 2006, Bhattacharya *et al.* 2007, Chizh *et al.* 2007, Gunthorpe *et al.* 2007, Tang *et al.* 2007). The promising therapeutic potential of these compounds is supported by data on various animal models of chronic pain, including bone cancer pain, complete Freund's adjuvant-induced thermal hyperalgesia, carageenan-induced inflammation, formalin test, capsaicin- and citric acid-induced cough, L-arginine-induced pancreatitis, UV-induced inflammation (Szallasi *et al.* 2007). Reduced inflammation and pain behavior was described in an arthritis model in TRPV1-/- mice, while TRPV1 antagonists were able to reduce both inflammation and pain in wild-type animals (Keeble *et al.* 2005, Kissin *et al.* 2005, Barton *et al.* 2006). There is also accumulating evidence that TRPV1 is involved in pain induced by lesions to the nerve itself (neuropathic pain). A number of TRPV1 antagonists as well as RNA interference or intrathecal application of antisense oligonucleotides were effective in inducing pain relief in several animal models of neuropathic pain such as chronic constriction injury and spinal nerve ligation (Walker *et al.* 2003, Jhaveri *et al.* 2005, Kanai *et al.* 2005, Christoph *et al.* 2006, 2007). Rapidly accumulating evidence for the analgesic and anti-inflammatory efficacy of TRPV1 antagonists has remarkably increased the research work for novel drug candidates.

The active substance MCS-18 is classified as a pure natural compound extracted from the roots of *Helleborus purpurascens* (Wissner and Kating 1974), and purified by repeated liquid-liquid extraction, selective solid phase adsorption-desorption, and by reversed phase liquid chromatography on preparative scale and finally by re-crystallization. Preliminary work has provided evidence that this compound modulates the immune system, inducing up-regulation of the cytokines IL-10 and TGF-β (Szegli *et al.* 2005). Recent investigations revealed that MCS-18 is a specific and potent suppressor of the maturation of human dendritic cells (DCs) *in vitro* (Horstmann *et al.* 2008) and it produces a strong down-regulation of T-cell-dependent antibody formation in mice (Kerek *et al.* 2008).

The aim of the present work was to test if MCS-18 could inhibit TRPV1 expressed in the native tissue (rodent DRG neurons in primary culture). Calcium microfluorimetry and the patch-clamp technique were applied to monitor the effects of MCS-18 on the responses mediated by TRPV1. The obtained data provide solid evidence for an antagonistic action of MCS-18 against the activation of native TRPV1 by capsaicin and acid solutions, but not by noxious heat.

Methods

Preparation of MCS-18

MCS-18 was prepared by the extraction of 1 kg dried roots of *Helleborus purpurascens* with a first extraction in 16 liters of methanol: water (90:10) at room temperature. Methanol was removed using a vacuum rotatory evaporator and the resulted 1.5 liter aqueous emulsion was extracted twice with dichlormethane/ hexane (1:2) to remove lipids. Subsequent purification steps were applied to remove the toxic bufadienolidic derivatives, like hellebrin and the hellethionins (Milbradt *et al.* 2003). The obtained crude MCS product was further purified through reversed phase HPLC on a preparative scale. A Kontron HPLC device equipped with a Pump-422, Autosampler-465, and DA-Detector-430 was used. Samples of 0.4-0.6 ml of a 5 % stock solution of crude MCS-18 in 20 % acetonitrile were applied to a preparative column filled with 5 µm Hypersil HS-C8 Thermoquest 250x21 mm. The elution was carried out with a 45 min linear gradient from 5 % to 50 % acetonitrile (gradient grade, Merck Darmstadt) in water with a flow-rate of 5.0 ml/min. Analytical HPLC was performed as described previously (Horstmann *et al.* 2008). The MCS-18 for therapeutic purposes is a chemically homogenous pure compound which contains several structurally very similar isomers which are not separable in individual species. The HPLC chromatogram (Fig. 1) evidences the purity of the drug substance by lack of any impurities detectable by HPLC. The content of MCS-18 in pure drug substance is assayed by the HPLC method with an internal standard such as 2,4-dimethoxybenzoic acid (DMBA). Standardization of the drug substance is according to specifications in conformity with methods of the European Pharmacopoeia including the UV-vis absorbance at specified wavelengths and their ratio, the fluorescence spectrum, the acid value, the complexation with transitional metal ions. Potential impurities of the final drug substance

MCS-18 are rigorously controlled in ppm amounts by ELISA methods. This refer to hellethionins (HT), the toxic cysteine rich 5kDa proteins described in *Helleborus purpurascens* (Milbradt *et al.* 2003) with a maximum tolerated limit of [HT] ≤ 0.05 % in the pure MCS-18. Second potentially toxic impurities of MCS-18 are the cardiac glycoside hellebrin (Hb) and derivatives with a maximum tolerated limit of [Hb] ≤ 0.01 %. The standard anti-hellethionin and anti-hellebrin antibodies may be purchased by the Anti-Prot GmbH Munich (www.antiprot.com).

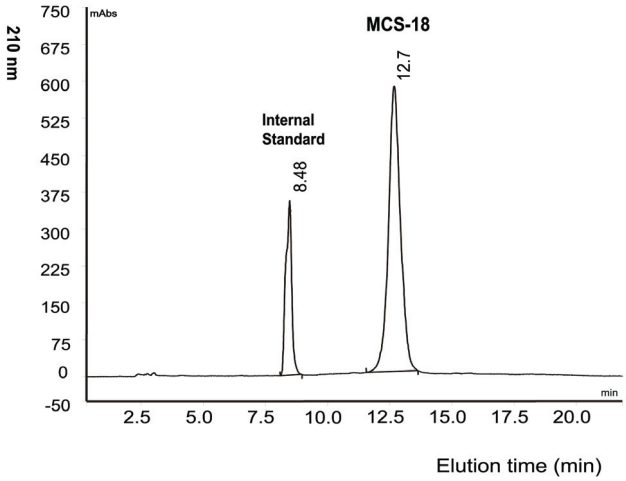

Fig. 1. HPLC chromatogram of MCS-18. Chromatographic conditions (reverse-phase system). Column: RP C8, e.g. Macherey-Nagel Nucleosil C8 (250 x 4 mm), 5 µm, 300 Å. Eluent A: 1 ml of phosphoric acid in 1000 ml of water. Eluent B: 1 ml of phosphoric acid in 1000 ml of ACN (Lichrosolv Merck). Gradient: 5 % B to 85 % B in 20 min. Flow rate: 1.0 ml/min. Detection: UV (210 nm). Injection vol.: 20 µL (0.2 % in water) Internal standard 2,4-dimethoxybenzoic acid (DMBA).

DRG culture

Dorsal root ganglion (DRG) neurons were obtained from all spinal levels of adult rats as described elsewhere (Reid *et al.* 2002). Wistar rats (150-200 g) were killed by CO_2 inhalation followed by decapitation, according to the European Guidelines on Laboratory Animal Care, with the approval of the institutional Ethics Committee of the University of Bucharest. DRGs were then removed and incubated in a mixture of 1 mg/ml collagenase (type XI, Sigma) and 3 mg/ml protease (Sigma) in IncMix solution (see Solutions and chemicals below) for 1 h at 37 °C. Neurons were mechanically dissociated by trituration and then plated on 25 mm borosilicate coverslips (0.17 mm thick) previously treated with poly-D-lysine (0.1 mg/ml, for 30 min), and cultured at 37 ºC and 5 % CO_2, in a 1:1 mixture of DMEM and Ham's F-12 medium with 10 % horse serum and 50 µg/ml gentamicin. The cells were used for recordings the day after the culture was made (between ~12 and 24 h after plating). All chemicals used for cell culture were from Sigma.

Intracellular calcium imaging

Before recording, coverslips with attached neurons were incubated for 30 min at 37 ºC in standard extracellular solution containing 2 µM Calcium Green-1 AM and 0.02 % Pluronic F-127 (both from Invitrogen), and then left for another 30 min to recover. Coverslips were then mounted in a Teflon chamber (MSC TD from Digitimer, Welwyn Garden City, UK) on the stage of an Olympus IX-70 inverted microscope. Most experiments involving chemical stimuli were carried out at room temperature (~22 ºC). Heat stimuli were applied by local superfusion, using a feedback controlled Peltier system previously described (Reid *et al.* 2001), and consisted in 15 s ramps from 32 to 45 ºC. Between heat stimuli the cells were kept at a holding temperature of 32 ºC. In order to measure the temperature experienced by the neurons, after the experiment, a miniature thermocouple (1T-1E, Physitemp, Clifton, NJ, USA) was placed where the cells had been and the same thermal protocol was applied. The reproducibility of the heat stimuli was very good. Fluorescence changes were recorded with a CCD camera (Cohu 4910, Pieper GmbH, Schwerte, Germany), while the cells were illuminated with 100 W halogen lamp and filter wheel (Cairn Research, Faversham, UK), controlled by the Axon Imaging 2.2 software, which was also used for image acquisition and analysis.

The stimulation protocol consisted of five successive application of the stimulus (chemical agents or heat), separated by 4 min intervals. MCS-18 was pre-applied for 1 min and during the third application of the stimulus. The response was measured as the maximal change in fluorescence ΔF ($F-F_0$, where F is the maximum level of fluorescence reached in the presence of the stimulus, and F_0 the initial fluorescence, immediately before the stimulus) divided by the initial fluorescence F_0. Cells with a $\Delta F/F_0 > 0.1$ were considered as having responded to the stimulus, and used for further analysis. Data were analyzed using the IDL 5.4 software (Research Systems, Boulder, Colorado, USA) and Microsoft Excel, and are presented as mean ± S.E.M. The statistical analysis was performed using one-way ANOVA with correlated samples. A value of $P < 0.05$ was considered to be statistically significant.

Patch-clamp recordings

Patch-clamp recordings were made in both the whole-cell and the outside-out configurations using borosilicate glass pipettes (GC150TF, Harvard Apparatus), heat polished to a resistance of 2-4 MΩ. Currents were recorded with an EPC-7 amplifier (HEKA Electronic GmbH), filtered at 3 kHz and digitized with a Labmaster 160 kHz DMA interface (Scientific Solutions, Mentor, Ohio, USA), using software written by Dr. Gordon Reid (University College Cork, Ireland, g.reid@ucc.ie). The experiments were carried out in extracellular calcium-free conditions to minimize TRPV1 desensitization induced by calcium entry. Analysis was carried out with the Origin 6.0 software (OriginLab Corporation, Northampton, MA, USA).

Solutions and chemicals

The IncMix solution for DRG incubation contained (in mM): NaCl 155, K_2HPO_4 1.5, HEPES 5.6, Na-HEPES 4.8, glucose 5. The antibiotic gentamycin was added to 50 μg/ml.

The standard extracellular solution contained (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, NaOH 4.55, glucose 5, pH 7.4 at 25 °C. For the acid solutions HEPES was replaced by MES (10 mM) and the pH was adjusted by adding 2 mM NaOH.

The extracellular calcium-free solution used in patch-clamp experiments contained (in mM): NaCl 140, KCl 4, $MgCl_2$ 3, HEPES 10, NaOH 4.5, glucose 5, pH 7.4 at 25 °C.

The pipette solution contained (in mM): KCl 135, $MgCl_2$ 1.6, EGTA 2, Mg-ATP 2.5, HEPES 10, with pH adjusted to 7.3 by adding NaOH.

Test substances were added from the following stock solutions: MCS-18, 1 mg/ml in H_2O, capsaicin (Sigma), 5 mM in ethanol, cinnamon oil (Sigma), 200 mM in ethanol. The same volume of pure solvent of the test substance was added in the standard extracellular solution to prevent any effects induced by the vehicle.

Results

MCS-18 inhibits capsaicin- and proton-, but not heat-evoked increases in intracellular calcium concentration in TRPV1-expressing DRG neurons in the rat

The experiments were aimed at monitoring the effect of the MCS-18 compound at 1 μg/ml on the polymodal receptor TRPV1. Three different modes of activation of TRPV1 were applied: capsaicin, acidic solutions and heat (> 43 ºC). The changes in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by application of these stimuli on rat DRG neurons in primary culture were recorded.

The first experimental protocol consisted in five consecutive applications of capsaicin (300 nM, for 15 s), at 4 min interval. MCS-18 was pre-applied 1 min before and during the third capsaicin challenge. In control conditions, in which only the vehicle (H_2O) was applied in the same conditions, a certain degree of desensitization of the response between the first and the second capsaicin applications was observed, such that some cells were completely desensitized and the response abolished. For data analysis only cells which responded to both of the first two capsaicin applications were considered. In this group the responses to the second, third, fourth and fifth applications of capsaicin were not statistically different (one-way ANOVA with correlated samples, $p > 0.05$) (Fig. 2B). In the presence of MCS-18 there was a substantial reduction (~86 %) in the amplitude ($\Delta F/F_0$) of the third response to capsaicin (0.03 compared to 0.21 for the second response, $n = 25$, $p < 0.0001$) (Figs 2A and 2B).

In a separate set of experiments MCS-18 (1 μg/ml) was pre-applied for 1 min and during the first application of capsaicin (300 nM, 15 s), in order to investigate the effect of the compound on the vanilloid receptor previously unexposed to its chemical agonist. Under these conditions, capsaicin could evoke substantial calcium transients, which were however reduced compared to those evoked by the same capsaicin stimulus in the absence of MCS-18 ($\Delta F/F_0$ was 0.43 ± 0.06, $n = 26$, compared to 0.55 ± 0.05, $n = 31$, Student's unpaired t test, $p < 0.05$). Moreover, 6 neurons which did not respond to capsaicin in the presence of MCS-18 became capsaicin-sensitive when exposed again to capsaicin. Such a situation (in which a neuron does not respond to the first capsaicin stimulation but does respond to subsequent stimuli) was never encountered in control conditions. It can be thus inferred that MCS-18 has a more pronounced effect on the desensitized state of TRPV1, but it does also inhibit, albeit to a lesser extent, the response to the first capsaicin exposure.

While investigating the effect of MCS-18 on the activation of TRPV1 by low pH in DRG neurons we had to take into account the fact that a solution of pH 5.5 would not only activate TRPV1 but also the members of the ASIC (acid-sensing ion channels) family which are all expressed in sensory neurons (ASIC1a and 1b, ASIC2a

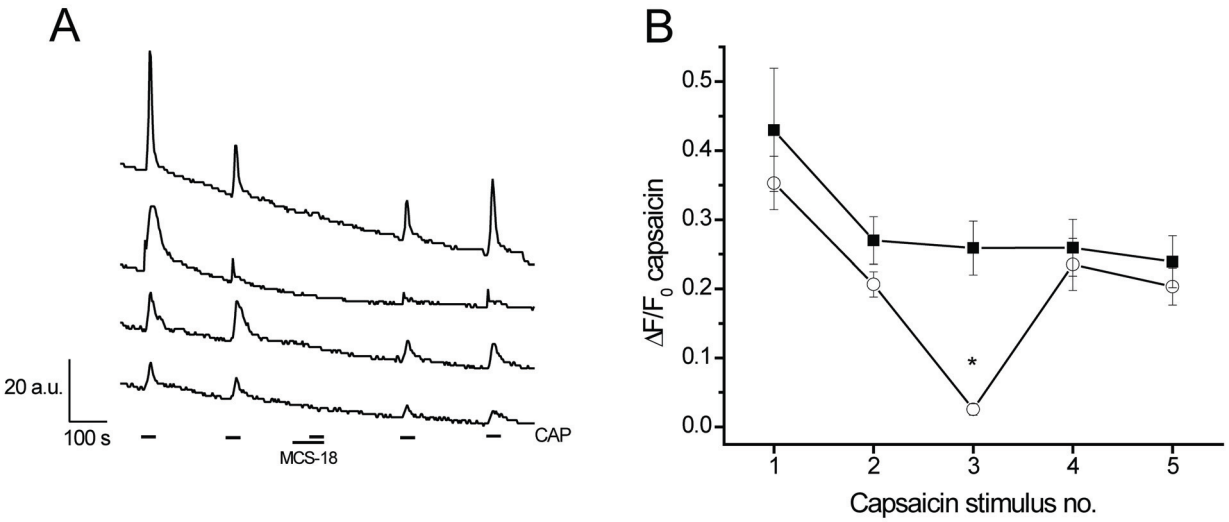

Fig. 2. A. Representative examples of Calcium Green-1 fluorescence changes induced by 5 consecutive applications of capsaicin (300 nM) for 15s at 4 min interval. The Y-axis represents fluorescence in arbitrary units, and the X-axis is time. MCS-18 (1 μg/ml) was pre-applied for 1 min and during the 3rd application of capsaicin. Note the almost complete inhibition of the response to capsaicin in the presence of MCS-18. **B.** Statistical analysis of the data illustrated in part A. The mean response ($\Delta F/F_0$) is plotted for each of the 5 stimuli in control conditions (black squares) and for the MCS-18 treated cells (open circles). Bars represent S.E.M. The mean response in the presence of MCS-18 (*) is significantly reduced compared to the responses to stimuli 2, 4 and 5 (one-way ANOVA with correlated samples, $n = 25$, $p < 0.0001$).

and 2b, ASIC3 and ASIC4) (Voilley *et al.* 2001). In order to separate the two acid detectors, a protocol was used consisting in five consecutive application of an acidic solution (pH 5.5 for 15 s at 4 min interval), followed by the application of a high dose of capsaicin (2 μM, 30 s). MCS-18 was pre-applied for 1 min before and during the third acid stimulus. Acid-sensitive neurons were then divided into capsaicin-sensitive (and thus TRPV1-expressing) and capsaicin-insensitive (probably displaying a pure ASIC response). As TRPV1 and the ASIC channels are known to be co-expressed in a fraction of sensory neurons, it is quite likely that in the first population of neurons (acid-sensitive, capsaicin-sensitive), low pH evokes a complex response, with contributions from both TRPV1 and the ASIC channels. MCS-18 had no significant effect on capsaicin-insensitive, acid-sensitive neurons, which suggests that the drug has no pharmacological action on the ASIC channels. However, in capsaicin-sensitive, acid-sensitive neurons, the response to low pH in the presence of MCS-18 was significantly decreased (by 44 %, $\Delta F/F_0$ was 0.14 ± 0.03, compared to 0.25 ± 0.02 for the second response, $n = 32$, $p < 0.01$) (Figs 3A and 3B). Interestingly, following MCS-18 application and wash, the fifth response to low pH was also significantly decreased, compared to the previous one. This may reflect increased desensitization of the response, following a stronger response (the fourth) after MCS-18 was removed (Figs 3A and 3B).

Activation of TRPV1 by heat was not prevented by MCS-18. In control conditions five consecutive heat stimuli (15 s heat ramps from 32 to 45 °C) were applied, following which neurons were challenged with a high dose of capsaicin (2 μM). In a separate set of experiments, MCS-18 was pre-applied for 1 min before and during the third heat stimulus. The compound had no significant effect on the amplitude of the response to heat in capsaicin-sensitive DRG neurons (Figs 4A and 4B). A very small fraction of heat-sensitive neurons were capsaicin-insensitive (3 of 28 in control conditions and 1 of 30 in the group on which the drug was tested).

MCS-18 has no effect on the increases in $[Ca^{2+}]_i$ evoked by the TRPA1 agonist cinnamaldehyde

TRPA1 is a non-selective cation channel expressed in cutaneous and visceral nociceptive endings, activated by a variety of irritant chemicals (mustard oil, cinnamaldehyde, allicin) and inflammatory agents (bradykinin) (Story *et al.* 2003, Bandell *et al.* 2004). To investigate the specificity of the MCS-18 action on TRPV1 the responses of cultured sensory neurons from the rat to the application of a saturating concentration (200 μM) of the specific TRPA1 agonist cinnamaldehyde (CA) were monitored, in the absence and in the presence of 1 μg/ml MCS-18. In control conditions, cinnamaldehyde was applied two times for 2 min at 10 min interval. In another

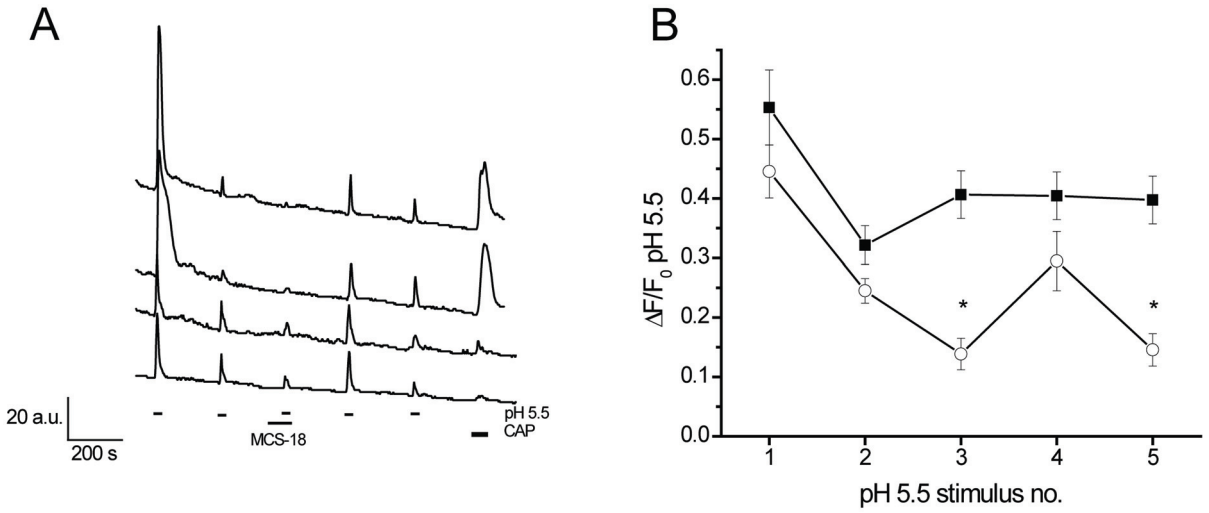

Fig. 3. A. Representative examples of Calcium Green-1 fluorescence changes induced by 5 consecutive applications of acid solutions (pH 5.5) for 15s at 4 min interval in capsaicin-sensitive DRG neurons. The Y-axis represents fluorescence in arbitrary units, and the X-axis is time. MCS-18 (1 μg/ml) was pre-applied for 1 min and during the 3rd application of low pH. Capsaicin (2 μM) was applied at the end of the experiment to separate capsaicin-sensitive from capsaicin-insensitive neurons. Note the partial inhibition of the response to extracellular acidity in the presence of MCS-18. **B.** Statistical analysis of the data illustrated in part A. The mean response ($\Delta F/F_0$) is plotted for each of the 5 stimuli in control conditions (black squares) and for the MCS-18 treated cells (open circles). Bars represent S.E.M. The mean response in the presence of MCS-18 and the response to the 5th stimulus (*) are significantly reduced compared to the responses to stimuli 2 and 4 (one-way ANOVA with correlated samples, n = 32, $p < 0.01$).

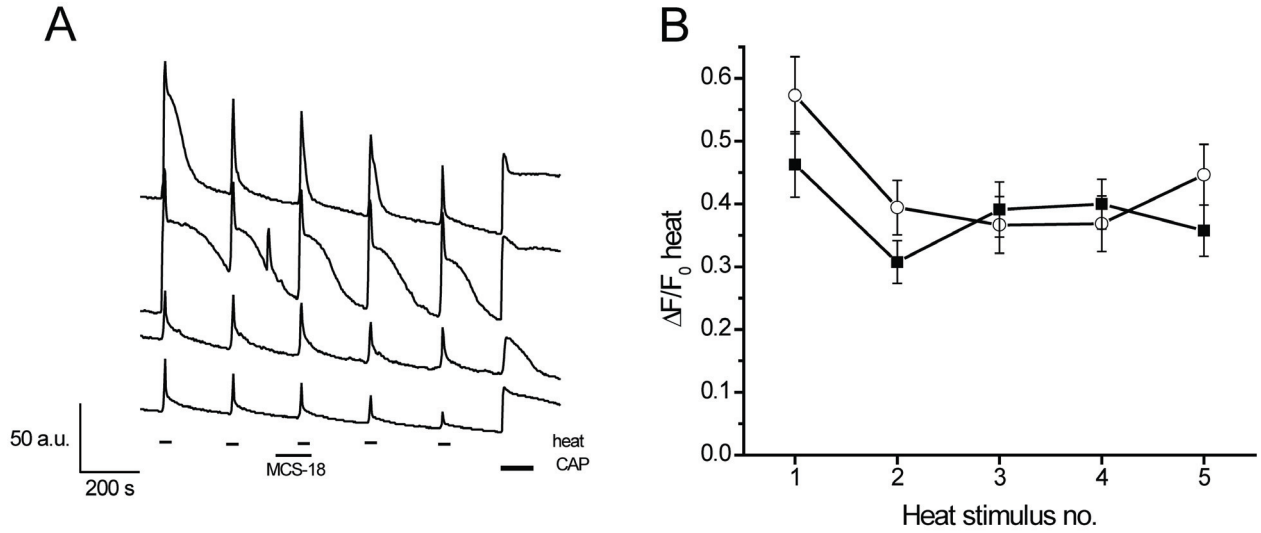

Fig. 4. A. Representative examples of Calcium Green-1 fluorescence changes induced by 5 consecutive applications of a heat ramp (15 s, from 32 to 45 °C) at 4 min interval. The Y-axis represents fluorescence in arbitrary units, and the X-axis is time. MCS-18 (1 μg/ml) was pre-applied for 1 min and during the 3rd heat stimulus. Capsaicin (2 μM) was applied at the end of the experiment to separate capsaicin-sensitive from capsaicin-insensitive neurons. Note the lack of an effect of MCS-18 on the heat-induced increase in $[Ca^{2+}]_i$. **B.** Statistical analysis of the data illustrated in part A. The mean response ($\Delta F/F_0$) is plotted for each of the 5 stimuli in control conditions (black squares) and for the MCS-18 treated cells (open circles). Bars represent S.E.M.

set of experiments, MCS-18 was pre-applied for 1 min and during the second application of cinnamaldehyde. The drug had no significant effect on the amplitude of the increase in $[Ca^{2+}]_i$ evoked by CA in cultured rat DRG neurons (data not shown). In control conditions, the response to the second application of CA was significantly reduced compared to the first, such that the ratio between the second and the first response was 0.76 ± 0.07 (n = 30). For MCS-18-treated cells the ratio was 0.62 ± 0.06 (n = 16), and the difference was not statistically significant ($p > 0.05$, Student's unpaired t test).

MCS-18 inhibits capsaicin-induced currents in rat DRG neurons in both the whole-cell and the outside-out patch-clamp configurations

Whole-cell capsaicin-induced currents were

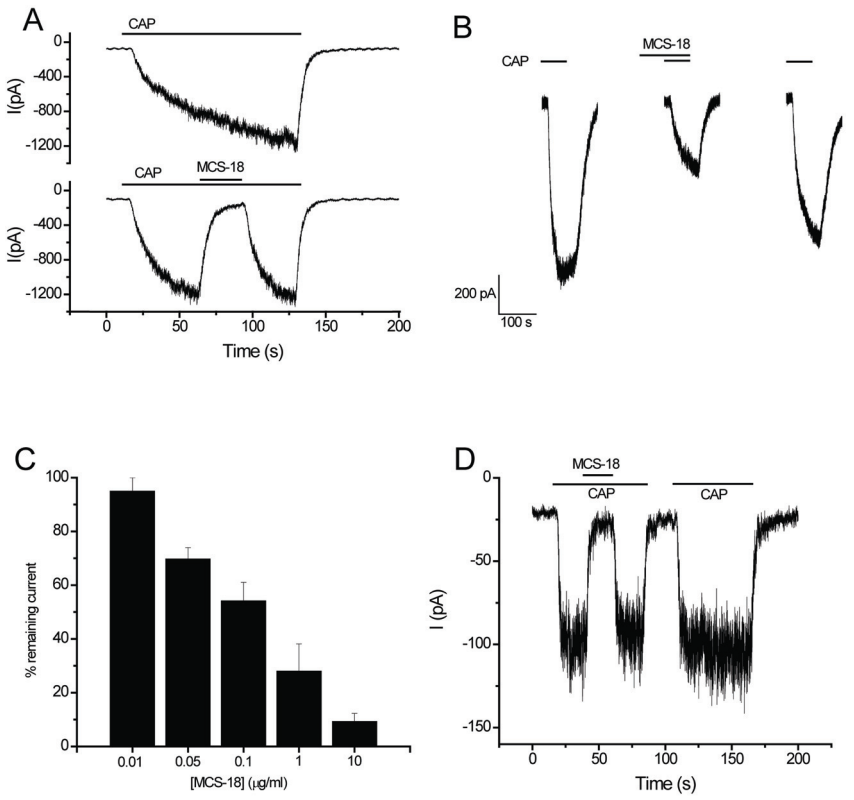

Fig. 5. A. Representative example of the capsaicin-induced current in calcium-free extracellular solution in a rat DRG neuron in the whole-cell configuration. Capsaicin (2 μM) was applied for 120 s in control conditions (upper trace). In the lower trace, MCS-18 (10 μg/ml) was co-applied with capsaicin for 30 s after the capsaicin-induced current reached a steady-state. Note the almost complete inhibition of the capsaicin-induced current in the presence of MCS-18. **B.** Inward currents induced by repetitive capsaicin (2 μM) application for 60 s at 3 min interval. The middle trace was recorded in the presence of MCS-18 (10 μg/ml). Note the strong and partly reversible reduction of the capsaicin-evoked current by MCS-18. **C.** Concentration dependence of the inhibitory action of MCS-18 on capsaicin-induced currents recorded in the whole-cell mode using the protocol illustrated in part A. The Y-axis shows the residual current in the presence of MCS-18 as a percentage of the steady-state current before the application of the drug. **D.** Capsaicin-induced current in an outside-out patch excised from a DRG neuron. Capsaicin was applied two times at 2 μM. During the first application, MCS-18 (10 μg/ml) was co-applied with capsaicin. Note the almost complete and reversible inhibition of the capsaicin-induced current in the isolated patch by MCS-18.

recorded at a holding potential of –60 mV in cultured rat DRG neurons in conventional whole-cell mode. The experiments were carried out in the absence of extracellular calcium in order to reduce TRPV1 desensitization induced by calcium entry and activation of calcineurin (Mohapatra and Nau 2005). Two experimental protocols were used. In the first protocol capsaicin (2 μM) was first applied for 120 s, during the second capsaicin application, the drug MCS-18 was applied together with capsaicin for 30s (Fig. 5A). In the second protocol capsaicin was applied at 2 μM for 60 s at 3 min interval, until a reproducible response was obtained (i.e. there was no further desensitization); following two consecutive capsaicin applications that yielded responses of similar amplitude MCS-18 was pre-applied for 1 min and during the following application of capsaicin, the drug was then washed out and the response to capsaicin further recorded to monitor recovery from the inhibition induced by MCS-18 (Fig. 5B). In both experimental designs the drug induced a profound and concentration-dependent inhibition of the whole-cell inward current evoked by capsaicin in rat DRG neurons. Figure 5C shows the concentration-dependence of the inhibitory effect of MCS-18 measured using the first protocol (a very similar dependence was obtained using the second protocol, data not shown).

Outside-out excised patches were obtained by withdrawing the pipette from the cell after entering the whole-cell configuration. In 4 such cell-free patches we could measure capsaicin (2 μM)-evoked currents which were reversibly inhibited by application of 10 μg/ml MCS-18 (Fig. 5D).

Discussion

MCS-18 is a novel natural compound isolated from the roots of *Helleborus purpurascens*, a plant which has long been used in traditional medicine for pain treatment in the Balkan area. Since 1980 *Helleborus* extracts have been approved and successfully used in Romania as injections and ointments for anti-rheumatic therapy (brand name Boicil®). Recent work has demonstrated that MCS-18 has a significant immunosuppressive action, by attenuating antibody production (Kerek *et al.* 2008), inhibiting dendritic cell activation and preventing autoimmunity in a mouse autoimmune encephalomyelitis model (Horstmann *et al.* 2008).

The present study shows that, in addition to its immunosuppressive activity, MCS-18 also acts as a

strong, specific and reversible antagonist of the TRPV1 receptor in the native tissue (rat DRG neurons in culture). The lack of any effect on the changes in $[Ca^{2+}]_i$ induced by extracellular protons in capsaicin-insensitive neurons (most likely mediated by ASIC channels) or on those evoked by the TRPA1 agonist cinnamaldehyde suggest that MCS-18 is not a non-selective ion channel blocker, but its action on TRPV1-mediated responses is specific. Moreover, the fact that MCS-18 failed to inhibit the increase in $[Ca^{2+}]_i$ induced by cinnamaldehyde demonstrates that it does not exert its inhibitory action by targeting voltage-gated sodium or calcium channels, or by reducing the overall excitability of sensory neurons. Interestingly, MCS-18 inhibits the activation of TRPV1 by capsaicin (Fig. 2), and partly by protons (Fig. 3), but not by heat (Fig. 4), and it is also less effective on the non-desensitized state of the channel, which indicates that the effects of MCS-18 are state-dependent. The weaker effect of the drug on proton-induced responses, compared to the very strong inhibition of the capsaicin-evoked increases in $[Ca^{2+}]_i$, may reflect an intrinsic difference in the effect of the drug on the two modes of TRPV1 activation, or may be due to a contribution of the ASIC channels to the neuronal responses to extracellular acidity.

The direct action of MCS-18 on TRPV1 was confirmed by patch-clamp recordings in both the whole-cell mode and in excised patch configuration. A strong reduction of the capsaicin-induced inward current in the presence of the drug was recorded using two experimental protocols. In both cases MCS-18 inhibited the capsaicin-induced whole-cell current, and the effect was both reversible and dose-dependent (Fig. 5C). At 1 μg/ml, MCS-18 blocked almost completely and reversibly the capsaicin-induced currents in excised outside-out membrane patches (Fig. 5D), indicating that its action is most likely a direct one on the channel protein itself, and not mediated by intracellular signaling pathways requiring soluble second messengers.

In conclusion, MCS-18 is a strong, selective, concentration-dependent and reversible inhibitor of the polymodal receptor TRPV1 expressed in cultured rat DRG neurons, it inhibits the activation of TRPV1 by capsaicin and protons (partially), but not by heat. MCS-18 has no effect on the activity of the acid-sensing ion channels (ASICs) or TRPA1. MCS-18 inhibits capsaicin-evoked inward currents in DRG neurons both in the whole-cell and the outside-out configurations, suggesting a direct action on the TRPV1 channel.

Conflict of Interest

There is no conflict of interest.

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