



## Volatile anaesthetics inhibit the thermosensitive nociceptor ion channel transient receptor potential melastatin 3 (TRPM3)



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### ARTICLE INFO

#### Keywords:

Volatile anaesthetics  
TRP ion channels  
TRPM3  
Nociception  
Thermosensation

### ABSTRACT

**Background:** Volatile anaesthetics (VAs) are the most widely used compounds to induce reversible loss of consciousness and maintain general anaesthesia during surgical interventions. Although the mechanism of their action is not yet fully understood, it is generally believed, that VAs depress central nervous system functions mainly through modulation of ion channels in the neuronal membrane, including 2-pore-domain K<sup>+</sup> channels, GABA and NMDA receptors. Recent research also reported their action on nociceptive and thermosensitive TRP channels expressed in the peripheral nervous system, including TRPV1, TRPA1, and TRPM8. Here, we investigated the effect of VAs on TRPM3, a less characterized member of the thermosensitive TRP channels playing a central role in noxious heat sensation.

**Methods:** We investigated the effect of VAs on the activity of recombinant and native TRPM3, by monitoring changes in the intracellular Ca<sup>2+</sup> concentration and measuring TRPM3-mediated transmembrane currents.

**Results:** All the investigated VAs (chloroform, halothane, isoflurane, sevoflurane) inhibited both the agonist-induced (pregnenolone sulfate, CIM0216) and heat-activated Ca<sup>2+</sup> signals and transmembrane currents in a concentration dependent way in HEK293T cells overexpressing recombinant TRPM3. Among the tested VAs, halothane was the most potent blocker (IC<sub>50</sub> = 0.52 ± 0.05 mM). We also investigated the effect of VAs on native TRPM3 channels expressed in sensory neurons of the dorsal root ganglia. While VAs activated certain sensory neurons independently of TRPM3, they strongly and reversibly inhibited the agonist-induced TRPM3 activity.

**Conclusions:** These data provide a better insight into the molecular mechanism beyond the analgesic effect of VAs and propose novel strategies to attenuate TRPM3 dependent nociception.

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<https://doi.org/10.1016/j.bcp.2020.113826>

Received 25 November 2019; Accepted 22 January 2020

Available online 25 January 2020

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## 1. Introduction

Volatile anaesthetics (VAs) are the most commonly used compounds to maintain general anaesthesia during operation both in human therapeutic interventions and in animal experiments [1]. Although the exact mechanisms, whereby VAs cause a reversible loss of consciousness are not yet fully understood, it is generally accepted that they suppress the activity of the central nervous system by specifically targeting cellular proteins, including several ion channels, among others. A few of these ion channels, especially GABA<sub>A</sub> receptors and various K<sup>+</sup> channels, are considered to play a central role in the general depression of the central nervous system functions resulting in reversible loss of the consciousness initiated by VAs [2–4]. However, VAs can also influence several other ion channels, including voltage-gated Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> channels [5,6]. Moreover, in recent years, their action on sensory transient receptor potential (TRP) channels has also been reported.

TRP channels form a heterogeneous and multifunctional group in the voltage gated-like superfamily of ion channels. The 6-transmembrane domain containing TRP proteins form non-specific, mostly Ca<sup>2+</sup> permeable cationic channels, and are functional as homo- or heterotetramers [7–9]. Most TRP channels are considered to function as polymodal “cellular sensors” sensitive to diverse changes in the physico-chemical environment (e.g. temperature, pH, osmolarity, ionic concentrations, endogenous mediators, external chemical irritants, etc.) [10,11] and increasing body of evidence indicates their emerging roles in various diseases [12,13]. In primary sensory neurons, the most widely studied sensory TRPs are the thermosensitive TRPV1, TRPA1 and TRPM8 channels. Their versatile role in diverse (patho)physiological sensory processes, including thermosensation, itch, pain (together with different forms of hyperalgesia and allodynia), and inflammation has inspired a plethora of research studies [14–21]. This in turn has urged several pharmaceutical companies to find effective tools targeting these thermosensitive TRP channels as potential novel drugs to manage several, mostly pain related, clinical syndromes [22]. Recently, VAs were also reported to activate TRPA1 [23,24], to sensitize TRPV1 [25] and also to modulate TRPM8 [26], and these results may explain some adverse effects related to general anaesthesia.

TRPM3 was recently introduced as a novel thermosensitive nociceptor TRP channel expressed by a large subset of primary sensory neurons of the dorsal root and trigeminal ganglia (DRG and TG). TRPM3 has an essential role in acute heat pain sensation [27], it contributes to the development of inflammatory heat hyperalgesia and transmits chemical pain sensation evoked by its endogenous steroid ligand pregnenolone sulfate (PregS) [28]. The stimulation of TRPM3 by certain ligands e.g. CIM0216, or special ligand combinations, e.g. co-application of PregS and the antifungal agent clotrimazole revealed an alternative ion permeation pathway beyond the main pore, which resulted in a strong inward current at negative membrane potentials. This unique current displays the general characteristics of the so-called omega currents reported earlier in mutant K<sup>+</sup> and Na<sup>+</sup> channels, and may have a high (patho)physiological relevance exacerbating TRPM3 related pain sensation [29–31]. The above results, together with the fact that modulation of TRPM3 activity, in contrast with TRPV1, did not induce any change in the core body temperature of experimental animals [28], introduce TRPM3 as an appealing drug target for novel therapeutic interventions in pain related syndromes. However, in order to exploit the putative therapeutic potential of TRPM3 as a potential drug target, a better understanding of its regulation and pharmacological interactions are of greatest importance.

In this study, we investigated the effect of VAs on recombinant and native TRPM3 ion channels and found that they potently inhibit TRPM3 function. These results further enhance our knowledge about VAs' mechanism of action and may contribute to the development of novel analgesics targeting TRPM3.

## 2. Materials and methods

### 2.1. Cell culturing and isolation of sensory neurons

Native HEK293T cells, and HEK293T cells stably overexpressing the mouse TRPM3 $\alpha$ 2 variant (HEK-M3 cells) were cultured as described before [28]. In brief, cells were cultured in DMEM medium (Invitrogen, Paisley, UK), supplemented with 10% foetal bovine serum, (Invitrogen), 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin (both from ThermoFisher, Waltham, MA, USA), 10 mM Glutamax, Non-Essential-Amino-Acids and 200  $\mu$ g/ml Hygromycin (all from Invitrogen) at 37 °C. Sensory neurons of dorsal root ganglia (DRGs) were obtained from adult (8–12 week old) C57BL6 mice, as described before [28]. Briefly, mice were euthanized by CO<sub>2</sub>, DRGs were isolated and digested with collagenase and dispase (both from Invitrogen). Suspension of sensory neurons was seeded on poly-L-lysine HBr (Sigma Aldrich, St.Louis, MO, USA) coated glass bottom culture dishes (MatTek, Ashland, MA, USA) and cultured in Neurobasal medium supplemented with 2% B-27 supplement (both from Invitrogen), 2 mM L-glutamine, 100  $\mu$ g/ml penicillin/streptomycin (both from ThermoFisher), and 100 ng/ml  $\beta$ -NGF (Pepro Tech EC, Ltd., London, UK) at 37 °C in 5% CO<sub>2</sub> containing atmosphere. Neurons were used for experiments within 24 to 36 h following isolation. We aimed at obtaining high number of sensory neurons yielding n  $\geq$  50 neurons in each experimental group for highly reliable statistical analysis. Therefore, respecting the 3R principles, we established multiple primary sensory neuron cultures from an animal and used them in independent measurements. Cultures were randomly recruited into the different experimental groups, and cultures from 3 individual animals were recruited into each group. Altogether, 12 animals were used for the study. All experimental procedures and animal husbandry were conducted following the European Parliament and the Council Directive (2010/63/EU) and national legislation.

### 2.2. Fluorescent Ca<sup>2+</sup> measurements

Fluorescent measurement of cytoplasmic Ca<sup>2+</sup> concentration in HEK cells and in individual DRG neurons were performed according to our previously optimized protocols: HEK-M3 cells were seeded in Poly-L-Lysine HBr (Sigma-Aldrich) coated 96-well/clear-bottom plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 100,000 cells per well in normal cell culture medium and incubated overnight. Next day, cells were loaded with 2  $\mu$ M Fura-2-AM (Invitrogen) at 37 °C for 30 min, and washed three times with Ca<sup>2+</sup>-buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>·x6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>·x2H<sub>2</sub>O, 10 mM glucose xH<sub>2</sub>O, 10 mM HEPES, pH 7.4 (all from Sigma-Aldrich)). The plates were then placed into a FlexStation 3 fluorescent microplate reader (Molecular Devices, Sunnyvale, CA, USA) and cytoplasmic Ca<sup>2+</sup> concentration (reflected by the ratio of fluorescence measured at  $\lambda_{\text{EX1}}$ : 340 nm,  $\lambda_{\text{EX2}}$ : 380 nm,  $\lambda_{\text{EM}}$ : 516 nm (F<sub>340</sub>/F<sub>380</sub>)) was monitored during application of compounds in various concentrations. During the measurements, cells in a given well were exposed to only one given concentration of the agents. Measurements were carried out at ambient temperature.

To determine the VAs' effect on the temperature-evoked activation of TRPM3, we used a Fluo-4 based assay and a QPCR system. HEK-M3 and non-transfected HEK293T cells were loaded with 2  $\mu$ M Fluo-4-AM (Invitrogen) for 30 min, then they were trypsinised and re-suspended in Ca<sup>2+</sup>-buffer in the presence or absence of VAs and transferred into PCR tubes with optical transparent cover (200.000 cells/tube, 200  $\mu$ L). Fluorescence was measured with a Stratagene Mx3005P QPCR instrument (Agilent Technologies Santa Clara, CA, USA) using an appropriate filter set while the well temperature was increased from 25 °C to 46 °C in steps of 3 °C.

To measure cytoplasmic Ca<sup>2+</sup> concentration in individual DRG neurons, we used a microscope based calcium imaging system. On the day after the isolation, DRG neurons were loaded with 2  $\mu$ M Fluo-4-AM

(Invitrogen) dissolved in normal  $\text{Ca}^{2+}$ -buffer, then placed on the stage of a Zeiss LSM 5 Live confocal fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany) and Fluo-4 loaded cells were captured with constant settings in every 1 s ( $\lambda_{\text{EX}}$ : 488 nm,  $\lambda_{\text{EM}}$ : 516 nm). During the measurements, cells were continuously perfused with  $\text{Ca}^{2+}$ -buffer and different compounds were applied via the perfusion. Data were presented as  $F_1/F_0$ , where  $F_0$  is the average fluorescence of the baseline (before the first compound application) and  $F_1$  is the actual fluorescence. Experiments were performed at room temperature (21–22 °C).

### 2.3. Electrophysiology

HEK-M3 cells were seeded to 12 mm glass coverslips previously coated with poly-L-lysine HBr (Sigma-Aldrich) and whole cell patch clamp measurements were carried out by using an Axopatch 1.D amplifier and Clampex 10.2 software (Molecular Devices). Pipettes with final resistances of 2–5 M $\Omega$  were fabricated and filled with intracellular solution containing 100 mM aspartic acid, 45 mM CsCl, 1.144 mM MgCl<sub>2</sub>, 10 mM HEPES, and 10 mM EGTA (all from Sigma-Aldrich). pH was adjusted to 7.2 using CsOH (VWR, Radnor, PE, USA). Experiments were performed in a bath solution composed of 150 mM NaCl, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES buffered to pH 7.4 (NaOH) (all from Sigma-Aldrich). To record TRPM3 mediated currents, the holding potential was 0 mV and cells were ramped every 2 s from –150 to +150 mV over the course of 200 ms. Recorded data were analysed and plotted using Origin 9.0 software (OriginLab Corporation, Northampton, MA, USA).

### 2.4. Preparation of working solutions of VAs

10 mM stock solutions of VAs were prepared in extracellular solution by a rigorous overnight stirring in air-tight closed vials. From these stock solutions, fresh dilutions of the final working solutions were prepared and used for measurements within 45 min. If needed, new working solutions were diluted in every 30 min.

### 2.5. Gas chromatography/mass spectrometry (GC/MS)

The stability of the stock solutions of VAs in an open, freely ventilating system was checked by GC/MS. Stock solutions prepared as described above were kept in an open vial at room temperature for 45 min simulating the conditions in an open perfusion system used for patch clamp and  $\text{Ca}^{2+}$  measurements. During this incubation time, samples were taken at different time points and subjected for analysis immediately. The active agents in the samples were identified by a GC/MS method using an Agilent 7980B-5977A instrument (Agilent Technologies, Santa Clara, CA, USA) at the Toxicology Laboratory of the Institute of Forensic Medicine of the University of Debrecen. The injection volume was 0.2  $\mu\text{L}$ , and the injector temperature was 250 °C. The applied capillary column was a J&W DB-35MS UI, 30 m  $\times$  0,25 mm  $\times$  0,25  $\mu\text{m}$ . The detection parameters were the followings: Sampling: split; split ratio: 20:1. Oven temperature program: 65 °C (2 min. hold time), heating: 50 °C/min to 230 °C; Interface: 280 °C; MS source: 230 °C; Ionization: EI; Detection mode: SCAN. The 'area under curve' of the specific peaks were determined in arbitrary units and normalized to that of obtained from the sample taken at the beginning (0 min) of the incubation.

### 2.6. Chemicals

Chloroform and halothane were obtained from Sigma Aldrich, isoflurane (sold as Vetflurane) was from Virbac (Carros, France) and sevoflurane from AbbVie Inc. (North Chicago, IL, USA). Pregnenolone sulphate (PregS), allyl isothiocyanate (AITC), capsaicin, thymol, CIM0216 were obtained from Tocris Bioscience (Bristol, UK). Isosakuranetin was obtained from Carl Roth (Karlsruhe, Germany).

Working solutions of VAs were prepared as described above. All other drugs were prepared as stock solutions in dimethyl sulfoxide (DMSO), and then diluted into extracellular solutions to reach the desired final concentration. Concentration of the vehicle (DMSO) in the final working solutions was 0.1%.

### 2.7. Curve fitting

Logistic dose-response curves were fitted using the equation  $y = A2 + (A1 - A2)/(1 + (x/x0)^p)$  where the calculated parameters are: A1: initial value ( $y_{\text{min}}$ ), A2: final value ( $y_{\text{max}}$ ),  $x0$ : center ( $\text{EC}_{50}$ ) and  $p$  is the calculated power. Fittings were carried out and parameters were calculated using Origin 9.0 (OriginLab Corporation).

### 2.8. Data and statistical analysis

Electrophysiological data were analysed using ClampFit 10.0 software (Axon Instruments, Foster City, CA, USA). IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA) and Origin 9.0 (OriginLab Corporation) were used for statistical analysis and data display. When testing antagonistic effect of isosakuranetin, agonist induced  $\text{Ca}^{2+}$  signals in the presence and absence of isosakuranetin were compared pairwise by two-tailed Student's  $t$ -test for independent samples. To control unwanted variances of measured currents between HEK-M3 cells, TRPM3 currents measured in the presence of VAs were normalized, and compared to the agonist induced current (considered as 100%) in the same cell and two-tailed Student's  $t$ -test for one sample was used for statistical evaluation. In case of  $\text{Ca}^{2+}$  signals recorded on sensory neurons, if it is not mentioned otherwise, signal amplitudes were normalized to the 1st agonist evoked signal, considered as 100%, to control variances experienced between individual neurons. Then, data were subjected for statistical analysis using one-way ANOVA with Dunnett post-hoc test to compare the effect of VAs to vehicle control. In every case,  $P < 0.05$  was regarded as showing significant differences between group means. All data are presented as mean  $\pm$  SD.

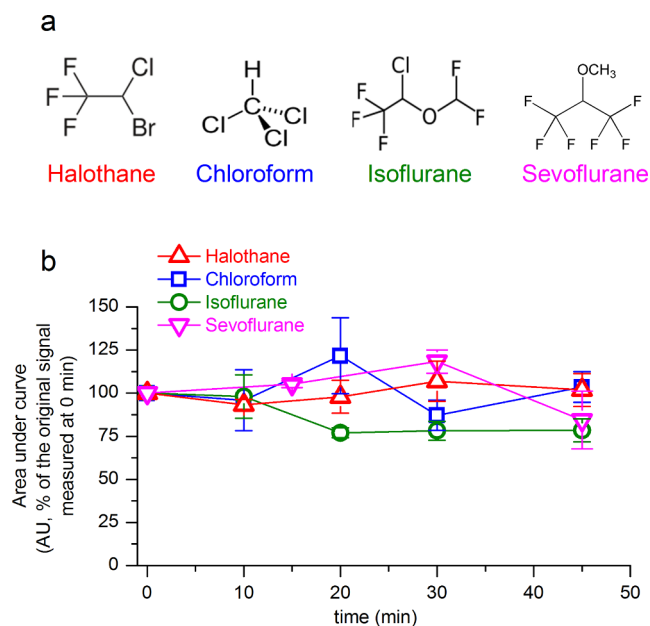
## 3. Results

### 3.1. VAs formed stabile solutions

In our study, we investigated the effect of VAs with various chemical structures (Fig. 1a) on TRPM3 ion channel functions. Due to their volatile and lipophilic nature, we assumed that it might be challenging to prepare a stabile aqueous solution of VAs suitable for measurements, although they reportedly can be solved  $> 10$  mM in water at 25 °C [32]. Therefore, we aimed at testing the stability of the stock solutions of VAs applied in an open, freely ventilating system mimicking the conditions in our perfusion and liquid handling systems used in the experiments. For this, we performed GC/MS measurements on 10 mM stock solutions of the VAs, which were prepared by rigorous overnight stirring in an airtight vial, as described in the Materials and methods. The concentration of the stock solutions in open vials did not change dramatically during 45 min (Fig. 1b). Therefore, working solutions were freshly prepared from 10 mM stock solutions stored in airtight vials and used for measurements within 30 min. After 30 min, new working solutions were prepared.

### 3.2. VAs inhibited chemical agonist induced activation of recombinant TRPM3

First, we investigated the effect of VAs on recombinant TRPM3 by carrying out  $\text{Ca}^{2+}$  measurements on HEK-M3 cells. During the measurements, we applied various concentrations of VAs followed by the application of TRPM3 agonists PregS or CIM0216 in the continuous presence of VAs, as shown in Fig. 2a. This experimental design enabled us to investigate both potential activating and inhibiting effect of VAs.



**Fig. 1.** The structure and stability of the investigated volatile anaesthetics (VAs). (a) Chemical structure of VAs investigated in this study: halothane (a halogenated alkene), chloroform (trichloromethane), isoflurane (a halogenated methyl-ethyl ether) and sevoflurane (fluorinated methyl-isopropyl ether). (b) The stability of the most concentrated 10 mM stock solutions in open, freely ventilating vials was checked by GC/MS in the given timeframe as described in the “Materials and methods” section ( $n = 3$  at each point).

We found that none of the investigated VAs activated the recombinant TRPM3, whereas each of them inhibited the TRPM3-mediated  $\text{Ca}^{2+}$  signals evoked by the endogenous TRPM3 agonist PregS and by the more potent synthetic TRPM3 agonist CIM0216 [30] in a dose dependent manner (Fig. 2a-c). The  $\text{IC}_{50}$  values for halothane, chloroform, isoflurane, and sevoflurane were (mean  $\pm$  SEM)  $0.52 \pm 0.05$  mM,  $1.67 \pm 0.03$  mM,  $1.09 \pm 1.49$  mM and,  $3.83 \pm 2.39$  mM, respectively, when TRPM3 was activated by  $10 \mu\text{M}$  PregS (Fig. 2b), while the  $\text{IC}_{50}$  values were  $1.52 \pm 0.05$  mM,  $1.64 \pm 0.25$  mM,  $2.71 \pm 0.88$  mM and,  $2.0 \pm 0.28$  mM when activated by  $3 \mu\text{M}$  CIM0216, respectively (Fig. 2c). Moreover, the  $\text{EC}_{50}$  value of PregS was shifted from  $0.37 \pm 11 \mu\text{M}$  to  $19.33 \pm 4.24 \mu\text{M}$  ( $\sim 51 \times$  fold),  $2.13 \pm 0.35 \mu\text{M}$  ( $\sim 6 \times$  fold),  $3.49 \pm 1.3 \mu\text{M}$  ( $\sim 8 \times$  fold), and  $6.14 \pm 6.39 \mu\text{M}$  ( $\sim 15 \times$  fold) by 1 mM halothane, chloroform, isoflurane, and sevoflurane, respectively (Fig. 2d).

Although halothane was found to be the most potent inhibitor of TRPM3 among the tested VAs, it also evoked robust  $\text{Ca}^{2+}$  transients when applied alone at high concentrations ( $\geq 5$  mM) (Fig. 3a). This stimulatory effect of halothane was found to be independent of TRPM3, because (i) it was equally observed in native HEK293T cells and HEK-M3 cells (Fig. 3a-b) and (ii) the halothane-induced  $\text{Ca}^{2+}$  signals were not inhibited by the TRPM3 antagonist isosakuranetin (Fig. 3c). It is important to mention that the commercially available halothane applied in the current study also contained ca. 150 ppm thymol, as stabilizer resulting in ca.  $0.75 \mu\text{M}$  thymol in the most concentrated (5 mM) halothane working solution tested. Since thymol was reported to be an activator of TRPV3 [33,34] and TRPA1 [35], other thermosensitive members of TRP family, we also investigated the effect of thymol on TRPM3, to clarify its potential contribution to the effect of halothane. Our results indicated that thymol applied at concentrations up to  $300 \mu\text{M}$  did not affect TRPM3 functions: it neither activated HEK-M3 cells nor inhibited their activation evoked by PregS (Fig. 3d).

### 3.3. VAs inhibited the heat-evoked activation of TRPM3

Next, we tested whether the VAs influence activation of TRPM3 by thermal stimuli, in addition to their effect on ligand-induced activation of the channel. HEK-M3 cells were challenged by precise temperature steps using a Quantitative real-time PCR instrument, and the intracellular  $\text{Ca}^{2+}$  concentration was monitored in the presence of VAs at various concentrations. Our results revealed that VAs also inhibited warming-induced activation of TRPM3: the obtained  $\text{IC}_{50}$  values of halothane, chloroform, isoflurane, and sevoflurane were  $1.08 \pm 0.58$  mM,  $1.50 \pm 0.63$  mM,  $1.64 \pm 0.59$  mM, and  $1.83 \pm 0.63$  mM, respectively, when cells were heated from  $37$  to  $43$  °C (Fig. 4).

### 3.4. VAs inhibited TRPM3 mediated transmembrane currents

In whole-cell patch-clamp measurements, PregS induced an outwardly rectifying whole-cell current in HEK-M3 cells (Fig. 5a-b). This TRPM3-mediated current was partially inhibited by 1 mM chloroform, halothane and isoflurane, and almost fully abolished when these VAs were applied at 5 mM. Sevoflurane was less effective: when applied at 1 mM, it only minimally inhibited the PregS-induced TRPM3 current, whereas at 5 mM it evoked a marked but incomplete inhibition (Fig. 5c). The effects of the VAs were fast and reversible (Fig. 5a).

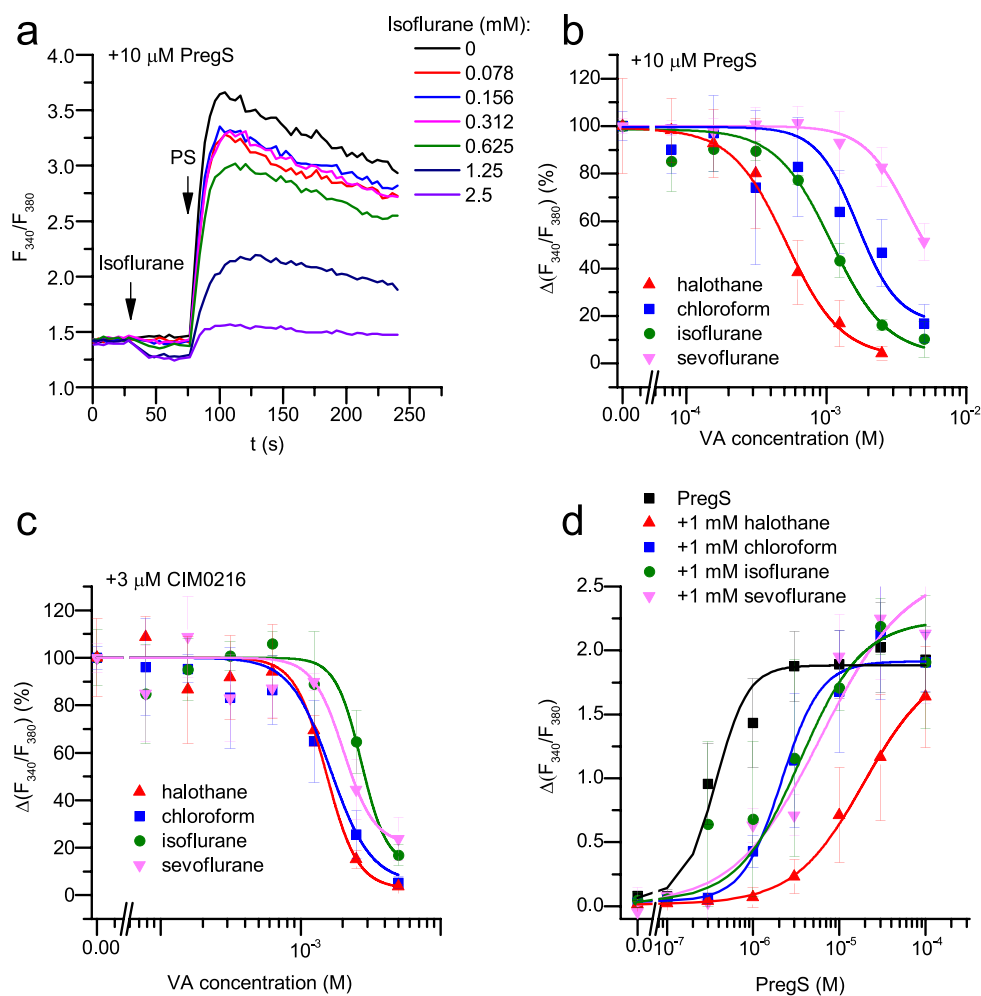
The PregS-induced current is conducted by the canonical pore of the TRPM3, but CIM0216 also induces the opening of an additional permeation pathway through the voltage-sensing domain of TRPM3 resulting in the appearance of a marked inward current at negative membrane potentials [29–31]. We found that VAs also inhibited the opening of the alternative ion permeation pathway, when activated by CIM0216 (Fig. 5d-f).

### 3.5. VAs inhibited native TRPM3 in sensory neurons of mouse dorsal root ganglia

To study the effect of VAs on native TRPM3, we investigated sensory neurons isolated from mouse DRGs. We used  $\text{Ca}^{2+}$  imaging to probe PregS responses in the presence and absence of VAs (Fig. 6a). We considered the PregS-responsive (PregS+) neurons as TRPM3 expressing (TRPM3+) neurons. As suggested in previous studies, our results also confirmed that some sensory neurons were directly stimulated by VAs: 15.3%, 21%, 5.1%, and 1.8% of the DRG neurons were activated by 1 mM halothane, chloroform, isoflurane and sevoflurane, respectively (Fig. 6b). The distribution of the neurons activated by VAs was similar in the TRPM3+ and TRPM3- population and, importantly, the large majority of TRPM3+ neurons was not activated by VAs (VA-neurons) (Fig. 6b). These results clearly argue for the conclusion that VAs activated a subset of sensory neurons independently of TRPM3. To investigate the VAs' potential inhibitory effect on native TRPM3, we compared the PregS evoked  $\text{Ca}^{2+}$  signals in the presence and absence of VAs. We analysed only the TRPM3+ but VA- sensory neurons to eliminate the influence of the TRPM3-independent  $\text{Ca}^{2+}$  signals evoked by VAs on PregS-induced responses. In our experiments, we applied repeated 2-minute-long pulses of PregS in the presence or absence of VAs, as shown in Fig. 6a. Only those cells that responded to a depolarizing pulse evoked by 25 mM KCl at the end of the measurement were considered as sensory neurons. We found that all of the investigated VAs applied at 1 mM decreased the PregS-induced, TRPM3-mediated  $\text{Ca}^{2+}$  transients. The inhibitory effect of VAs was reversible within 4 min after application (Fig. 6c).

Since activation of TRP channels results in depolarization of sensory neurons,  $\text{Ca}^{2+}$  signals evoked by activating TRP channels may be further amplified by the consequent activation of voltage-gated  $\text{Ca}^{2+}$  channels. Previous studies revealed that VAs inhibit some voltage-dependent  $\text{Ca}^{2+}$  channels, as well [36]. Therefore, we considered, that the inhibition of voltage-gated  $\text{Ca}^{2+}$  channels might contribute to the





**Fig. 2.** Identification of different VAs as inhibitors of the recombinant TRPM3. (a) Averaged  $\text{Ca}^{2+}$  responses ( $n = 5$ ) on HEK-M3 cells evoked by  $10 \mu\text{M}$  PregS (PS) in the presence of various concentrations of isoflurane, as indicated. Compounds were added at the indicated time points. (b) Concentration dependent inhibitory effect of VAs on the recombinant TRPM3 activated by  $10 \mu\text{M}$  PregS. The measurements were carried out as shown in panel (a), VAs were applied 30 s in advance to the agonist. (c) Concentration dependent inhibitory effect of VAs on the recombinant TRPM3 activated by CIM0216. The measurements were carried out as in panel (a) but  $3 \mu\text{M}$  CIM0216 was applied instead of PregS. (d) Dose-response relationship of PregS in the presence and absence of various VAs, as indicated in the figure. The measurements were carried out as in panel (a), but PregS was applied at various concentration and VAs were applied in  $1 \text{ mM}$ . Data are presented as mean  $\pm$  SD,  $n = 5$  in each group.

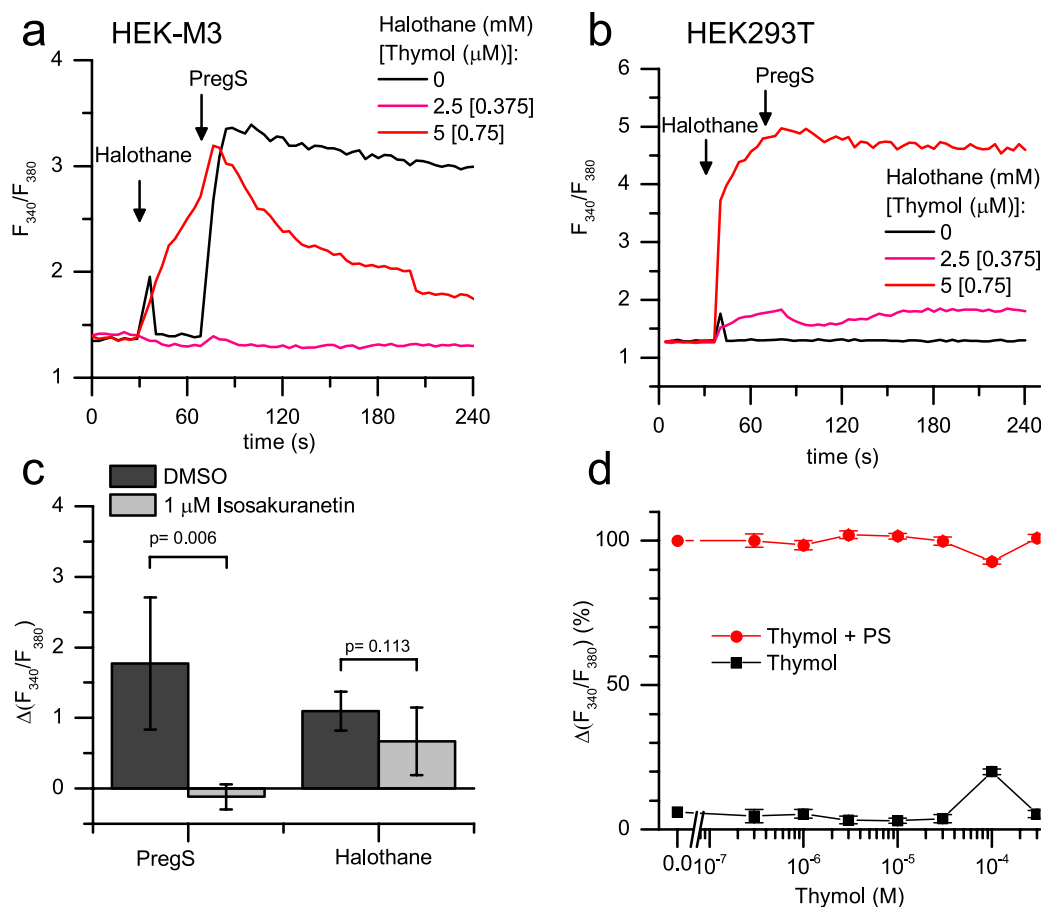
apparent inhibition of signals induced by PregS or agonists of other TRP channels. To challenge the role of voltage-gated  $\text{Ca}^{2+}$  channels, we tested whether or not VAs influence  $\text{Ca}^{2+}$  responses evoked by activating other native thermosensitive TRP channels expressed on sensory neurons. During these experiments, we slightly modified the previous protocol as illustrated in Fig. 6d. Because the effect of capsaicin and AITC is poorly reversible, we applied isoflurane before and together with the 1st agonist application, and the effect was compared to the 2nd agonist application after the wash out of the VAs. If the inhibitory effect of VAs experienced in our previous experiments were partially or totally mediated by some other voltage-gated  $\text{Ca}^{2+}$  channels, then VAs should also inhibit  $\text{Ca}^{2+}$  transients evoked by activating other TRP channels that cause depolarization of sensory neurons. Our results refuted this theory. The amplitudes of the  $\text{Ca}^{2+}$  transients activated by the TRPV1-specific agonist capsaicin and the TRPA1 agonist AITC did not decrease in the presence of isoflurane, whereas, the PregS-evoked  $\text{Ca}^{2+}$  transients were again significantly decreased (Fig. 6d-e). These results clearly argue for the conclusion that VAs inhibit PregS-induced responses via TRPM3, without influencing voltage gated  $\text{Ca}^{2+}$  channels.

#### 4. Discussion

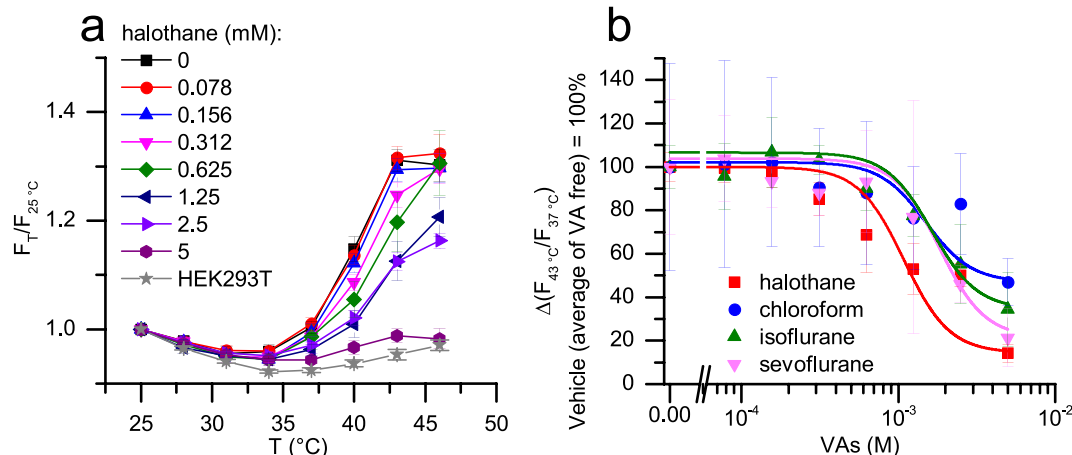
Activation of several members of the superfamily of the voltage-gated ion channels [37] is influenced by VAs, and a number of these channels, in particular the hyperpolarization activated and cyclic nucleotide gated channel 1 (HCN1) [38], shaker-related delayed rectifier  $\text{K}^+$  channels ( $\text{K}_v1$ ) [39] and two-pore-domain  $\text{K}^+$  channels (K2P) have been implicated in the induction of general anaesthesia. Voltage gated

$\text{Na}^+$  and  $\text{Ca}^{2+}$  channels can also be inhibited by VAs [36,40]. Moreover, recent studies reported the effect of VAs on thermosensitive TRP channels, as well. The cold- and menthol-activated TRPM8, after an initial activation, was inhibited by VAs. Likewise TRPC5 [41], another cold-sensitive family member, was inhibited by halothane and chloroform [42]. The warmth sensor TRPM2 was not influenced by halothane or chloroform [41], whereas the noxious heat sensor TRPV1 was sensitized [25], or, if applied at higher concentration, even activated by VAs [43]. Moreover, irritant VAs, isoflurane and desflurane directly activated TRPA1, a general target of several irritant chemicals, whereas the non-irritating halothane and sevoflurane did not induce TRPA1 activation [24]. These results can explain some adverse effects often associated with general anaesthesia induced by certain VAs. Indeed, irritant VAs evoke mechanical hyperalgesia and bronchoconstriction, impaired respiratory pattern, augmented laryngeal C-fiber activity and stimulate tracheal CGRP release mainly mediated by TRPA1 [24,44–46]. In our current study, we investigated the effect of VAs on TRPM3, a less characterized thermo-nociceptive TRP channel, which together with TRPV1 and TRPA1 play a crucial role in acute heat pain sensation [27,28].

In contrast to the other two heat-pain sensors TRPV1 and TRPA1, TRPM3 was found to be neither sensitized nor activated by any of the investigated VAs. In contrast, activation of TRPM3 by both chemical ligands and heat was markedly inhibited by the investigated VAs. Among those, halothane was found to be the most potent, inhibiting PregS-evoked TRPM3 activity with an  $\text{IC}_{50}$  of approximately  $0.5 \text{ mM}$ , equivalent to ca. 2-times the minimal alveolar concentration (2 MAC) that induces anaesthesia in different species [2,47]. Although the  $\text{IC}_{50}$



**Fig. 3.** High concentration of halothane induced Ca<sup>2+</sup> signals independently of TRPM3 and thymol used in 150 ppm as stabilizer in halothane. (a-b) Representative Ca<sup>2+</sup> signals evoked by halothane applied in high concentration in HEK-M3 (a) and HEK293T (b) cells. (c) Statistical analysis of PregS (PS, 10 μM) and halothane (5 mM) induced Ca<sup>2+</sup> signals in the presence and absence of isosakuranetin in HEK-M3 cells using two-tailed Student's *t*-test for independent samples. Amplitude was measured within the 1st minute after the application of the compounds. (d) Dose-response relationship of thymol on HEK-M3 cells, before and after applying 10 μM PregS, as shown in panel (a-b), but thymol was applied without halothane. Data are presented as mean ± SD, n = 5 in each group.

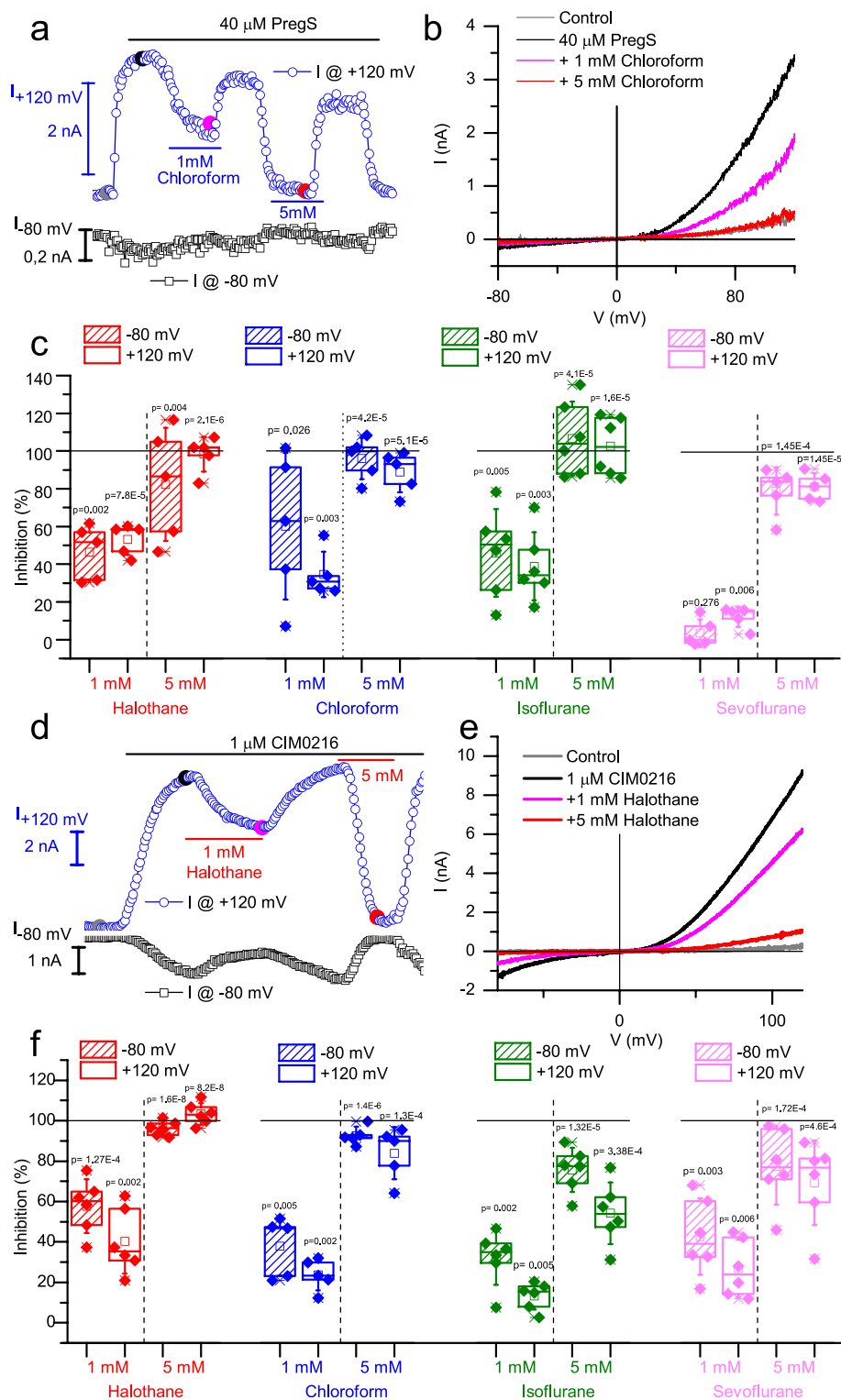


**Fig. 4.** VAs inhibited thermal activation of TRPM3. (a) Changes in intracellular Ca<sup>2+</sup> concentration of HEK-M3 cells upon elevating temperature in the presence of various concentrations of halothane as indicated in the figure. Native HEK293T cells were used as negative control. (b) Dose dependent inhibitory effect of VAs on the recombinant TRPM3 activated by temperature shift from 37 to 43 °C. The measurements were carried out as in panel (a). Data are presented as mean ± SD, n = 5 in each group.

of chloroform was slightly higher (ca. 1.67 mM), this value also corresponded to approximately 1.5 MAC [5]. Other VAs were less effective in clinically relevant concentrations: the IC<sub>50</sub> value for isoflurane (≈ 1.1 mM) is equivalent to about 3 MAC, whereas the IC<sub>50</sub> of sevoflurane approached 10 MAC against PregS-evoked activation. Applied at 1 mM, each VA shifted the PregS activation curve of TRPM3 toward higher concentrations. Again, halothane was found to be the most effective, as it increased the EC<sub>50</sub> of PregS approximately 50-fold. Importantly, at 1 mM, all investigated VAs inhibited the activation of native TRPM3 in sensory neurons of mouse DRGs, as well. Moreover,

VAs inhibited not only the activity of TRPM3 induced by PregS, but they also inhibited the effect of the synthetic agonist CIM0216 and the heat-induced TRPM3 responses with very similar potencies.

The sensitivity of TRPM3 toward some VAs seems to be slightly lower than sensitivity of ion channels generally believed to mediate anaesthesia. Clinically relevant concentrations (≈ 1 MAC) of volatile anaesthetics activate several members of the K2P channel family known to conduct background K<sup>+</sup> currents, which crucially contribute to the negative membrane potential [3]. For example, the EC<sub>50</sub> of halothane and sevoflurane that induce TASK-1 mediated K<sup>+</sup> currents were

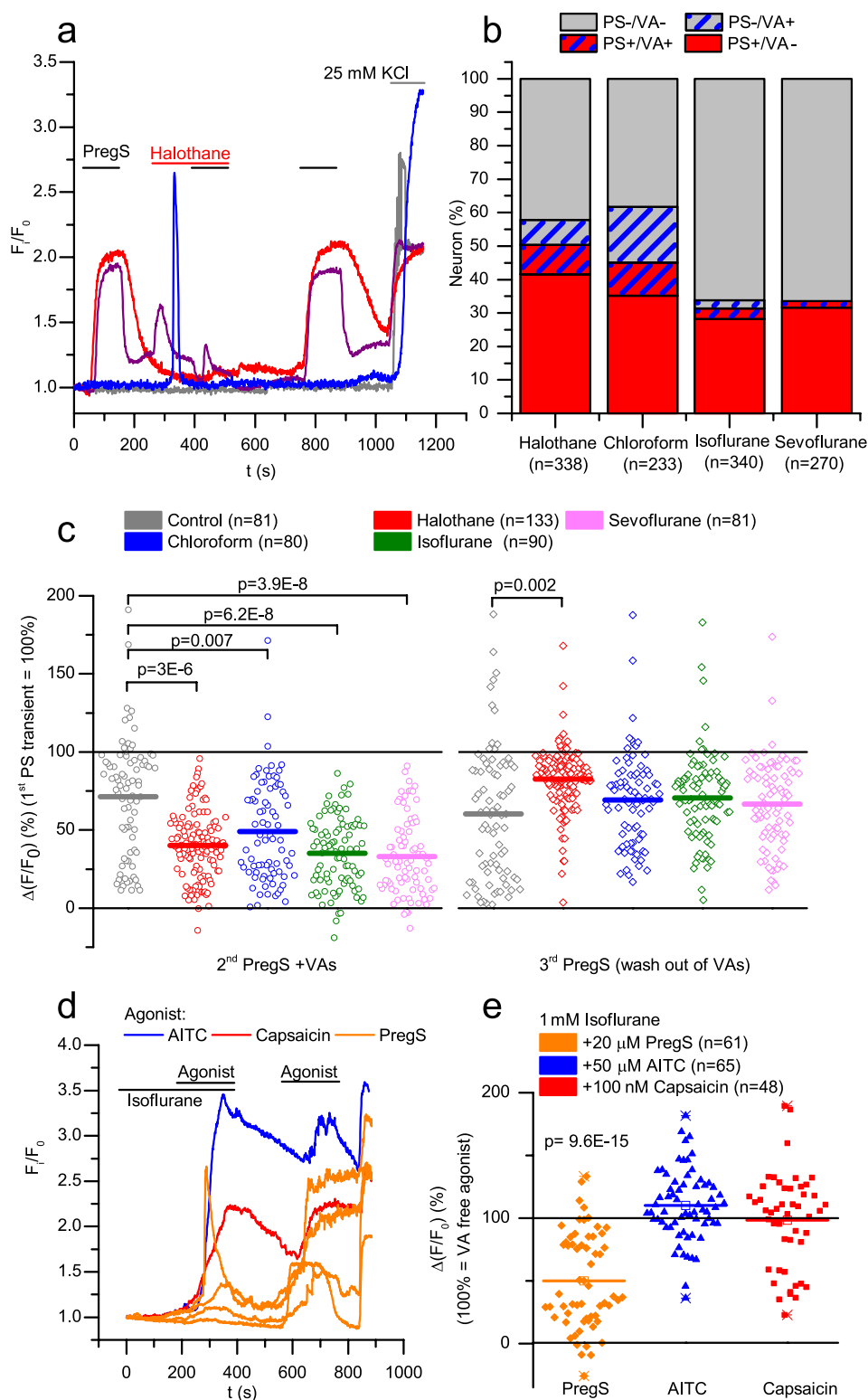


**Fig. 5. Reversible inhibition of whole-cell TRPM3 currents by various VAs.** (a) Representative time courses illustrating the effect of PregS and chloroform on TRPM3 mediated transmembrane currents at  $-80$  and  $+120$  mV. Currents were measured during a voltage ramp from  $-150$  to  $+150$  mV applied at every 2 s. (b) I/V relationship of the transmembrane currents at different time points as indicated in panel (a), illustrating the concentration dependent inhibition of PregS induced currents by chloroform. (c) Statistical analysis of VAs' effect on PregS induced transmembrane currents ( $n = 5$  in each group, except in case of isoflurane, where  $n = 6$ ). The measurements were carried out as shown in panel (a). (d) Representative time courses illustrating the effect of CIM0216 and halothane on TRPM3 mediated transmembrane currents at  $-80$  and  $+120$  mV. A voltage ramp from  $-150$  to  $+150$  mV was applied at every 2 s. (e) I/V relationship of the transmembrane currents at different time points as indicated in panel (d), illustrating the concentration dependent inhibition of CIM0216 induced currents by halothane. (f) Statistical analysis of VAs' effect on CIM0216 induced transmembrane currents ( $n = 6$ , except in case of chloroform, where  $n = 5$ ). The measurements were carried out as in panel (d). Effect of VAs was compared to the agonist (PregS or CIM0216) induced current before applying the VAs (one sample t-test, control agonist induced current is considered as 100%). Box plots represent the range between 25 and 75 percentile values, horizontal lines mark the median values, square symbols mark the means and whiskers represent SD.

0.23 mM and 0.29 mM (near to 1 MAC), respectively [48]. Moreover, NMDA receptor mediated currents were also effectively inhibited by isoflurane and sevoflurane with reported  $IC_{50}$  values between 0.25 and 1.3 MAC and ca. 1.25 MAC, respectively [49,50]. The  $EC_{50}$  of isoflurane and sevoflurane potentiating GABA induced activity of GABA<sub>A</sub> receptors was found also around 1 MAC (0.29 mM and 0.33 mM, respectively) [49,51]. However, the potency of halothane inhibiting NMDA receptor mediated postsynaptic excitatory currents ( $IC_{50} = 0.57$  mM, equivalent with ca. 2 MAC) [52] and potentiating

GABA induced GABA<sub>A</sub> currents ( $EC_{50} = 0.67$  mM, ca. 2 MAC) [53] were very close to the value we found for TRPM3.

Although we analysed the  $\alpha 2$  variant and native channels from mice, we strongly believe that our findings can be easily extrapolated to native human channels which is not characterized functionally. Until today, the best characterized variant is the mTRPM3 $\alpha 2$  [54] which functionally seem to be hardly distinguishable from the native mouse and the recombinant human TRPM3: each of these variants form  $Ca^{2+}$ -permeable cation channels [55–57], are activated by PregS [58,59],



**Fig. 6.** VAs inhibited TRPM3 dependent  $Ca^{2+}$  transients in somatosensory neurons isolated from mouse DRGs. (a) Representative traces showing typical changes of intracellular  $Ca^{2+}$  concentration in DRG neurons from wild-type mice in response to 20  $\mu$ M PregS, 1 mM halothane, and their combination as indicated in the panel. 25 mM KCl was used as positive control to depolarize the neuronal cell membrane. (b) Percentage of DRG neurons responding to PregS (PS+) and various VAs applied in 1 mM (VA+) . The measurements were carried out as in panel (a). (c) Statistical analysis of VAs' effect (1 mM each) on PregS induced  $Ca^{2+}$  transients in PregS +/VA- sensory neurons. Values are given as percentage of the first PregS-induced  $Ca^{2+}$  transient without VAs. Dots represents individual neurons, and horizontal lines indicate mean values. Effect of VAs was compared to the vehicle treated control group by ANOVA and Dunnett's post-hoc test. (d) Representative traces showing typical changes in intracellular  $Ca^{2+}$  concentration in DRG neurons in response to PregS (multiple traces), AITC, and capsaicin in the presence and absence of 1 mM isoflurane, as indicated in the figure. (e) Statistical analysis on the amplitude of PregS, AITC and capsaicin induced  $Ca^{2+}$  in the presence and absence of 1 mM isoflurane. Measurements were carried out as in panel (d). Effect of isoflurane was compared to transients evoked by isoflurane free agonist as 100% by one-sample *t*-test. Dots represents individual neurons, and horizontal lines indicate mean values.

and undergo very similar regulation by phosphatidylinositol 4,5-bisphosphate [60,61] and by the  $\beta\gamma$  subunit of G protein coupled receptors [62–65]. Heat sensitivity is also a shared feature of the recombinant (mTRPM3 $\alpha$ 2) and native TRPM3 channels [27,28]. Importantly, both mouse and human TRPM3 were effectively blocked by diclofenac and primidone [59,66]. These close functional and pharmacological similarities suggest that our findings on mouse TRPM3 can be generalized to native human channels.

While our results indicate that VAs inhibit homotetrameric TRPM3,

potential heteromerization between different TRP channel subunits can influence the functional characteristic of the tetramer channels, as described for example for the thermosensitive members of the TRPV family [67,68]. However, available data do not suggest significant participation of TRPM3 in forming heteromeric TRP channels. Although the closest relative TRPM1 was shown to be able to form heteromultimeric channels with TRPM3, TRPM4 was found not to interact with TRPM3 [69]. Considering that expression of TRPM1 is mainly restricted to melanocytes and retinal bipolar cells [70,71], and it is not



expressed in mouse DRGs [72], we do not assume significant heteromerization which would influence the responsiveness of TRPM3 to VAs. However, PregS and VAs induced responses may be influenced by the presence of alternative targets expressed by sensory neurons. Indeed, although ca. half of the sensory neurons isolated from wild type DRG are activated by PregS and considered as TRPM3+ neurons, about 10% of the neurons obtained from TRPM3-/- mice are also activated by PregS [27,28] indicating the presence of TRPM3 independent mechanisms. This has to be considered when interpreting our experiments carried out on sensory neurons: a low percentage of the PregS induced responses is probably independent of TRPM3. However the exact nature of additional target(s) is mainly elusive. Although sensory neurons of the DRG express both NMDA and GABA<sub>A</sub> receptors, their role is questionable in PregS induced responses without their primary ligands.

Although an emerging body of evidence supports the direct action of VAs on TRP channels, we have only a limited knowledge about the underlying mechanisms and the potential binding sites. Studies on TRPA1 and TRPV1 highlighted the role of the pore domain in forming the binding pocket for VAs [43,73], although molecular dynamics simulation suggested multiple binding sites on TRPV1 [74]. Our experiments did not directly address the mechanism of action of VAs on TRPM3, but we observed in our electrophysiological measurements that VAs inhibited not only the canonical pore currents but also the alternative pore currents induced by CIM0216 at negative membrane potential [30]. These currents are mediated by the opening of an alternative permeation pathway established by the voltage sensing domain of the channel [29,31,75]. The finding that both the canonical and the alternative pore mediated currents are blocked by VAs indicates that VAs do not act as classical pore blockers, but rather inhibit channel gating via a conformational change affecting activation by various mechanisms.

Our results not only reveal an additional ion channel affected by VAs, but also extend our knowledge about the pharmacological interactions of TRPM3 that potentially modulate sensory functions mediated by this channel. TRP channels are promising targets pursued by several pharmaceutical companies for the development of novel drugs to manage several, mostly pain related, clinical syndromes [22,76,77]. Despite significant efforts, several TRP-targeting drugs, in particular the first generation of antagonists targeting TRPV1, failed in clinical trials due to undesirable side effects such as hyperthermia and impaired noxious heat sensation [22,78,79]. From this point of view, TRPM3 may be a safer target, since its activation by PregS, in contrast to capsaicin, a potent and selective activator of TRPV1 [80], did not affect core body temperature [28]. To date, only a few blockers of TRPM3 have been characterised, and in animal models some of these were indeed found to inhibit TRPM3-mediated pain, including the flavanone derivative isosakuranetin [81] or the antiepileptic drug primidone [66]. Our results introduce VAs as a new class of TRPM3 inhibitors. A potential advantage of VAs is that they are well established, approved compounds and large amount of data available about their safety in human applications. However, their slightly lower affinity to TRPM3 than to several other targets, e.g. to GABA<sub>A</sub> and K2P channels, can limit their usage in clinical applications as TRPM3 targeting blockers. In spite of concerns about specificity of VAs, our data provide important new information contributing to the characterization of TRPM3 as potential pharmacological target of novel analgesics. The knowledge about the general mechanism of action of VAs may help identify targetable regions and basic biophysical and molecular interactions which can be utilized in future studies applying rational drug design approach. In the other hand, characterizing VAs, as a new class of chemicals inhibiting TRPM3 may also advance the field of pharmacology of TRP channels.

In conclusion, we characterised TRPM3 on sensory neurons as a potential target of VAs. These findings may contribute to the better understanding of the analgesic effect of VAs, and may aid in the further development of TRPM3 modulators as novel analgesics.

## 5. Authors' contributions

Balázs Kelemen, Thomas Voets, Tamás Bíró and Balázs István Tóth conceptualized the project and designed the study. Balázs Kelemen, Erika Lisztes, Anita Vladár, Martin Hanyicska, János Almássy, Attila Oláh, Attila Gábor Szöllösi, Zsófia Péntes, János Posta, Balázs István Tóth conducted the research presented in the manuscript. Balázs Kelemen, János Almássy, Attila Oláh, Attila Gábor Szöllösi, János Posta, Thomas Voets, Tamás Bíró, and Balázs István Tóth performed formal analysis of the results. All authors contributed significantly to the writing, editing and review of the final version of the manuscript. Tamás Bíró and Balázs István Tóth were responsible for funding acquisition.

## CRediT authorship contribution statement

**Balázs Kelemen:** Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Erika Lisztes:** Investigation, Writing - original draft. **Anita Vladár:** Investigation, Writing - original draft. **Martin Hanyicska:** Investigation, Writing - original draft. **János Almássy:** Investigation, Formal analysis, Writing - original draft. **Attila Oláh:** Investigation, Formal analysis, Writing - original draft. **Attila Gábor Szöllösi:** Investigation, Formal analysis, Writing - original draft. **Zsófia Péntes:** Investigation, Formal analysis, Writing - original draft. **János Posta:** Investigation, Formal analysis, Writing - original draft. **Thomas Voets:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Tamás Bíró:** Funding acquisition, Formal analysis, Writing - original draft, Writing - review & editing. **Balázs István Tóth:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

TB and AO provide consultancy services to Phytects Inc. (TB) and Botanix Pharmaceuticals Ltd. (AO). TV is co-inventor on patents entitled "Treatment of pain" derived from WO2012149614, and his lab has received research funding for pain-related research from industrial parties. Botanix Pharmaceuticals Ltd, Phytects Inc., and the founding sponsors had no role in conceiving the study, designing the experiments, writing of the manuscript, or in the decision to publish it. Other authors declare no conflict of interest.

## Acknowledgements

The presented work was supported by research grants of the National Research, Development and Innovation Office (K\_120187, PD\_121360, PD\_128034, FK\_125053, FK\_125055, GINOP-2.3.2-15-2016-00050). The project has received funding from the EU's Horizon 2020 research and innovation program under grant agreement No. 739593. The work of AO, AGSz, MH and BIT was supported by the New National Excellence Program of the Ministry for Innovation and Technology (ÚNKP-19-4-DE-287, ÚNKP-19-4-DE-142, ÚNKP-19-3-I-DE-140, and ÚNKP-19-4-DE-285). The work of AO, AGSz, JA, and BIT was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences and JA was supported by the Lajos Szodoray Research Scholarship of the Faculty of Medicine, University of Debrecen.

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