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CrediT author statement

The authors made the following contributions:

Noémie Luyts: Conceptualization, Methodology, Investigation, Formal analysis, Writing- Original draft preparation.

Jan Daniluk: Investigation, Formal analysis, Writing-Reviewing and Editing.

Bahar Bazeli: Investigation, Formal analysis, Writing-Reviewing

Annelies Janssens: Investigation, Formal analysis, Writing-Reviewing.

Ana Freitas: Investigation, Formal analysis, Writing-Reviewing, Marie Mulier Investigation, Formal analysis, Writing-Reviewing

Wouter Everaerts: Conceptualization, Methodology, Supervision, Funding acquisition, Writing.

Thomas Voets: Conceptualization, Methodology, Investigation, Formal analysis Supervision, Funding acquisition, Writing.

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Inhibition of TRPM8 by the urinary tract analgesic drug phenazopyridine

Noémie Luyts[1,2], Jan Daniluk[1,2], Ana Freitas[1,2], Bahar Bazeli[1,2], Annelies Janssens[1,2], , Marie Mulier[1,2], Wouter Everaerts[2,3,#], Thomas Voets[1,2,#]

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Key words

Phenazopyridine, TRPM8, urinary tract, analgesia, molecular target

Declarations of interest:

None

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Abstract

Background and purpose: Phenazopyridine (PAP) is an over-the-counter drug widely used to provide symptomatic relief of bladder pain in conditions such as cystitis or bladder pain syndrome (BPS). Whereas the analgesic effect of PAP has been attributed to a local effect on the mucosa of the lower urinary tract (LUT), the molecular targets of PAP remain unknown. We investigated the effect of PAP on pain-related Transient Receptor Potential (TRP) channels expressed in sensory neurons that innervate the bladder wall.

Experimental approach: The effects of PAP on the relevant TRP channels (TRPV1, TRPA1, TRPM8, TRPM3) expressed in HEK293 or CHO cells was investigated using Fura-2-based calcium measurements and whole-cell patch-clamp recordings. Activity of PAP on TRPM8 was further analysed using Fura-2-based calcium imaging on sensory neurons isolated from lumbosacral dorsal root ganglia (DRG) of mice.

Key results: PAP rapidly and reversibly inhibits responses of TRPM8 expressed in HEK293 cells to cold and menthol, with IC_{50} values between 2 and 10 µM. It acts by shifting the voltage dependence of channel activation towards positive potentials, opposite to the effect of menthol. PAP also inhibits TRPM8-mediated, menthol-evoked calcium responses in lumbosacral DRG neurons. At a concentration of 10 µM, PAP did not significantly affect TRPA1, TRPV1, or TRPM3.

Conclusion and implications: PAP inhibits TRPM8 in a concentration range consistent with PAP levels in the urine of treated patients. Since TRPM8 is expressed in bladder afferent neurons and upregulated in patients with painful bladder disorders, TRPM8 inhibition may underlie the analgesic activity of PAP.

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1. Introduction

Phenazopyridine (PAP) is an over-the-counter available urinary analgesic that provides symptomatic relief of pain caused by irritation of the lower urinary tract (LUT) (Hill et al., 2008; Hooton, 2012; Lusty et al., 2018). It is clinically used in the context of urinary tract infections (UTIs) (Davidov et al., 2021; Hooton, 2012), bladder pain syndrome (BPS)(Lusty et al., 2018) or as peri-operative urinary analgesic (Stewart et al., 2022) to reduce symptoms of frequency, urgency or pain. Phenazopyridine was first synthesized by Chichibabin and Zeide in 1914 and was subsequently introduced into the American medical pharmacopoeia in 1928 as a urinary tract antiseptic, before the arrival of the first antibiotics (Johnson and Chartrand, 1976). It is now generally accepted that PAP has no significant bactericidal activity (Amabile-Cuevas and Arredondo-Garcia, 2013; Zelenitsky and Zhanel, 1996) and, despite the long and extensive use, the mechanisms underlying the analgesic effects of PAP are largely elusive and its molecular targets in the LUT remain unknown.

The Transient Receptor Potential (TRP) channel superfamily represents a group of cation-permeable ion channels that respond to a wide variety of physical and chemical stimuli, allowing them to function as polymodal cellular sensors and making them indispensable for many homeostatic and sensory processes (Nilius et al., 2007). Several TRP channels have been described to participate in sensory processes in the urinary bladder. In particular, TRPA1, TRPV1, TRPM8, and TRPM3 are expressed on sensory neurons innervating the bladder wall and have been implicated in bladder sensation and pain (Everaerts et al., 2010; Vanneste et al., 2021b; Wu et al., 2020). The goal of this study was therefore to explore the effects of PAP on these TRP channels, as a possible mechanism for PAP-induced analgesia.

2. Materials and methods

All experiments were conducted in accordance with the European Union Community Council guidelines. Research using animals was approved by the Animal Ethics Committee of KU Leuven (P184/2021).

Cell culture

We used Chinese Hamster Ovary cells (CHO) stably expressing mouse TRPA1, HEK 293 cells stably expressing human TRPM8 or human TRPM3, as well as HEK293 cells transiently transfected with 2 μg of pCAGGS/IRES-GFP vector encoding mouse TRPM8 or human TRPV1, linked to expression of GFP allowing identification of transfected cells. Cells were transfected using TransIT-293 transfection reagent (BioMirus) 24h before the measurement. All cells were cultured in DMEM high glucose (Thermo fisher) supplemented with 50 mL foetal bovine serum (Gibco), 10 mL penicillin/streptomycin

(Gibco), 10 mL glutamax (Thermo Fisher) and 5 mL non-essential-amino-acids (Thermo Fisher). In addition to this, 1 mL hydromycin and 500 µL blasticidin were added to the culture medium of the CHO cells. Cells were kept at 37°C in 10% (HEK293 cells) or 5% (CHO cells) CO_2.

DRG culture

Bilateral L5-S1 DRGs were isolated from 10-12-week-old female C57BL/6 mice, as described previously (Mulier et al., 2020).In brief, the spinal column was isolated, cleaned, and split sagittal. The spinal cord, meninges covering the DRG, and the distal axon bundles were removed. DRGs were washed in neurobasal A medium (Invitrogen) supplemented with 10% fetal calf serum (basal medium), and then incubated for 1 h at 37°C in a mix of 1.5 mg /mL collagenase and 2.5 mg /mL dispase (Gibco). Digested ganglia were gently washed twice with basal medium and subsequently washed twice in neurobasal A medium supplemented with B27 (Invitrogen), 2 ng /mL GDNF (Invitrogen), 10 ng /mL NT4 (Peprotech), 1% (v/v) Glutamax (Gibco) and penicillin 50 units /mL—streptomycin 50 µg /mL (Gibco) (complete medium). 1 mL of complete medium plus 1 mL of bovine serum albumin (BSA) solution (16% w/v in PBS) were added to the ganglia. Cells were mechanically dissociated by passage through syringes fitted with increasing needle gauges. The cell suspension was added on top of a 3 mL BSA solution (16% w/v in PBS) and centrifuged at 500 × g for 6 min. The supernatant was discarded, and the pellet was resuspended in complete medium. Neurons were seeded on poly-L-lysine/laminin coated glass-bottom chambers (Fluorodish, WPI) and cultured overnight at 37°C in 5% CO_2 in complete medium.

Measurement of intracellular Ca^{2+} concentration.

For intracellular Ca^{2+} measurements, cells were incubated with 2 µM Fura-2 acetoxymethyl (AM) ester for 30 min at 37°C. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored through the ratio of fluorescence measured upon alternating illumination at 340 and 380 nm, using an MT-10 illumination system and cell^M software (Olympus). Absolute calcium concentrations were calculated from the ratio of the fluorescence signals at both wavelengths (F_{340}/F_{380}) after correction for the individual background fluorescence signals, using the Grynkiewicz equation (Grynkiewicz et al., 1985)

$$[\mathrm{Ca}^{2+}] = K_{eff}\,\frac{R - R_0}{R_1 - R}$$

where the calibration constants R_0, R_1 and K_{eff} were determined from *in vitro* calibrations using droplets of Fura-2AM-containing solution with known calcium concentrations. Increases of intracellular Ca^{2+} concentration of $\geq$ 50nM were defined as responses in DRG neurons.

Patch-clamp experiments.

Membrane currents were measured in the whole-cell and inside-out modes of the patch-clamp technique using an EPC-10 amplifier and Patchmaster software (HEKA Elektronik). Patch electrodes had a direct current (DC) resistance between 2 and 4 MΩ when filled with intracellular solution. Currents were sampled at 20 kHz and digitally filtered at 2.9 kHz. In whole-cell recordings, between 50 and 70% of the series resistance was electronically compensated to minimize voltage errors. The holding potential in all experiments was 0 mV. A ramp protocol was applied consisting of 500-ms linear voltage ramps, applied at 0.5 Hz, from -100 mV to +100 mV (TRPM8), -100 mV to +150 mV (TRPM3), or -100 mV to +160 mV (TRPV1). For TRPM8, we also used a voltage step protocol, consisting of 100-ms steps to voltages ranging from -120 to +160 mV, followed by a 50-ms step to +60 mV. The standard extracellular solution for electrophysiological measurements contained (in mM) 150 NaCl, 1 $MgCl_2$ 10 HEPES (pH 7.4 with NaOH). Depending on the type of investigated channel, the pipette solution was composed of (in mM) 100 CsAsp, 45 CsCl, 10 EGTA, 10 HEPES, 1 $MgCl_2$ (pH 7.2 with CsOH) (TRPM3), 150 NaCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA (pH 7.2 with NaOH) (TRPV1), or 150 NaCl, 5 $MgCl_2$, 5 EGTA, 10 HEPES (pH 7.2 with NaOH) (TRPM8). For inside-out measurements of TRPM8, this latter solution was used as bath solution, whereas the standard extracellular solution was used as pipette solution. The time course of TRPM8 current in inside-out measurements is represented as $N \times P_{open}$, where N represents the number of channels in a patch and P_{open} the channel open probility. $N \times P_{open}$ was calculated as the current amplitude divided by the single-channel amplitude. All measurements were carried out at room temperature (22–25°C).

Statistical analysis

Statistical analysis and graphing were performed in OriginPro. Results were considered significant if the two-sided p-value was smaller than 0.05. Except where indicated otherwise, all summary data are reported as mean ± SEM. Concentration-response curves representing the effect of PAP on TRPM8 activity were fitted to modified Hill curve of the following forms:

$$\text{Inhibition}(\%) = \frac{1}{1+\left(\frac{IC_{50}}{[PAP]}\right)^{n_H}} \text{ or } \mathrm{I/I_{max}} = \frac{1}{1+\left(\frac{[PAP]}{IC_{50}}\right)^{n_H}}$$

where IC_{50} represents the concentration for half-maximal inhibition, and n_H the Hill coefficient.

3. Results

Several studies have demonstrated an increased expression of TRPM8 on bladder sensory neurons in patients with BPS, suggesting a correlation between TRPM8 and bladder pain (Mukerji et al., 2006; Wu et al., 2020). To investigate whether PAP affects TRPM8 activity, we performed intracellular Ca^{2+} imaging experiments on HEK293 cells expressing murine TRPM8. We observed a concentration-

dependent and reversible inhibition of menthol-induced TRPM8 responses by PAP, with an IC_{50} value of 9.6 ±1.2 µM (fig. 1A-B). Similarly, PAP (30 µM) strongly suppressed TRPM8-dependent calcium responses to cold stimulation (normalized response: 16 ± 4%) (fig. 1C-D).

In patch-clamp recordings in HEK293 cells expressing human TRPM8, PAP caused a rapid and reversible inhibition of the whole-cell currents (fig. 2A-B). The inhibitory effect of PAP was more pronounced for inward than for outward currents, with IC_{50} values of 2.0 ± 0.2 µM and 12.0 ± 0.8 µM at -80 mV and +80mV, respectively (fig. 2C).

To test whether the inhibitory action of PAP on TRPM8 depends on intracellular signaling, we recorded TRPM8 currents in cell-free inside out patches. In these inside-out patches, PAP (50µM) induced a very rapid, complete (100% inhibition; n=5), and rapidly reversible block of menthol-induced TRPM8 current. These results indicate that PAP acts in a direct, membrane-delimited manner to inhibit TRPM8 channels.

TRPM8 is a voltage-dependent, depolarization-activated channel, and stimuli such as cold and menthol activate the channel by shifting the voltage dependence of activation towards more negative potentials (Voets et al., 2004; Voets et al., 2007). To analyse the effects of PAP on voltage-dependent gating, we measured whole-cell currents during voltage steps ranging from -120 mV to +160 mV in the absence or presence of menthol (50 µM) and various concentrations of PAP. These experiments revealed that PAP shifts the voltage dependence of TRPM8 towards more positive potentials in a concentration-dependent manner, thus opposing the effects of menthol (fig. 3A-C). Taken together, these findings reveal that PAP is an antagonist of TRPM8.

Next, we evaluated the effects of PAP on other TRP channels that are expressed in bladder-innervating sensory neurons (Vanneste et al., 2021a; Vanneste et al., 2021b). In whole-cell recordings on HEK293 cells expressing human TRPM3, PAP (10 µM) did not noticeably affect currents activated by the neurosteroid agonist pregnenolone sulphate, whereas full inhibition was observed with the flavonoid antagonist isosakuranetin (fig. 4A-C). Similarly, capsaicin-activated whole-cell currents in HEK293 cells expressing human TRPV1 were unaffected by PAP (10 µM) but fully abolished by the TRPV1 antagonist AMG517 (fig. 4D-F). Intracellular Ca^{2+} imaging experiments on mouse TRPA1-expressing CHO cells further showed that application of PAP at a concentration of 10 µM did not inhibit the response to the TRPA1 agonist AITC (100 µM) (fig. 5A). In contrast, we observed a small increase in intracellular Ca^{2+} when PAP was applied following stimulation with AITC (fig. 5A), suggesting that it may have an agonistic effect on the channel. Indeed, when applying PAP to mouse TRPA1-expressing CHO cells, we observed sizeable and concentration-dependent increases in cytosolic calcium at concentrations ≥30 µM (fig. 5B-C). However, at the highest test concentration of PAP (100 µM), the amplitude of the

calcium response amounted to only 42.4 ± 8.1% of the response to AITC (50 μM), suggesting that PAP is a relatively weak TRPA1 agonist.

Finally, we evaluated the effect of PAP in isolated dorsal root ganglion neurons (DRGs) from mice, harvested from the spinal levels that innervate the bladder (L5-S1). Since cold and menthol can activate both TRPM8 and TRPA1 (Karashima et al., 2007; Karashima et al., 2009), we used allyl isothiocyanate (AITC; 100 μM) to identify TRPA1 positive DRG neurons. In the AITC-negative, cold-responsive neurons (n = 33/757 neurons (4.4%) from 3 mice), preincubation with PAP (50 μM) cause a robust reduction of cold-evoked calcium responses (48 ± 7 % inhibition), which recovered upon PAP wash-out (fig. 6A,B). Similarly, menthol responses in AITC-negative neurons (n = 34/1181 neurons (2.9%) from 3 mice) were partially and reversible inhibited by preincubation with PAP (49 ± 6 % inhibition; Fig. 6C,D). These data indicate that cold-evoked and menthol-evoked responses in AITC-negative neurons, which we identify as TRPM8-positive neurons, are inhibited by PAP. Finally, PAP (50 μM) by itself evoked a calcium response in only a very limited subset of DRG neurons (3/1181; 0.3%), compared to the 595/1181 neurons (50%) that responded to AITC. These data indicate very little agonistic effect of PAP (50μM) on TRPA1 in DRG neurons. In conclusion, our results demonstrate that PAP acts as an inhibitor of TRPM8 activity, both in a heterologous expression system and in primary sensory neurons.

4. Discussion

In this study, we have demonstrated that PAP has an inhibitory effect on the sensory ion channel TRPM8, with IC_{50} values of in the range of ~10 μM. This inhibitory effect was observed for menthol- and cold-induced responses in cells heterologously expressing TRPM8 as well as in TRPM8-positive sensory DRG neurons. Furthermore, this effect showed specificity for TRPM8 amongst sensory TRP channels expressed in bladder-innervating sensory neurons: TRPM3 and TRPV1 were not noticeably affected by PAP at 10 μM, whereas PAP had a mild agonistic effect on TRPA1 at concentrations >10 μM in an overexpression system, but little effect on TRPA1-positive DRG neurons.

Several selective TRPM8 antagonists have been developed for clinical testing as analgesic drugs or to treat bladder dysfunction. In preclinical studies, such compounds were found to efficiently inhibit TRPM8-dependent responses such as icilin-induced wet-dog shakes and cold-induced urinary frequency in rats (Kobayashi et al., 2021; Uvin et al., 2015). However, clinical development of one of the most advanced antagonists, PF-05105679, was halted after phase 1 studies revealed significant adverse effects as periorally hot sensations and dysgeusia (Winchester et al., 2014). Most likely, the adverse effects of hot sensations are related to the recently identified role of TRPM8 in warmth sensation (Paricio-Montesinos et al., 2020), where reduced activity of TRPM8-positive sensory neurons is perceived as warming. Since these adverse effects are likely to be on-target and were observed at

free plasma concentrations of only three times the IC_{50}, levels that are also required for analgesic efficacy, it appears that systemically available TRPM8 antagonists may have a relatively low therapeutic index, hampering their clinical implementation (Weyer and Lehto, 2017; Winchester et al., 2014). In this respect, PAP has a favourable safety profile: the maximal plasma levels of PAP after oral dosing of a therapeutic dose of 200 mg in health male volunteers amounted to only ~300 nM (Li et al., 2008), well below the concentrations needed to have a systemic effect on TRPM8 activity. The low plasma exposure can be explained by the highly efficient excretion via the urine, of which a large fraction (41%) as the unchanged drug, resulting in PAP concentrations in the bladder in the range of 100–300 μM (Amabile-Cuevas and Arredondo-Garcia, 2013; Johnson and Chartrand, 1976). As such, PAP can act as a topical TRPM8 antagonist in the bladder wall, without the unwanted side effects of systemic TRPM8 inhibition.

In addition to TRPM8, other TRP channels, like TRPV1, TRPM3 and TRPA1 are expressed in bladder sensory neurons (Vanneste et al., 2021a; Vanneste et al., 2021b). We tested the effect of 10μM PAP on these ion channels, but could not detect any inhibitory effect. At concentrations >10μM, however, PAP had a mild agonistic effect in cells heterologously expressing mouse TRPA1. Although TRPA1 does not seem to play a role in normal voiding, it acts as a detector of noxious stimuli that can contribute to bladder pain perception and nociceptive behaviour (Vanneste et al., 2021). However, even at higher doses (100μM) the effects of PAP were limited compared to the potent TRPA1 agonist AITC. Moreover, in cultured DRG neurons PAP (50μM) induced calcium responses in only 0.3% of cells. These data suggest that PAP likely has minimal effect on TRPA1-expressing sensory neurons *in vivo*.

Earlier research had demonstrated inhibition of Aδ fibre activity by PAP, but the molecular PAP target on these nerve endings was not identified (Aizawa and Wyndaele, 2010). Based on molecular modelling experiments, Preynat-Seauve and colleagues identified PAP as a kinase inhibitor, with high affinity for PIP4K2C, a crucial enzyme for phosphatidylinositol 4,5-bisphosphate (PIP_2) synthesis (Preynat-Seauve et al., 2021). PIP_2 is a known TRPM8 modulator, so it can be envisaged that inhibition of PIP4K2C could decrease TRPM8 activity due to PIP_2 depletion (Liu and Rohacs, 2020; Liu et al., 2018). However, it appears unlikely that this is the primary underlying mechanism of TRPM8 inhibition in our experiments, since we observed a rapid and reversible inhibition, unlike the slower time course of activity for PIP_2 depletion and resynthesis upon kinase inhibition (Liu and Qin, 2005). Moreover, TRPM8-mediated currents were rapidly inhibited by PAP in cell-free inside-out patches, providing evidence for direct inhibition of TRPM8 by PAP. PAP is often prescribed to treat bladder pain syndrome (BPS) (Rovner et al., 2000), and was found to improve the symptoms in 61% of all patients (Hill et al., 2008; Lusty et al., 2018). BPS is defined as “the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased day and night-time frequency, in the absence of proven urinary infection or other obvious pathology” (van de Merwe et al., 2008). There is a lack of

knowledge about the underlying disease mechanism (Rovner et al., 2000). Wu et al. have found a higher relative density of nerve fibres and an increased expression of TRPM8 mRNA and protein in bladder tissues from patients with BPS, compared to control biopsies (Wu et al., 2020). Other groups have confirmed the increased TRPM8 expression and the correlation with clinical symptoms (Homma et al., 2013; Mukerji et al., 2006). Our finding that PAP at therapeutically relevant concentrations inhibits TRPM8 in these afferent pathways thus provides a straightforward explanation for the local analgesic effect on the lower urinary tract.

5. Conclusion

PAP, at therapeutically relevant concentrations, exerts an inhibitory effect on TRPM8. Considering that TRPM8 is expressed in bladder afferent neurons and upregulated in patients with painful bladder disorders, local TRPM8 inhibition may contribute to the analgesic activity of PAP.

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Author contribution statement

Noémie Luyts: Conceptualization, Methodology, Investigation, Formal analysis, Writing- Original draft preparation. Jan Daniluk: Investigation, Formal analysis, Writing-Reviewing and Editing. Bahar Bazeli: Investigation, Formal analysis, Writing-Reviewing, Annelies Janssens: Investigation, Formal analysis, Writing-Reviewing. Ana Freitas: Investigation, Formal analysis, Writing-Reviewing, Marie Mulier Investigation, Formal analysis, Writing-Reviewing, Wouter Everaerts: Conceptualization, Methodology, Supervision, Funding acquisition, Writing. Thomas Voets: Conceptualization, Methodology, Investigation, Formal analysis Supervision, Funding acquisition, Writing.

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Figures

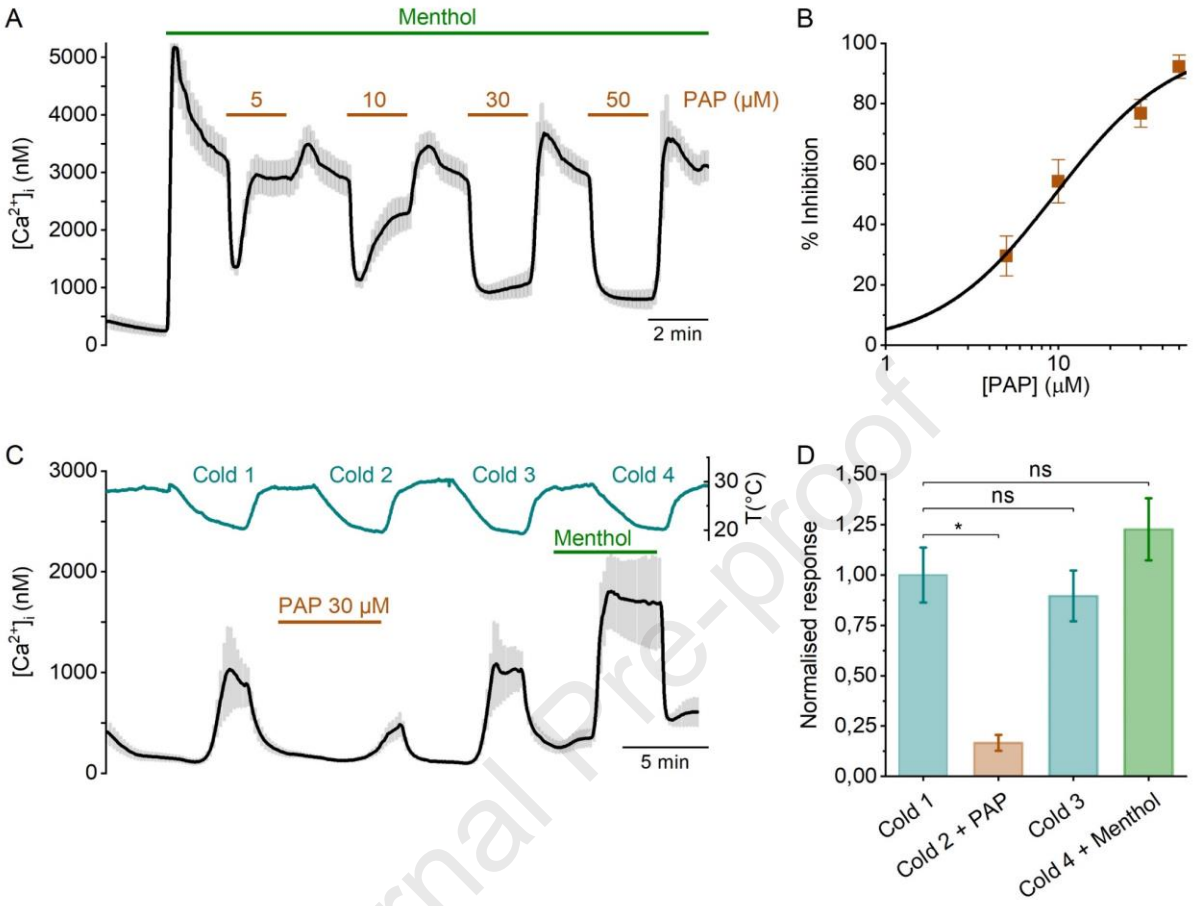

Figure 1. PAP inhibits menthol and cold-induced TRPM8-mediated calcium responses.

A Representative average trace of HEK293 cells expressing murine TRPM8 during continuous stimulation with menthol (50 μM) and upon application of increasing PAP concentrations. **B** Concentration dependences of the percentage of inhibition of PAP on menthol (50 μM)-evoked intracellular Ca^{2+} responses in mTRPM8 HEK293 cells. The solid line represents the fit using the Hill equation (n = 23 cells, IC_{50} 9.6 ± 1.2 μM, Hill coefficient 1.3 ± 0.2). **C** Representative average trace of HEK293 cells expressing murine TRPM8, stimulated with cold solution (20 °C) in the absence or presence of either PAP (30 μM) or menthol (50 μM). **D** Calcium amplitudes normalised to the first cold application. Upon application of PAP (30 μM) the responses were significantly reduced. Repeated Measures One-Way ANOVA, Dunnett test pairwise comparison of the first cold stimulus compared to cold + PAP, cold and cold + menthol ($p < 0.0001$, $p = 0.69$, $p = 0.13$. respectively, n= 40).

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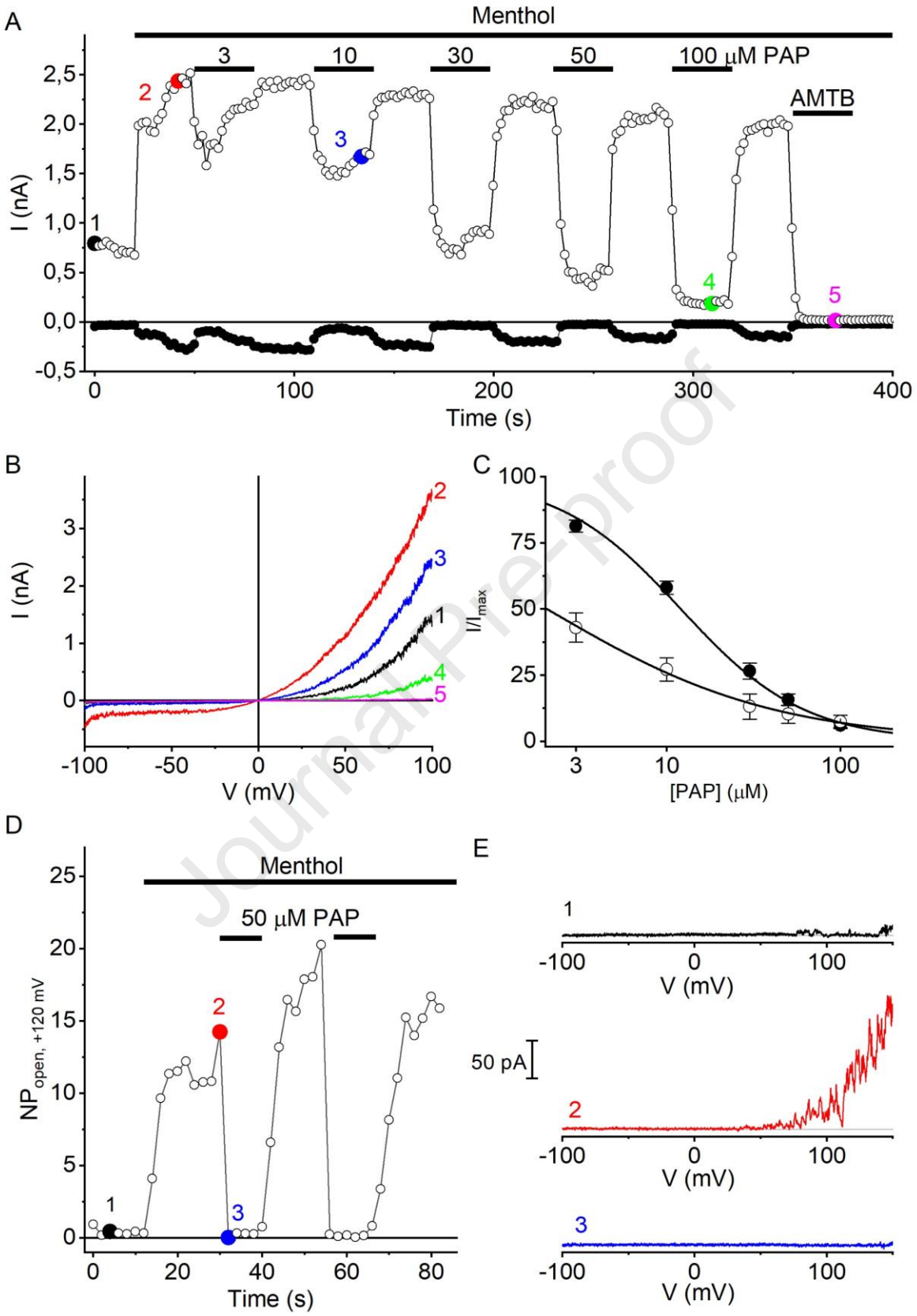

Figure 2. Inhibitory effect of PAP on whole-cell TRPM8 currents. A Representative trace of the whole cell TRPM8 current recorded in HEK293 cells expressing human TRPM8. Shown are currents at +80 mV

and –80 mV measured during 500-ms linear voltage ramps from -100 to +100 mV upon application of menthol (50 µM) and subsequent treatment with increasing concentrations of PAP (1, 10, 30, 50, and 100 µM). AMTB (10 µM) was used as positive control. **B** Current-voltage relationship at the time points indicated in A. **C** Concentration-response relationship for the action of PAP on TRPM8. Data points are represented as mean normalized current values ± SEM at +80 mV (black) and –80 mV (red), fitted to a Hill curve , resulting in values of IC_{50} 12.0 ± 0.8 µM, n_H = 1.22 ± 0.06 at +80mV and IC_{50} 2.0 ± 0.2 µM, n_H = 0.66 ± 0.03 at -80mV (n = 5 cells). **D** Representative time course of TRPM8 channel activity in an inside-out patch at +120mV stimulated with menthol (50 µM), showing rapid, full and reversible inhibition by PAP (50 µM). **E.** Current-voltage relationship at the time points indicated in D**.**

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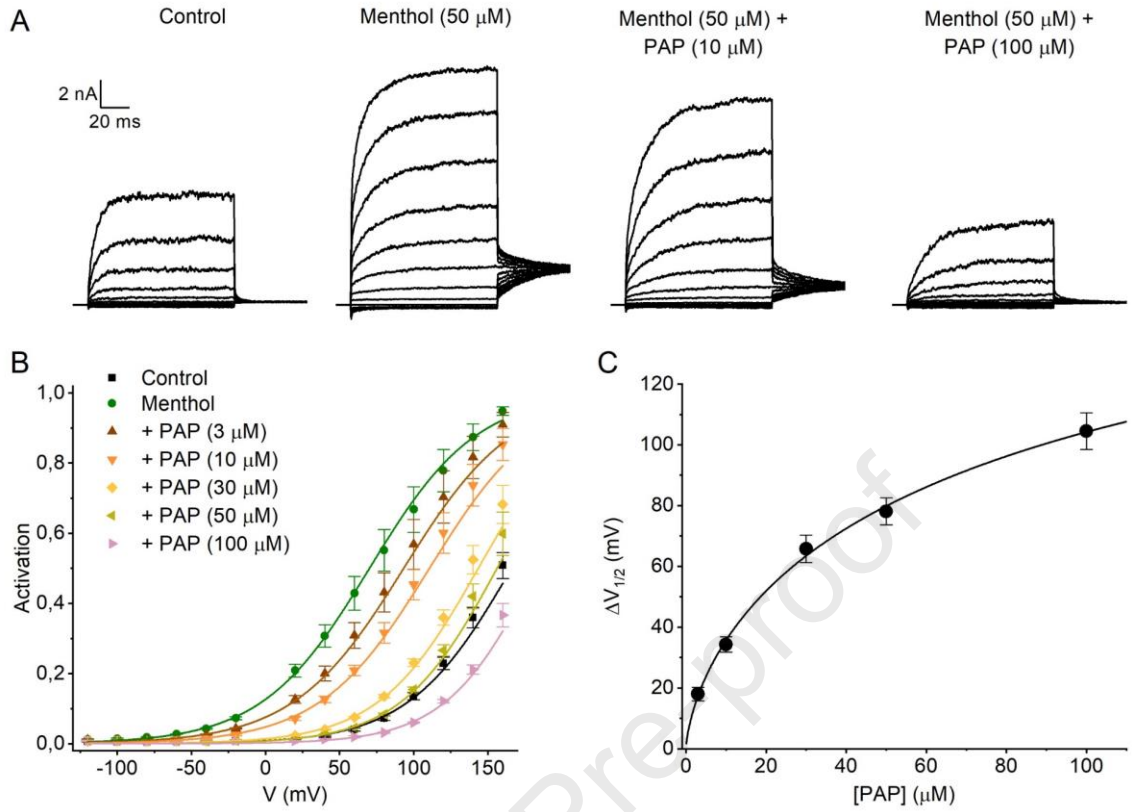

Figure 3. Effect of PAP on the voltage dependence of TRPM8 activation. **A** TRPM8 currents in response to a step protocol, consisting of 100-ms steps to potentials ranging from -120 to +160 mV followed by a step to +60 mV, illustrating the effect of menthol and PAP on voltage-dependent gating. **B** Voltage-dependent activation curves in the indicated conditions (n=5), determined as the steady-state conductance from traces as in (a), normalised to the maximal conductance. Solid lines indicate fits using the Boltzmann equation. **C** Concentration dependence of the PAP-induced shift of the midpoints of the voltage-dependent activation curves presented in B.

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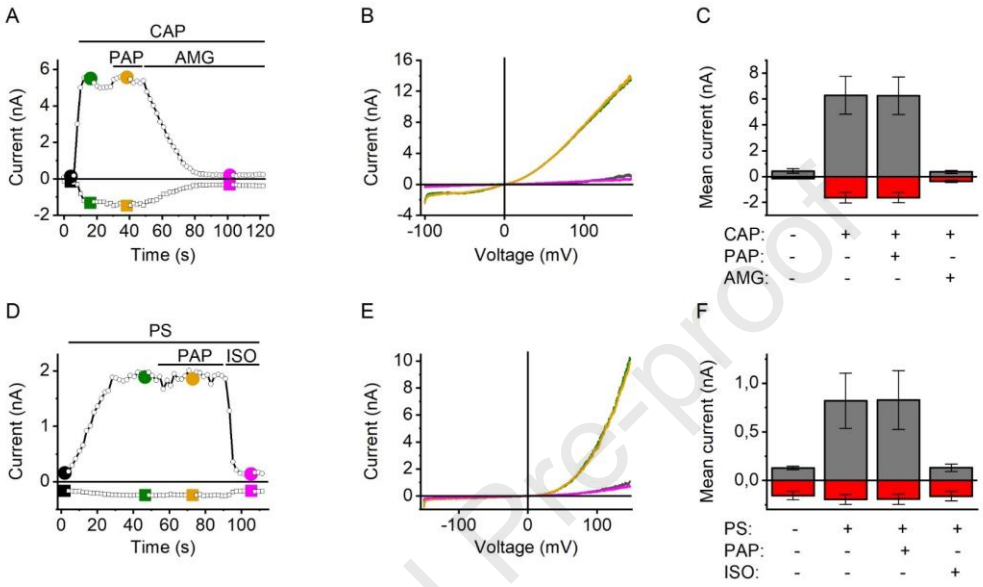

Figure 4. The effects of PAP on other sensory TRP channels. A Representative time course of the whole-cell TRPV1 current at +80 mV and –80 mV measured during voltage ramps upon application of capsaicin (CAP, 50 nM) and subsequent treatment with 10 µM of PAP. AMG 517 (10 nM) was used as positive control. **B** Current-voltage relationship at the time points indicated in (a). **C** Mean whole-cell TRPV1 current at +80 mV (grey) and –80 mV (red). Data are represented as mean ± SEM (n = 5). **D** Representative time course of the whole-cell TRPM3 current at +80 mV and –80 mV measured during voltage ramps upon application of pregnenolone sulfate (PS, 40 µM) and subsequent treatment with 10 µM of PAP. Isosakuranetin (ISO, 5 µM) was used as positive control. E Current-voltage relationship at the time points indicated in (d). **F** Mean whole-cell TRPM3 current at +80 mV (grey) and –80 mV (red). Data are represented as mean ± SEM (n = 5).

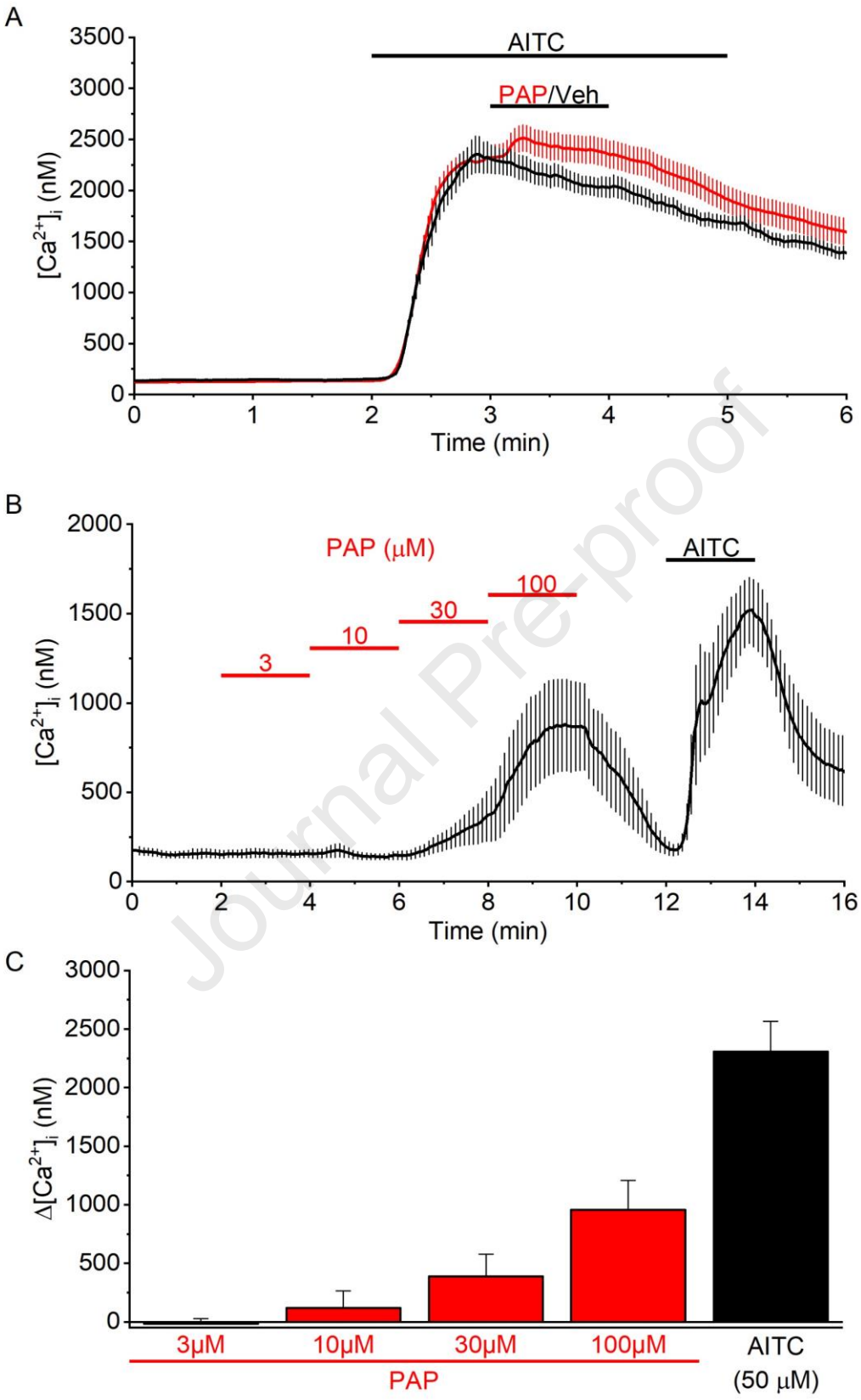

Figure 5. PAP has a small agonistic effect on TRPA1.

A Representative average trace of intracellular Ca^{2+} responses upon application of AITC (100 µM) alone (black trace), or upon application of AITC (100 µM) and PAP (10 µM) (brown trace). **B** Representative average trace of CHO cells stably expressing TRPA1 upon application of increasing PAP concentrations (3, 10, 30, 100 µM) and application of AITC (50 µM), as a positive control. **C** Calcium amplitudes upon application of PAP (3, 10, 30, 100 µM) and AITC (50 µM). Bars represent mean ± SEM (n = 20).

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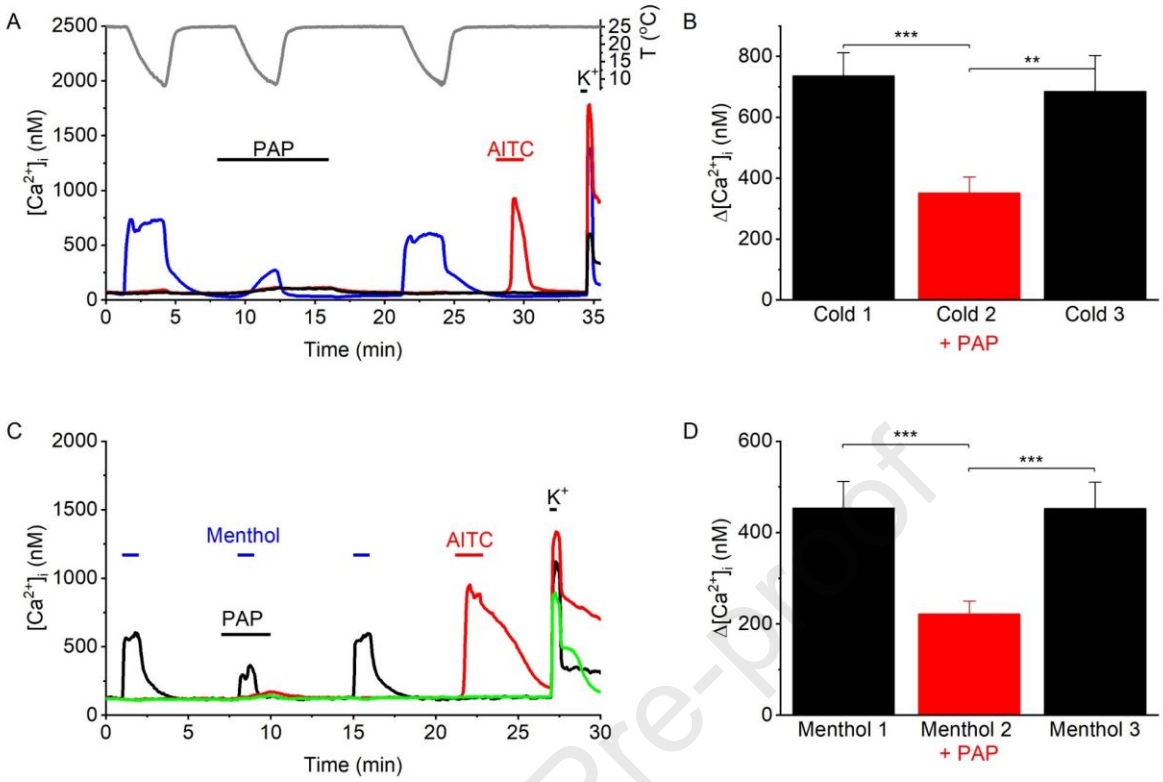

Figure 6. PAP inhibits menthol-induced TRPM8 responses in DRG neurons. A Representative time course of the intracellular calcium concentration in murine DRGs from vertebral levels L5-S1, stimulated with cold solution (10 °C) in the absence or presence of PAP (50 µM) . AITC (100 µM) was used to identify TRPA1-expressing neurons. A high K^+-solution (50 mM) was used to distinguish neurons from non-neuronal cells. **B** Calcium amplitudes in DRG neurons responding to cold stimuli in the presence or absence of PAP (50 µM). Bars represent mean ± SEM (n = 33 cells, from 3 mice). **C** Representative time course of the intracellular calcium concentration in murine DRGs from vertebral levels L5-S1, stimulated with menthol (50 µM) in the absence or presence of PAP (50 µM). **D** Calcium amplitudes in AITC-insensitive DRG neurons responding to menthol (50 µM) in the presence or absence of PAP (50 µM). Bars represent mean ± SEM (n = 34 cells, from 3 mice).

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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