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# Propacetamol-induced injection pain is associated with activation of TRPV1

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**Running title**:

**Propacetamol activates TRPV1 to evoke pain**

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abbreviations: BCTC (4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide); CGRP calcitonin gene-related peptide; HEK 293 human embryonic kidney 293; hTRPA1 human TRPA1; hTRPV1 human TRPV1; LA local anesthetic; Nav voltage-gated sodium channel; PCM paracetamol; PPCM propacetamol; QST quantitative sensory testing ;TRP transient receptor potential;

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**Abstract**

Propacetamol (PPCM) is a prodrug of paracetamol (PCM) which was generated to increase water solubility of PCM for intravenous delivery. PPCM is rapidly hydrolyzed by plasma esterases to PCM and diethylglycine, and it shares some structural and metabolic properties with lidocaine. While PPCM is considered to be comparable to PCM regarding its analgesic properties, injection pain is a common side effect only described for PPCM. Injection pain is a frequent and unpleasant side effect of numerous drugs in clinical use, and previous reports have indicated that the ligand gated ion channels TRPA1 and TRPV1 on sensory neurons can mediate this effect. The aim of this paper was to investigate molecular mechanisms by which PPCM, in contrast to PCM, causes injection pain. Therefor human TRPV1 and TRPA1 receptors were expressed in HEK 293 cells and investigated by means of whole cell patch clamp and ratiometric calcium imaging. PPCM, but not PCM activated TRPV1, sensitized heat-induced currents and caused an increase in intracellular calcium. In TRPA1 expressing cells however, PPCM and PCM both evoked calcium responses but failed to induce inward currents. Intracutaneous injection of PPCM, but not of PCM, in human volunteers induced an intense and short-lasting pain and an increase in superficial blood flow indicating activation of nociceptive C-fibers and subsequent neuropeptide release. In conclusion, activation of human TRPV1 by propacetamol seems to be a relevant mechanism for induction of pain upon intracutaneous injection and thus also for pain reported as an adverse side effect upon intravenous administration.

## Introduction

Paracetamol (PCM, acetamionphen) is a popular analgesic drug for light to medium pain. However, due to its poor solubility and stability in water-based solutions at physiological pH-values, it is less suitable for intravenous application. Therefore, propacetamol (PPCM), a prodrug of PCM, was generated by esterification of PCM and the carboxylic acid diethylglycine to improve solubility (see Figure 1 for comparison of molecular structures) (Barbaud *et al.*, 1995). PPCM was effectively used to treat fever and pain in several European countries when oral application was not appropriate. Intravenous infusion leads to rapid hydrolysis of PPCM within seven minutes by plasma esterases in PCM and diethylglycine (Bannwarth *et al.*, 1992). While analgesic properties of PPCM have been proven to be superior to placebo and comparable to the effects of PCM (McNicol *et al.*, 2011) it has become evident that injection pain occurs quite frequently (up to 39%) upon intravenous application of PPCM (Depre *et al.*, 1992). In addition to this pain and a local irritation at the site of injection, health care associated cases of allergic reactions have been reported for PPCM (Barbaud *et al.*, 1995).

Injection pain is a common problem causing discomfort and distress during induction of general anesthesia with propofol (Tan and Onsiong, 1998; Doenicke *et al.*, 1996) or etomidate and even after local infiltration with local anesthetics such as lidocaine. Many of these proalgesic substances were described to activate TRP channels expressed in nociceptive nerve endings, and today TRPV1 and TRPA1 seem to be the most relevant candidates mediating activation and/or sensitization of sensory neurons by both propofol and local anesthetics (Matta *et al.*, 2008; Leffler *et al.*, 2008; Leffler *et al.*, 2011). Both TRPV1 and TRPA1 non-selective cation channels are expressed in peripheral nerve endings of nociceptive C- and Aδ-fibers, but also along the peripheral axon and in central nerve terminals in the spinal cord. As polymodal receptors TRPA1 and TRPV1 can be activated by a multitude of different agonists related to pain, inflammation and oxidative stress. Among several mechanisms resulting in activation the modification of N-terminal cysteines has been shown as the underlying mechanism of how reactive agents sensitize and activate these ion channels (Hinman *et al.*, 2006; Chuang and Lin, 2009). We noted that PPCM indeed shows some structural similarities to lidocaine, also involving the cleavage of diethylglycine (PPCM), respectively ethylglycine (lidocaine), during hydrolysis. Therefore, we hypothesized that activation of TRPV1 and/or TRPA1 may account

for the injection pain caused by PPCM as well. We thus aimed to investigate whether activation of human (h) TRPV1 and/or TRPA1 channels could account for injection pain caused by intravenous PPCM application. We combined cellular methods including whole-cell patch clamp and calcium imaging. Furthermore, we wanted to indirectly explore activation of epidermal C-fibers by PPCM and PCM in human volunteers by quantifying injection pain and flare reaction measured by laser Doppler imaging.

## Materials and Methods

*Chemicals*

Chemicals were purchased and solved as follows: acetaminophen/paracetamol, GSK1016790A and probenecid from Sigma-Aldrich (Taufkirchen, Germany), BCTC 4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide, TRPV1 blocker, 100 mM stock solution in ethanol) and HC030031 (TRPA1 blocker, 100 mM stock solution in DMSO) both from Tocris (Bio-Techne, Wiesbaden-Nordenstadt, Germany), Propacetamol MolPort (Riga, Latvia). Paracetamol and propacetamol solutions were prepared in external solution before each experiment.

*Cell culture*

For experiments on human TRPV1 and TRPA1 channels stable cell lines were used. The hTRPV1-expressing HEK 293 cell line was a kind gift from Dr. Peter Zygmunt (Clinical Chemistry & Pharmacology, Department of Laboratory Medicine, Lund University Hospital, Lund, Sweden) and the hTRPA1-expressing cell line was established in our laboratory (de la Roche *et al.*, 2013). For some experiments HEK 293T cells were transfected with plasmids of hTRPV1, hTRPV2, hTRPV4 and hTRPA1 (1 µg each) and co-transfected with EGFP (0.25 µg) for patch clamp experiments using Nanofectin (PAA, Pasching, Austria). Cells were cultured at 37°C and 5% $CO_2$ for 24 – 48 hours before use for patch clamp or calcium imaging.

*Patch Clamp*

Cells were examined by whole-cell patch clamp with an EPC10 USB HEKA amplifier (HEKA Electronics, Lamprecht, Germany), low-passed at 1 kHz and sampled at 2 kHz. Pipettes were pulled from borosilicate glass tubes (TW150F-3; World Precision Instruments, Berlin, Germany) to give a resistance of 2- 5 MΩ. The external calcium-free solution included: NaCl 140 mM, KCl 5 mM, $MgCl_2$ 2 mM, EGTA 5 mM, HEPES 10 mM and glucose 10 mM (pH 7.4 was adjusted with tetramethylammonium hydroxide). The internal solution contained: KCl 140 mM, $MgCl_2$ 2 mM, EGTA 5 mM and HEPES 10 mM (pH was adjusted with KOH). Cells were either held at -60 mV or currents evoked by 500 ms long voltage ramps from -100 to +100 mV were measured. All experiments were performed at room temperature.

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$Na^+$ currents were recorded from the ND7/23-hybrid cell line derived from neonatal rat spinal sensory neurons fused with mouse neuroblastoma cells (Wood *et al.*, 1990). For these experiments data were sampled at 20 kHz, and filtered at 5 kHz. The series resistance was compensated by 60-70% and the capacitance artefacts were cancelled using the Patchmaster software. Pipettes were pulled with a resistance of 1 - 2 MΩ and filled with internal solution containing CsF 140 mM, $MgCl_2$ 2 mM, EGTA 5 mM, HEPES 10 mM with pH adjusted to 7.4 by KOH. The external solution contained NaCl 140 mM, KCl 5 mM, $MgCl_2$ 2 mM, $CaCl_2$ 1.2 mM, HEPES 10 mM and glucose 10 mM (pH 7.4 was adjusted by tetramethylammonium hydroxide). Sodium currents were elicited by depolarizing pulses to -10 mV at 0.1 or 10 Hz using -120 mV as holding potential. The Fitmaster Software (HEKA Electronics, Lambrecht, Germany) and the Origin 8.5.1 Software (Origin Lab, Northampton, MA, U.S.A) were used for data analysis.

*Ratiometric $[Ca^{2+}]_i$ measurements*

Cells were stained by 3 µM Fura-2-AM and 0.01% pluronic for about 45 min. After wash out to allow Fura-2-AM deesterification coverslips were mounted on an inverse microscope with a 20x objective (Axio observer D1, Zeiss). Cells were constantly superfused with extracellular solution at room temperature containing NaCl 145 mM, KCl 5 mM, $CaCl_2$ 1.25 mM, $MgCl_2$ 1 mM, glucose 10 mM, HEPES 10 mM using a software-controlled 7-channel gravity-driven common-outlet superfusion system. Fura-2 was excited using a microscope light source and an LEP filter wheel (Ludl electronic producs Ltd.) to switch between 340 and 380 nm wavelengths. Images were exposed for 40 and 20 ms respectively and acquired at a rate of 1 Hz with a CCD camera (Cool SNAP EZ, Photometrics). Data were recorded using VisiView 2.1.1 software (all from Visitron Systems GmbH, Puchheim, Germany). Background fluorescence was subtracted before calculation of ratios. Either 0.3 µM capsaicin or 250 µM carvacrol were used to identify hTRPV1- or hTRPA1-expressing cells on a functional level and 5 µM ionomycin was applied as a control at the end of each experiment. Experiments were repeated at least five times and performed on different days. Averaged results are reported as means (± S.E.M.) of area under the curve (AUC delta ratio F340/380 nm) for regions of

interest adapted to the cells. Calculation of the AUC included 60 s from the beginning of PPCM/PCM application to include the maximum of calcium responses and baseline calcium levels were subtracted.

*Psychophysics:*

For studies on human volunteers experimental procedures were approved to fulfil the requirements of the Declaration of Helsinki by the ethics committee of Hannover Medical School. The recommended dose to treat postoperative pain in adults is an intravenous infusion of 1 g PCM usually applied in 100 ml isotonic saline solution, resulting in a concentration of 66.2 mM (Sinatra *et al.*, 2005). As an equivalent dose 2 g of PPCM are clinically used resulting in 66.5 mM if applied in 100 ml isotonic saline infusion. Therefore, either 50 mM PCM or PPCM were intradermally injected in 75 µl volume to the volar forearm of the volunteer (*n*= 7 each; 3 male and 4 female) in a double-blinded manner. Pain and itch were assessed on a numerical rating scale (NRS), ranging from 0 (no sensation) to 10 (maximum pain/itch imaginable) in 15 s intervals for a period of 10 min. For quantitative sensory testing (QST) numerical ratings (0 - 10) to a 10 s-lasting heat stimulus of 47°C or to a 10 s-lasting cold stimulus of 0°C, applied by 0.8 cm in diameter metal rods heated by a water bath or cooled with ice, were compared before and after application of substances. The skin was tested for dynamic mechanical allodynia by stroking with a fine brush. A laser Doppler imager (moorLDI2-IR, Moor, London, UK) was used to record changes in superficial blood flow. Two baseline scans of 0.6 mm spatial resolution were taken, followed by 7 scans every 2 min starting right after the injections and a final one after 20 min. The areas of superficial vasodilation were analyzed with moorLDI software 5.3 and defined as pixels in which intensity exceeded the mean of basal values plus two standard deviations.

*Statistics:*

For analysis of patch clamp data and intraindividual QST data from human volunteers two groups were compared by non-parametric Wilcoxon matched pairs test for $n < 10$. A comparison of more than two groups for calcium imaging and psychophysics data was performed by analysis of variance following honestly significant difference (HSD) *post hoc* tests as stated using Statistica 7 software

(StatSoft, Tulsa, U.S.A.). Differences were considered significant at *$p < 0.05$. *** denotes $p < 0.001$.

All data are displayed as mean ± S.E.M.

## Results

### PPCM activates membrane currents and induces rise in $[Ca^{2+}]_i$ through TRPV1

To investigate whether PPCM exhibits hTRPV1-activating properties, HEK 293 cells expressing wild type hTRPV1 were examined by standard whole-cell voltage clamp. Measuring ramp currents by 500 ms long depolarizations from -100 to +100 mV, we applied PPCM at increasing concentrations (1 µM to 10 mM). In hTRPV1-expressing cells 100 µM and higher concentrations activated membrane currents with a strong outward rectification in all cells which also generated capsaicin (1 µM)-induced currents (Figure 2A, n = 9).

PPCM applied at a concentration of 20 mM to hTRPV1-expressing cells constantly held at -60 mV induced large inward currents which could completely be blocked by 100 nM of the TRPV1-blocker BCTC (Figure 2B, n = 8; p = 0.01, Wilcoxon matched pairs test). The same concentration of PCM (20 mM) did not evoke any currents in hTRPV1-expressing HEK cells (Figure 2C, n = 8).

Sensitization of TRPV1-mediated heat responses has been described for many TRPV1 agonists such as capsaicin or lidocaine (Tominaga *et al.*, 1998; Leffler *et al.*, 2008). We investigated heat-induced currents by repetitively applying 5 s lasting ramps from 25°C to 45°C. Indeed PPCM at 10mM enhanced heat-induced currents from 3392 ± 795 pA to 7313 ± 661 pA (p = 0.028, n = 6, Figure 2D). In contrast to this PCM had no effect on heat-induced inward currents (4999 ± 1353 pA vs 4657 ± 1138 pA, p = 0.48, n = 8, Figure 2E). Consequently, the threshold for heat-induced currents was shifted from 42 ± 1°C to 37 ± 2°C by PPCM 10 mM (p = 0.028, n = 6, Figure 2D), while the temperature threshold of hTRPV1 remained unchanged by application of PCM (42 ± 1°C vs 41 ± 1°C, p = 0.12, n = 8, Figure 2E, all Wilcoxon matched pairs test).

To confirm the results obtained by patch clamp recordings, we applied ratiometric calcium imaging to Fura-2-stained hTRPV1-expressing HEK 293 cells. PPCM was applied at a concentration of 20 mM for 40 s and evoked a robust increase in intracellular calcium in 68% of hTRPV1-expressing cells also responding to 0.3 µM capsaicin (Figure 2F upper panel and Figure 2H blue bar, n = 475). Again, this PPCM-induced effect could be abolished by the TRPV1-blocker BCTC (100 nM, Figure 2F lower panel and 2H white bar) and PCM 20 mM did not evoke any increase in intracellular calcium in

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hRPV1-expressing cells (Figure 2G and H cyan bar, n = 427, ANOVA F(2, 1157)=457,5; $p < 0.001$; following HSD post hoc tests $p < 0.001$ each).

**Both PPCM and PCM induce small membrane currents and rise in $[Ca^{2+}]_i$ through TRPA1**

In hTRPA1-expressing cells responding to the TRPA1-agonist carvacrol (250 µM), outwardly rectifying ramp currents were evoked by high concentrations (≥10 mM) of PPCM (Figure 3A, n = 8). In contrast to this 20 mM PPCM completely failed to induce membrane currents in hTRPV2- and hTRPV4-expressing HEK293 cells (n = 5 each, "Supplemental Figure 2A, B"). PPCM and PCM applied at a concentration of 20 mM to hTRPA1-expressing cells constantly held at -60 mV did not lead to inward currents ("Supplemental Figure 2C,D", n = 5 and 6). However, 50 mM PPCM evoked small inward currents through activation of hTRPA1 and these inward currents could reliably be inhibited by 50 µM of the TRPA1-blocker HC030031 (Figure 3B, n = 6, $p < 0.05$, Wilcoxon matched pairs test). Furthermore, 50 mM PCM evoked comparably small inward currents in hTRPA1-expressing cells (Figure 3 C, n = 6).

Using ratiometric calcium imaging 20 mM PPCM induced calcium responses in 86% of all cells also responding to 250 µM carvacrol (Figure 3D upper panel and F blue bar, n = 218). According to the notion that this effect is mediated by hTRPA1, these PPCM-induced responses were completely blocked by HC030031 (50 µM; Figure 3D lower panel and F white bar; ANOVA F (1, 381)=408.0; $p < 0.001$; following HSD post hoc tests $p < 0.001$). Following the washout of HC030031 we observed an immediate recovery of calcium signals in 82% of PPCM-responsive cells indicating a sustained activation of hTRPA1 by PPCM. Similar to PPCM, 20 mM PCM induced increases in intracellular calcium in 92% of hTRPA1-expressing HEK cells (Figure 3E upper panel, and G cyan bar, n = 299). Again, this effect could be inhibited by HC030031 (Figure 3E lower panel and H white bar; ANOVA F F(1, 465)=590.6; $p < 0.001$; following HSD post hoc tests $p < 0.001$).

**High concentrations of PPCM induce tonic block of neuronal voltage-gated sodium channels**

To test the hypothesis that PPCM might block voltage-gated sodium channels due to structural similarities to lidocaine "(Supplemental Figure 1)", we investigated whether PPCM or PCM inhibit

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$Na^+$ currents in neuroblastoma ND7/23 cells known to functionally express tetrodotoxin-sensitive $Na^+$ channels. Cells were held at -120 mV in order to examine the block of resting channels and currents were elicited by depolarizing pulses to -10 mV. Tonic block by PPCM was determined by application of 10 µM, 100 µM, 1 mM and 10 mM PPCM or PCM respectively. Only high concentrations of PPCM (> 10mM) induced a substantial block of $Na^+$ currents. While 10 mM PPCM reduced current amplitude to 66.4 ± 1.4% ($p = 0.0002$, $n = 6$, Figure 4A), 10 mM PCM decreased current amplitude only to 93.3 ± 1.7% ($p = 0.002$, $n = 7$, Figure 4B). These data indicate that PPCM induces a stronger tonic block of $Na^+$ currents as compared to PPCM ($p = 0.0002$, Figure 4C, all ANOVA $F(9, 55)=81.37$; $p < 0.0001$, following HSD post hoc tests). No use-dependent block of $Na^+$ channels could be observed at 10 mM PPCM or 10 mM PCM when currents were activated at 10 Hz "(Supplemental Figure 3)".

**Injection of PPCM evokes pain and an increase in cutaneous blood flow in humans**

Activation of TRPV1 and TRPA in endings of nociceptive nerve fibers has been proposed to be responsible for the injection pain caused by many clinically used drugs such as lidocaine and propofol. Depolarization of the membrane following TRP channel activation gives rise to action potentials which are propagated to the central nervous system where they lead to the experience of pain. However, also vasoactive neuropeptides, like the potent vasodilator calcitonin gene-related peptide (CGRP), are released from the activated nerve endings and from collaterals of wide-branching peptidergic C-fibers by antidromic action potential propagation. This so-called "axon-reflex" flare leads to a widespread and sometimes patchy reddening of the skin. As infusion of PPCM, but not PCM, causes pain and skin irritations in some patients, we hypothesized that intracutaneous injection of PPCM might lead to pain and axon reflex flare in human skin. This was tested in human volunteers ($n$= 7; 3 male and 4 female) by a double-blinded intracutaneous injection of 75 µl of either PPCM or PCM at 50 mM (e.g. concentrations slightly below the concentrations of infusions of PPCM and PCM used in clinical settings). Indeed, the injection of PPCM caused an immediate pain with a mean maximum pain rating of 6.3 ± 0.7 (on a numerical rating scale 0 - 10). This intense pain was rapidly declining over approximately 5 minutes (Figure 5A), and was described as burning or stinging. Apart

from the initial pain caused by the injection needle, application of PCM was not painful. Furthermore, neither PPCM nor PCM caused itch within 15 minutes following the injection. No allodynia was reported by any subject, and neither responses to noxious heat (10 s, 47°C) nor to cold (10 s, 0°C) changed significantly after injection of PPCM or PCM ($n = 7$ each; $p > 0.05$; Wilcoxon matched pairs test; data not shown). In addition to injection pain, PPCM induced a large axon-reflex erythema visualized by laser Doppler scanning of superficial blood flow of the forearm (Figure 5B). In contrast, apart from the irritation caused by the injection needle, we could not detect any erythema following injection of PCM (Figure 5B, C; "Supplemental Figure 3"; ANOVA $F(9, 4)=10.37$; $p < 0.02$; following HSD post hoc tests; $p \leq 0.04$).

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## Discussion

TRPV1 and TRPA1 are co-expressed in a large population of nociceptive sensory neurons (Story *et al.*, 2003). Our data suggest that PPCM activates hTRPV1 more than hTRPA1 in cellular models by means of patch clamp and calcium imaging. PPCM, but not PCM also sensitized heat-induced currents and lower the temperature-threshold for heat-induced currents through hTRPV1. Therefore, it seems plausible that activation of nociceptive C-fibers by PPCM likely to cause injection pain is predominantly mediated by TRPV1. Accordingly, we could show activation of human nociceptive C-fibers by means of pronounced PPCM-evoked pain and axon reflex erythema in healthy volunteers.

Injection pain due to the activation of TRP channels has been shown for propofol (Fischer *et al.*, 2010), etomidate (Matta *et al.*, 2008) and lidocaine as well as for other local anesthetics (Leffler *et al.*, 2008; Leffler *et al.*, 2011). While TRPA1 is the channel that seems to be predominantly activated by etomidate and propofol (Matta *et al.*, 2008), the latter at least also activates TRPV1 after receptor sensitization in mouse DRG neurons (Fischer *et al.*, 2010). Local anesthetics activate TRPV1, and lidocaine induces a strong sensitization to heat and capsaicin. The $EC_{50}$ value for lidocaine to activate rat TRPV1 was determined to be approximately 12 mM (Leffler *et al.*, 2008), and subsequently published an $EC_{50}$ value of 24 mM was found for the activation of rat TRPA1 (Leffler *et al.*, 2011). While TRPV2 and TRPV4 are expressed in sensory neurons we could not detect a sensitizing or activating effect by PPCM on these channels. While activation of TRP channels on nociceptive nerve endings induces pain, sensitization and activation of spinal TRPA1 receptors was proposed to contribute to the analgesic effects of PCM (Andersson *et al.*, 2011). To induce this effect, however, PCM has to be metabolized to the reactive metabolites N-acetyl-p-benzoquinoneimine (NAPQI) and parabenzoquinone (pBQ) in the spinal cord. Several reactive molecules have been shown to modify N-terminal cysteines of both TRPA1 and TRPV1, leading to channel conformation changes and sometimes to a sustained receptor activation (Hinman *et al.*, 2006; Chuang and Lin, 2009; Eberhardt *et al.*, 2014).

Activation of TRP-channels in the periphery can account for injection pain induced by PPCM. Due to rapid hydrolysis of PPCM, an additional analgesic effect caused by the activation of spinal TRP channels seems unlikely. However, once being metabolized to PCM and further to NAPQI and pBQ,

again TRP channel mediated effects might account for analgesic properties of PPCM. Although PCM induced only minimal membrane currents in TRPA1-expressing cells, it induced a robust increase in intracellular calcium. We can only speculate on why this calcium-influx does not translate into inward currents, but a higher sensitivity of TRPA1 to agonists in calcium imaging compared to patch clamp has been observed repeatedly (Andersson *et al.*, 2015; Eberhardt *et al.*, 2012). One reason might be calcium itself which has been described to sensitize and desensitize TRPA1 (Wang *et al.*, 2008; Zurborg *et al.*, 2007). Another issue could be sensitization of TRPA1 by UV-light, which might be the case using Fura-2 (Hill and Schaefer, 2009). Nevertheless, injection of PCM neither caused pain, nor a significant increase in cutaneous blood flow. Therefore, the observed activation of TRPV1 by PPCM seems to be the more relevant mechanism for injection pain.

In addition to PCM-induced analgesic effects, TRP channel activation has also been discussed to account for adverse effects of PCM. In several epidemiological studies, the exposure to therapeutic doses of PCM was reported to predispose children to asthma and eczema (Eneli *et al.*, 2005; Beasley *et al.*, 2008). An important role for TRPA1 in airway hyperreactivity was demonstrated in animal models of asthma (Caceres *et al.*, 2009), and the application of therapeutic dosages of PCM in mice induced a TRPA1-dependent airway inflammation (Nassini *et al.*, 2010).

PPCM has been shown to induce contact dermatitis in health care personal with occupational exposure to the drug (Barbaud *et al.*, 1995; Berl *et al.*, 1998). Importantly, activation of TRPA1 and TRPV1 has been discussed to be involved in skin sensitizing in contact hypersensitivity. Dermatitis associated with direct handling of capsaicin-containing chili peppers is one of the most obvious examples (Williams *et al.*, 1995). The widely used phthalate esters accused of promoting cutaneous allergic responses, also exhibit TRPA1- and TRPV1-activating properties (Shiba *et al.*, 2009). The increase in intracellular calcium following TRP channel activation and concomitant release of CGRP could play an important role in inducing and modulating sensitizing immune responses (Shiba *et al.*, 2009). Furthermore, the deletion of TRPA1 in mice revealed a crucial role for TRPA1 in hapten-induced contact hypersensitivity. In this model TRPA1 in the skin controls levels of inflammatory cytokines (IL4, IL6) and nerve growth factor as well as substance P and serotonin which induces pruritogen responses (Liu *et al.*, 2013).

Berl and colleagues proposed a mechanism of how PPCM induces occupational contact dermatitis in sensitized nurses, who had positive patch tests to PPCM but negative patch tests to PCM (Berl *et al.*, 1998). It remains to be investigated whether TRPA1 or TRPV1 are directly involved in these sensitization, or whether they indirectly aggravate skin responses by mediating neurogenic inflammation and itch (Bessac and Jordt, 2008).

In our cellular models, PPCM but not PCM induced a substantial sensitization of heat-evoked inward currents through TRPV1. In contrast, in our psychophysical study only four out of seven volunteers showed a small increase in pain rating to noxious heat. Expectedly PPCM was much less potent than capsaicin to activate TRPV1 in vitro. But pain ratings of our volunteers to PPCM injections were also moderate in comparison to capsaicin injections frequently used to cause sensitization in human pain models (Gerber *et al.*, 2007). However, heat as well as mechanical hyperalgesia following capsaicin injections have been shown to be concentration dependent and therefore to depend on the extent of TRPV1 activation (Simone *et al.*, 1987; Simone *et al.*, 1989). Therefore not to observe a significant sensitization against heat in our psychophysics studies is without contradiction to involvement of TRPV1 in PPCM-induced injection pain.

PPCM is rapidly hydrolysed to PCM and diethylglycine. Besides ethylglycine diethylglycine is also generated in the metabolism of lidocaine (Nelson *et al.*, 1977). Both were considered as inert compounds. However, recent studies have proposed that ethylglycine induces antinociceptive effects of intravenous lidocaine by being a co-substrate for the spinal glycine transporter GLY T1 (Werdehausen *et al.*, 2012). Thus ethylglycine was suggested to hamper spinal glycine uptake and consecutively to enhance spinal synaptic inhibition. In animal models of inflammatory and neuropathic pain, Werdehausen et al. demonstrated that systemic treatment with ethylglycine was sufficient to reduce hyperalgesia (Werdehausen *et al.*, 2015). Whether diethylglycine is further metabolized to ethylglycine or whether diethylglycine may also exhibit effects on glycine transporters remains to be investigated. However, the studies comparing the analgesic effects of PCM and PPCM apparently did not identify any relevant differences (McNicol *et al.*, 2011).

Despite similarities in molecular structure and metabolism, PPCM does not display properties of local anesthetics. While there was a small tonic block of $Na^+$ currents at 10 mM PPCM, we observed no

use-dependent block. However, we observed that PCM blocked $Na^+$ currents with lower potency as compared to PPCM. Nevertheless, considering the high concentrations of PPCM necessary to block $Na^+$ currents in our experiments and its rapid hydrolysis in vivo, this mechanism is probably not of clinical relevance. In addition quantitative sensory testing in human volunteers following intracutaneous injections also did not show any indications for a local anaesthetic effect of PCM or PPCM.

In this paper we show that PPCM activates human TRPV1 more than TRPA1 receptors in different cellular models. The hTRPV1-channel activation leads to an increase in intracellular calcium, to depolarizing inward currents and to sensitization of heat-induced currents. These properties of PPCM may be responsible for the injection pain reported as an adverse effect upon intravenous administration. Finally, in human volunteers this injection pain is followed by an increase in superficial blood flow, indicating that PPCM but not PCM excites nociceptors and a concomitant release of vasoactive neuropeptides.

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**Authorship contribution**

*Participated in research design:* Eberhardt M., Leffler.

*Conducted experiments:* Eberhardt E., Eberhardt M., Leffler, Schillers.

*Performed data analysis:* Eberhardt E., Eberhardt M., Leffler, Schillers.

*Wrote or contributed to the writing of the manuscript:* Eberhardt M., Leffler, Schillers

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## Footnotes

## Funding

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**Figure legends**

**Figure 1. Molecular structure of PCM and PPCM.** Ester hydrolysis of the water soluble prodrug propacetamol gives PCM and diethylglycine.

**Figure 2. PPCM activates hTRPV1.** (A) PPCM evoked concentration-dependently ramp currents in HEK 293 cells expressing hTRPV1. (B) Representative experiments at a holding potential of -60 mV 20 mM PPCM evokes large inward currents in hTRPV1-expressing cells which can be inhibited by BCTC, whereas 20 mM PCM (C) does not induce inward currents. PPCM (D) but not PCM (E) increases heat ramp (25-45°C)-induced inward currents and shifts temperature thresholds of evoked currents to lower temperatures (see red dotted lines). Bar diagrams show maximum of heat-induced inward currents (mean ± SEM) and temperature needed to evoke those currents before and after treatment with PPCM (D) and PCM (E). * $p = 0.028$ (PPCM) and $p > 0.05$ (PCM), all Wilcoxon matched pairs test, n = 6 and 8. (F) 20 mM PPCM applied for 40 s evoked an increase in intracellular calcium in hTRPV1-expressing Fura-2-stained HEK 293 cells (n = 475; 68% of capsaicin (0.3 µM)-responsive cells, upper panel). Responses were blocked by the TRPV1 antagonist BCTC (100 nM; n = 198, lower panel). (G) PCM 20 mM did not induce increases in intracellular calcium in hTRPV1-expressing cells (n = 427). Responses are shown as mean ± SEM (bold black trace and dashed lines) and calcium measurements from representative cells (thin grey traces). (H) area under the curve (AUC) of evoked calcium signals. PPCM-responses (blue bar) were significantly reduced by the TRPV1 channel blocker BCTC (white bar) and no responses could be evoked by PCM 20mM (cyan bar, ANOVA $F(2, 1157)=457,5$; $p < 0.001$; following HSD post hoc tests; $p < 0.001$ each).

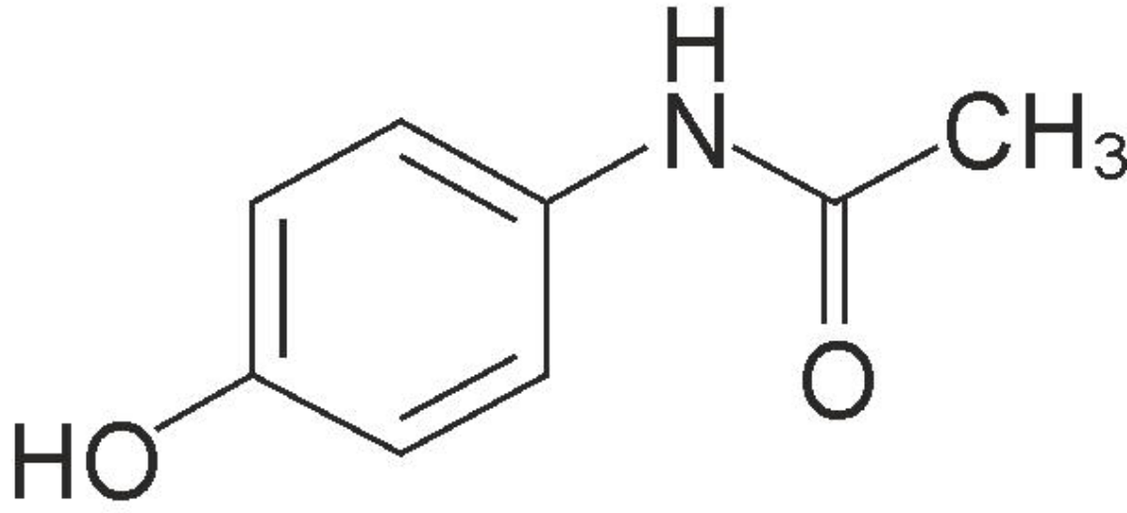
**Figure 3. PPCM and PCM induce rise in intracellular calcium by activating hTRPA1 but hardly evoke inward currents.** (A) PPCM sensitized ramp currents in hTRPA1-expressing HEK 293 cells but only at concentrations above 1 mM. (B) Representative experiments of PPCM 50 mM to evoke small inward currents in hTRPA1-expressing cells which could be inhibited by the TRPA1-blocker HC030331. Cells were held at -60 mV. (C) 50 mM PCM similarly induced small inward currents. (D)

In hTRPA1-expressing HEK 293 cells 20 mM PPCM induced a rise in intracellular calcium in 86% of cells which also responded to cavacrol (250 µM, upper panel, n = 178). Responses were prevented by the TRPA1 blocker HC030031 (50 µM; n= 123) for the duration of application, but reoccurred following washout of HC030031 (lower panel). (E) Similar to PPCM PCM 20mM evoked increases in intracellular calcium in hTRPA1-expressing HEK cells (92%, upper panel, n = 299). These responses were completely inhibited by HC030031 (lower panel, n = 168). Responses are shown as mean ± SEM (bold black trace and dashed lines) and calcium measurements from representative cells (thin grey traces). (F,G) areas under the curve (AUC) of evoked calcium signals: PPCM responses (blue bar) were significantly reduced by the TRPA1 channel blocker HC030031 (white bar) and so were PCM-evoked increases (cyan bar; both ANOVA (1, 381)=408.0; $p < 0.001$ for PPCM and F(1, 465)=590.6; $p < 0.001$ for PCM, following HSD post hoc tests; $p < 0.001$ each).

**Figure 4. At high concentrations PPCM blocks $Na^+$ channels more potently than PCM.** (A) Representative current traces displaying tonic block of $Na^+$ currents by PPCM. Cells were held at -120 mV and currents were activated by test-pulses to -10 mV at 0.1 Hz. Increasing concentrations of PPCM were applied inducing block only at 10 mM. (B) Representative recording of PCM applied in increasing concentrations, barely affecting $Na^+$ currents evoked by the same protocol in ND7 cells. (C) 10 mM PPCM reduced current amplitude more than 10mM PCM (66% vs. 93%; $p < 0.001$; n = 6-7; ANOVA following HSD post hoc test).

**Figure 5. Injection of PPCM in human skin induces pain and axon-reflex erythema.** (A) Magnitude and time course of PPCM (50 mM)-evoked pain in human volunteers after intracutaneous injection to the volar forearm. Pain was rated on a numerical rating scale (NRS) from 0 to 10. PCM (50 mM) was used as a control and did not induce pain (n=7). (B) Laser Doppler scanning to measure superficial blood flow reveals increased flow after PPCM injection in comparison to PCM (ANOVA F(9, 4)=10.37; $p< 0.02$; following HSD post hoc tests; $p\leq 0.04$). (C) Representative pseudocolor image series of PPCM and PCM injections in one volunteer.

paracetamol




propacetamol

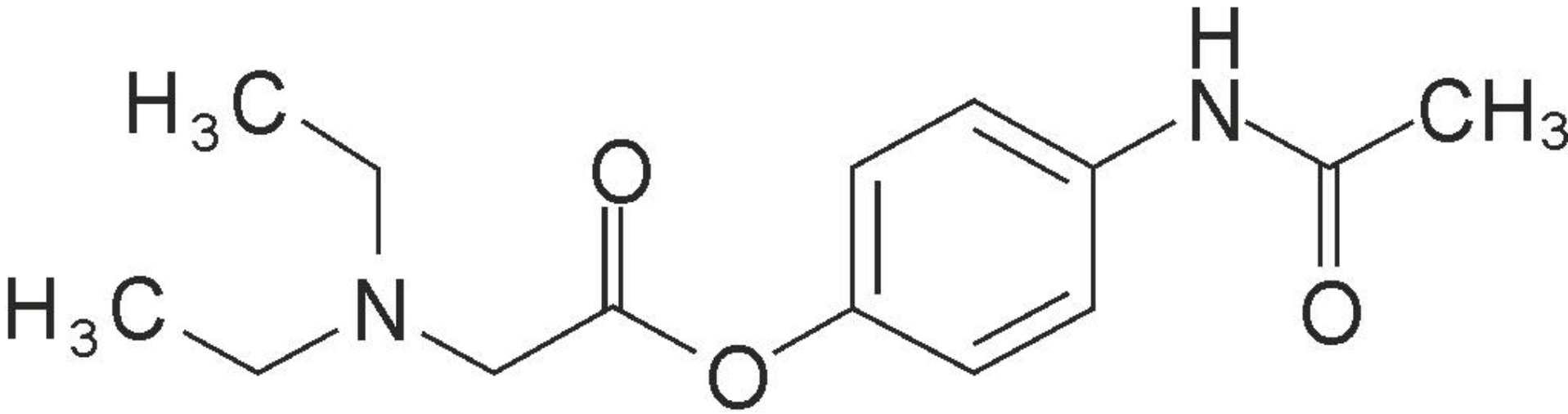
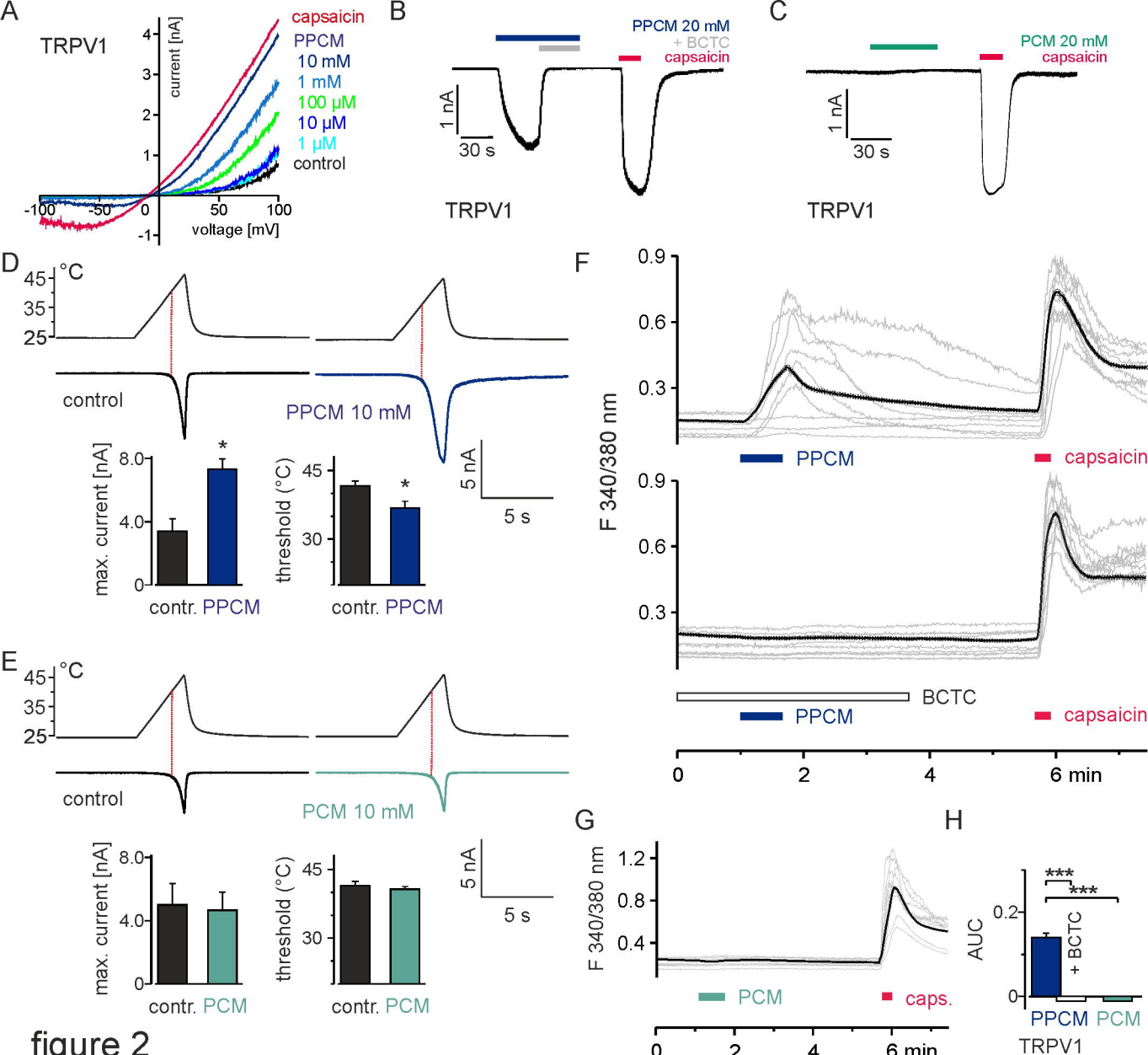



figure 1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TRPV1
capsaicin
PPCM
10 mM
1 mM
100 µM
10 µM
1 µM
control
current [nA]
voltage [mV]
B
PPCM 20 mM
+ BCTC
capsaicin
1 nA
30 s
TRPV1
C
PCM 20 mM
capsaicin
1 nA
30 s
TRPV1
D
°C
control
PPCM 10 mM
max. current [nA]
threshold (°C)
contr. PPCM
contr. PPCM
5 nA
5 s
E
°C
control
PCM 10 mM
max. current [nA]
threshold (°C)
contr. PCM
contr. PCM
5 nA
5 s
F
F 340/380 nm
PPCM
capsaicin
BCTC
PPCM
capsaicin
6 min
G
F 340/380 nm
PCM
caps.
6 min
H
AUC
+ BCTC
PPCM
PCM
TRPV1

figure 2

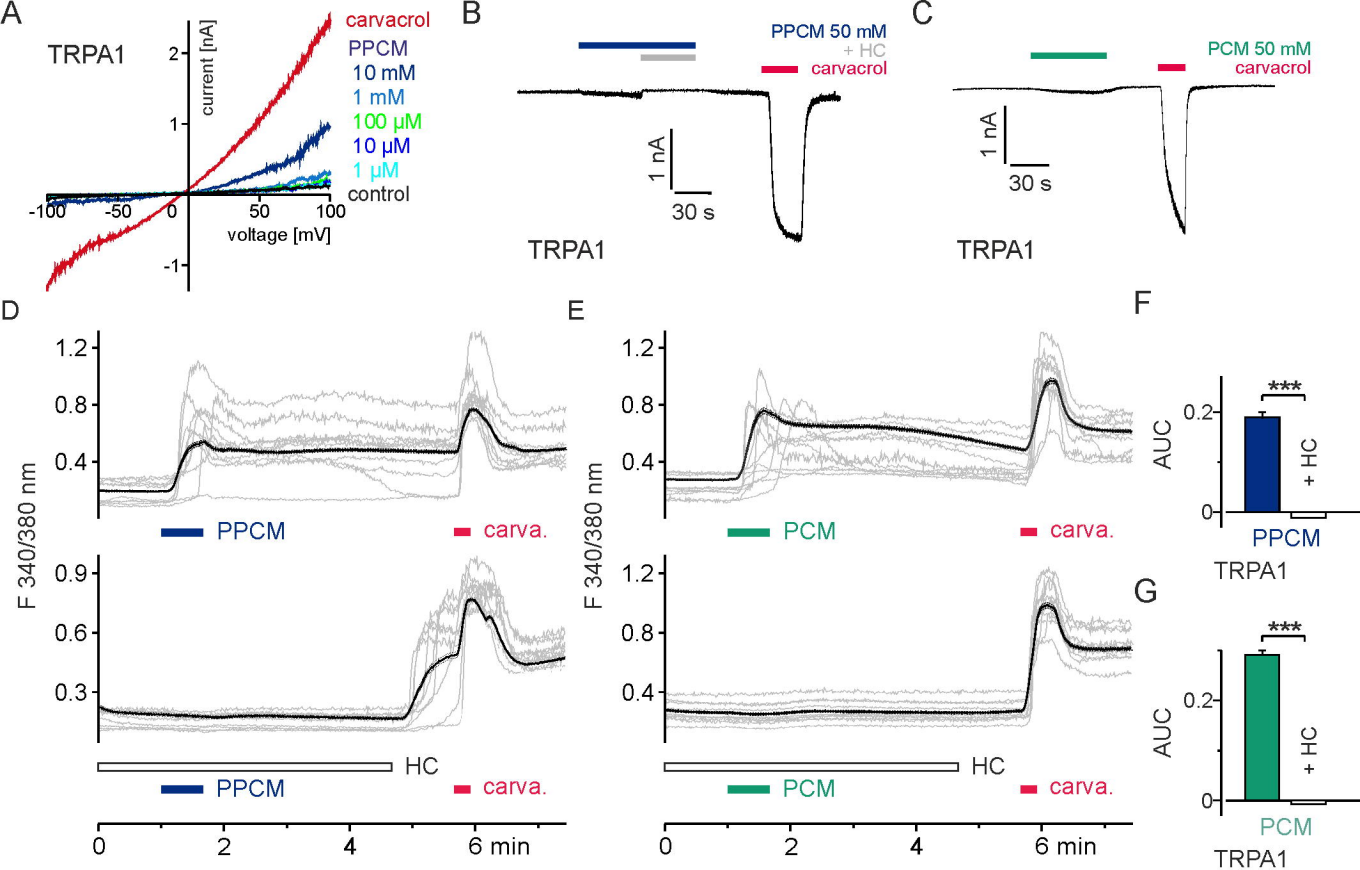
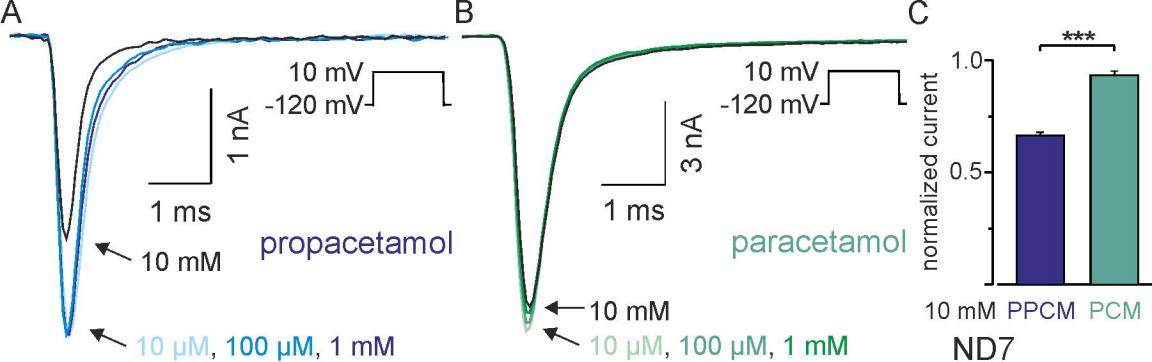

A
TRPA1
current [nA]
voltage [mV]
carvacrol
PPCM
10 mM
1 mM
100 µM
10 µM
1 µM
control
B
PPCM 50 mM
+ HC
carvacrol
1 nA
30 s
TRPA1
C
PCM 50 mM
carvacrol
1 nA
30 s
TRPA1
D
F 340/380 nm
PPCM
carva.
HC
PPCM
carva.
E
F 340/380 nm
PCM
carva.
HC
PCM
carva.
0
2
4
6 min
F
AUC
***
+ HC
PPCM
TRPA1
G
AUC
***
+ HC
PCM
TRPA1

figure 3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10 mV
-120 mV
1 nA
1 ms
propacetamol
10 mM
10 µM, 100 µM, 1 mM
B
10 mV
-120 mV
3 nA
1 ms
paracetamol
10 mM
10 µM, 100 µM, 1 mM
C
***
normalized current
1.0
0.5
10 mM PPCM PCM
ND7

figure 4

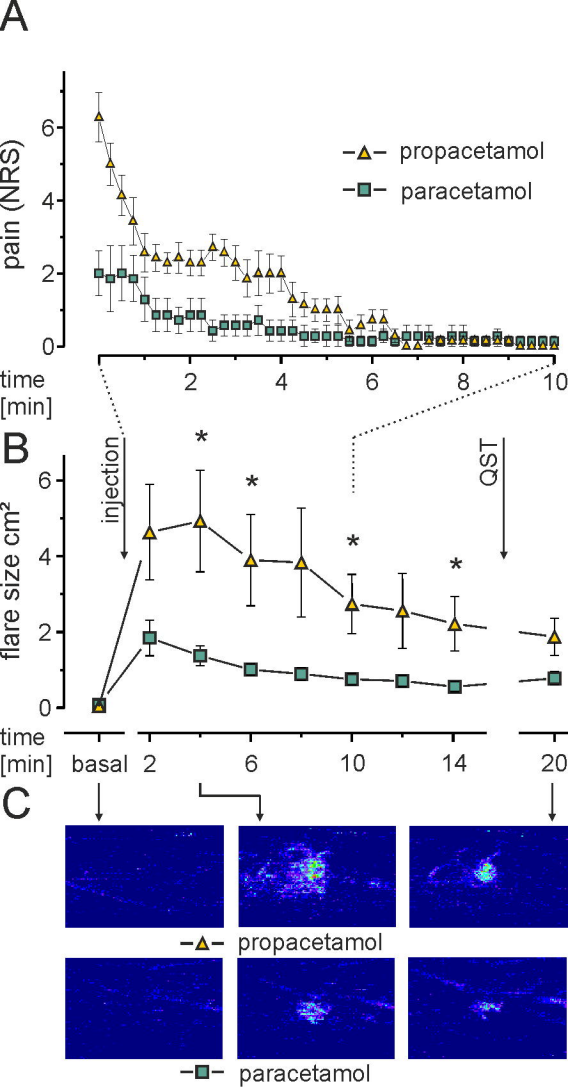

A
pain (NRS)
propacetamol
paracetamol
time
[min]
B
flare size cm²
injection
QST
time
[min]
basal
C
propacetamol
paracetamol

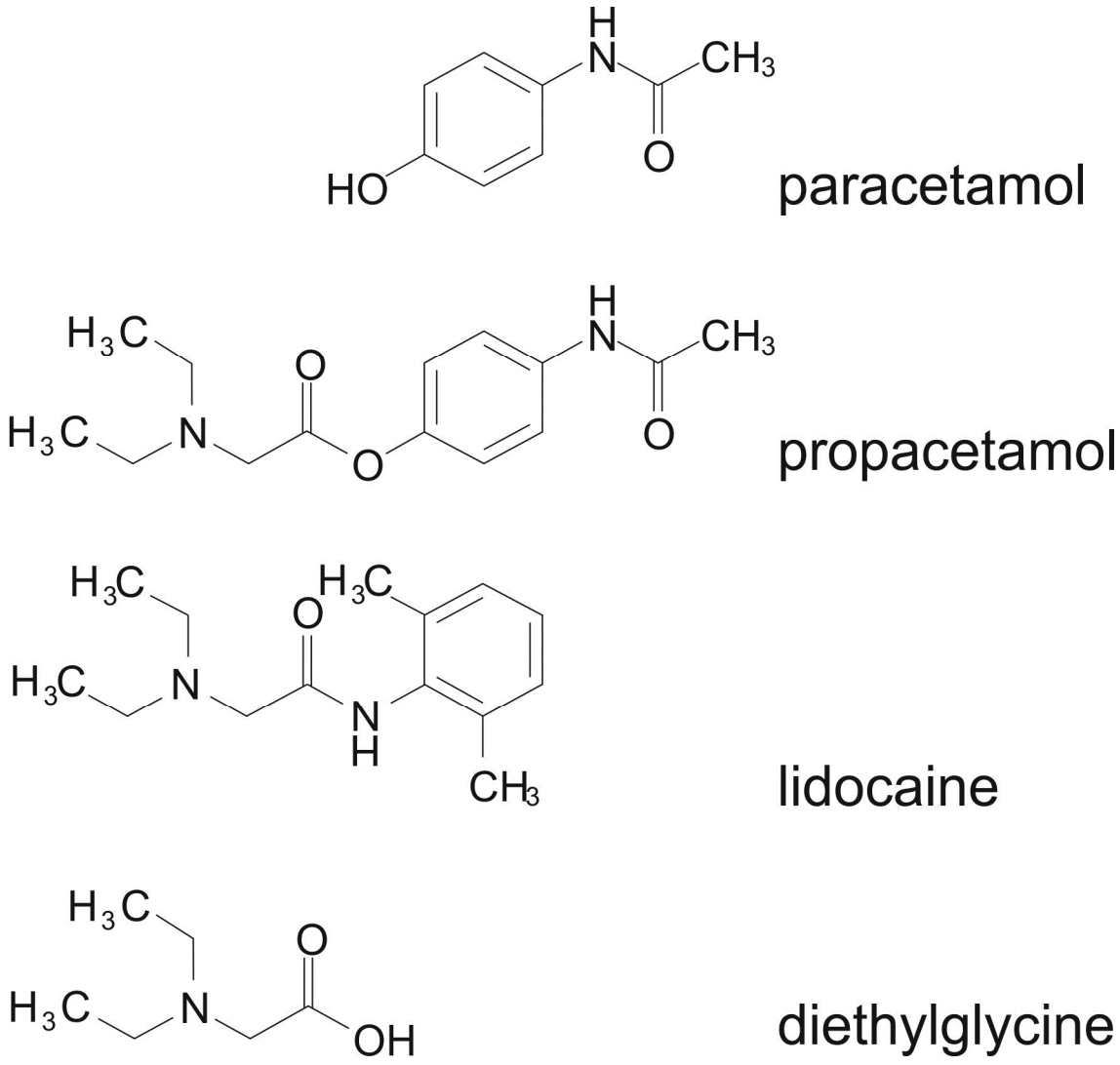
figure 5

**Propacetamol, but not paracetamol activates TRPV1 and TRPA1 and evokes pain**

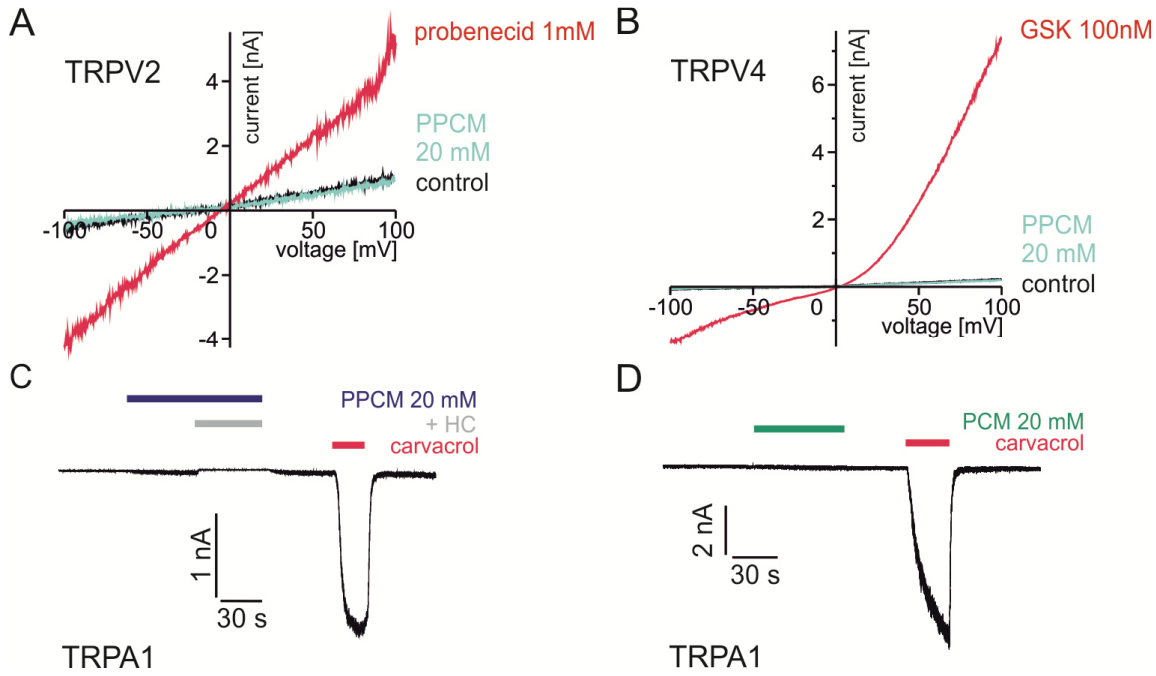
Florian Schillers, Esther Eberhardt, Andreas Leffler and Mirjam Eberhardt

Journal:
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS (JPET)

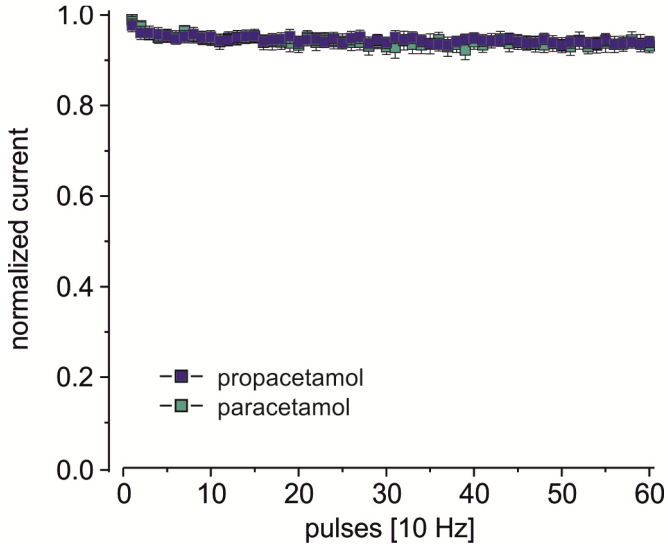
**Supplemental Figures:**



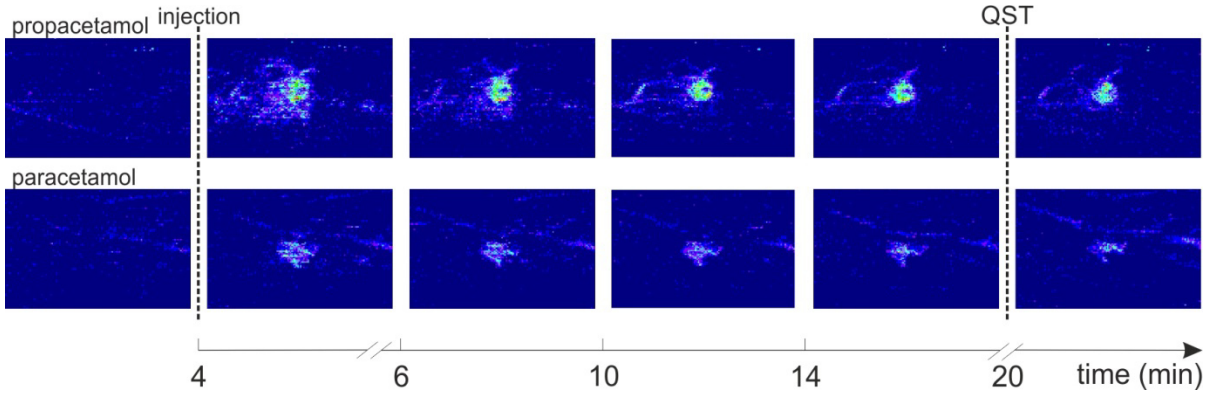

**Supplemental Figure 1. Molecular structures of PCM, PPCM, lidocaine and diethylglycine.** Ester hydrolysis of the water soluble prodrug propacetamol (PPCM) gives paracetamol (PCM) and diethylglycine




**Supplemental Figure 2. 20 mM PPCM does not activate TRPV2 or TRPV4, but induces minimal currents through TRPA1.** PPCM did neither sensitize ramp currents in hTRPV2-expressing cells (A, n = 5) nor in TRPV4-expressing cells (B, n = 5). 1 mM probenecid and 100 nM GSK were used to confirm functional expression. Ramp currents were evoked by 500 ms-long voltage ramps from -100 to +100 mV. 20 mM PPCM (C) and PCM (D) did not evoke inward currents in hTRPA1-expressing cells which were held at -60 mV. The TRPA1 blocker HC030031 (50 µM) also reduced basal TRPA1 activity "noise" in current trace (C) for the duration of its application. Representative recording of one cell (n = 5 - 6 each).




**Supplemental Figure 3. PPCM and PCM fail to induce use-dependent block of sodium channels in ND7/23 cells.** Currents of endogenous sodium channels in ND7/23 cells were activated at 10 Hz by 60 test pulses from the holding potential of -120 mV to -10 mV. Peak current amplitudes were normalized to the amplitude of the first pulse and plotted against the pulse number. Neither 10 mM paracetamol (PCM, cyan, n = 6) nor 10 mM propacetamol (PPCM, blue, n = 8) induced a use-dependent block.




**Supplemental Figure 4. PPCM-induced axon reflex erythema**. Complete series of laser Doppler scans taken before and every 2 minutes after double-blind intracutaneous injection of PPCM or PCM to the volar forearm of a human volunteer. While there is a strong axon reflex erythema induced by neuropeptide release following antidromic activation of wide branching C-fibers after PPCM injection, there is only a short-lasting weak erythema comparable to physiological saline injection after PCM injection.