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ORIGINAL ARTICLE

The phospholipase C inhibitor U73122 is a potent agonist of the polymodal transient receptor potential ankyrin type 1 (TRPA1) receptor channel

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Abstract

The aminosteroid U73122 is frequently used as a phospholipase C (PLC) inhibitor and as such was used to investigate PLC-dependent activation and modulation of the transient receptor potential ankyrin type 1 (TRPA1) receptor channel. However, U73122 was recently shown to activate recombinant TRPA1 directly, albeit this interaction was not further explored. Our aim was to perform a detailed characterization of this agonistic action of U73122 on TRPA1. We used Fura-2 calcium microfluorimetry and the patch clamp technique to investigate the effect of U73122 on human and mouse wild type and mutant (C621S/C641S/C665S) TRPA1 expressed in HEK293t cells, as well as native TRPA1 in primary afferent neurons from wild type and TRPV1 and TRPA1 null mutant mice. In addition, we measured calcitonin gene-related peptide (CGRP) release from skin isolated from wild-type and TRPA1 null mutant mice. Human and mouse TRPA1 channels were activated by U73122 in the low nanomolar range. This activation was only partially dependent upon modification of the N-terminal cysteines 621, 641, and 665. U73122 also activated a subpopulation of neurons from wild-type and TRPV1 null mutant mice, but this effect was absent in mice deficient of TRPA1. In addition, U73122 evoked marked calcitonin gene-related peptide (CGRP) release from skin preparations of wild type but not TRPA1 null mutant mice. Our results indicate that U73122 is a potent and selective TRPA1 agonist. This effect should be taken into account when U73122 is used to inhibit PLC in TRPA1-expressing cells, such as primary nociceptors. In addition, U73122 may present a novel lead compound for the development of TRPA1-targeting drugs.

Keywords Nociceptor · Dorsal root ganglion · TRP channel · Pain · Signal transduction

Introduction

TRPA1 and TRPV1 receptor channels are expressed in primary sensory neurons where they act as sensors for detecting potentially harmful stimuli, in particular products of oxidative stress. TRPA1 is activated by various irritant plant chemicals, such as the highly electrophilic allyl isothiocyanate (AITC, from horseradish and mustard oil), cinnamaldehyde (from cinnamon), allicin (from garlic) (Bandell et al. 2004; Bautista et al. 2005; Macpherson et al. 2007), as well as non-electrophilic compounds such as menthol and carvacrol (Karashima et al. 2007; Xu et al. 2006). TRPV1 is activated by noxious heat, extracellular protons, and the vanilloid capsaicin (from chili peppers) (Caterina et al. 1997; Tominaga et al. 1998). Both these TRP channels are positively modulated by phospholipase C (PLC) activation (Bandell et al. 2004; Chuang et al. 2001; Jordt et al. 2004; Kadkova et al. 2017; Wang et al. 2008a).

PLC is the primary effector of an important number of first messengers binding to G protein-coupled receptors. It degrades membrane phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) which further act as second messengers. Since the description of the aminosteroid U73122 (1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione) as a PLC inhibitor (Bleasdale et al. 1990; Smith et al. 1990), this compound has widely been used to explore PLC involvement in intracellular signaling

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mechanisms (Horowitz et al. 2005; Jin et al. 1994), including the modulation of TRPA1 by PLC (Bandell et al. 2004; Bellono et al. 2013; Karashima et al. 2008). The precise mechanism of PLC inhibition by U73122 has not been clarified but is thought to involve alkylation of PLC cysteine residues (Klein et al. 2011). U73122 inhibited several PLC-dependent processes with IC50 values between 0.1 and 10 μM. For example, diacylglycerol production in polymorphonuclear neutrophils stimulated with N-formyl-methionyl-leucyl-phenylalanine was inhibited by U73122 with a Ki of 2 μM (Bleasdale et al. 1990).

However, PLC inhibition is not the only described effect of U73122, as there are many reported actions of the compound which are clearly PLC-independent (Horowitz et al. 2005). U73122 blocks potassium channels like Kir 3.1, 3.2 and BK channels (Klose et al. 2008) and inhibits the sarcoplasmic reticulum calcium pump in smooth muscle cells (Macmillan and McCarron 2010). It also inhibits ACh-activated K+ channels in mouse atrial myocytes (Cho et al. 2001) and partially inhibits voltage-gated calcium channels in the neuronal cell line NG 108-15 (Jin et al. 1994). Modulation of two TRP channels, TRPM3 and TRPM4, by U73122 was recently shown not to involve PLC inhibition: U73122 directly inhibits TRPM3 and activates TRPM4 (Leitner et al. 2016). Some of the PLC-independent actions of U73122 have been attributed to alkylation of protein thiol groups (Horowitz et al. 2005). Interestingly, TRPA1 was shown to be activated by alkylating agents, such as 2-chloroethyl-ethylsulfide (Stenger et al. 2015).

U73122 shares an electrophilic character with certain TRPA1 activators, such as AITC and cinnamaldehyde, chemically being a substituted N-maleimide, similar to the potent TRPA1 agonist N-methyl maleimide (NMM) (Hinman et al. 2006; Ibarra and Blair 2013), which may suggest a similar mechanism of action on TRPA1. U73122 was indeed reported to activate mouse TRPA1 in expression systems at 1 μM concentration, but the involvement of TRPA1 N-terminal cysteines in this activation was not investigated, nor was the compound tested on native sensory neurons (Karashima et al. 2008). NMM does not activate the triple cysteine C621S/C641S/C665S TRPA1 mutant (TRPA1-3C), which is known to be less sensitive to electrophilic TRPA1 agonists (Hinman et al. 2006). Considering the structural analogy between NMM and U73122, and also the susceptibility of TRPA1 to alkylating agents, we decided to investigate if the same cysteine-dependent covalent mechanism is involved in the activation of TRPA1 by U73122. Moreover, as TRPV1 is also activated by electrophilic compounds, including AITC (Gees et al. 2013), and by reactive oxygen species such as H_2O_2 (DelloStritto et al. 2016), we also investigated the effect of U73122 on this receptor channel. We found that human TRPA1, but not hTRPV1, is activated by low nanomolar levels of U73122 and this activation is not fully dependent on the reactive N-terminal cysteines in positions 621, 641, 665.

Materials and methods

Solutions and reagents

The external solution employed in calcium imaging and some patch-clamp experiments contained (in mM) NaCl 140, KCl 4, $CaCl_2$ 1.25, $MgCl_2$ 1, HEPES 10, glucose 5; the pH was adjusted to 7.4 with NaOH. The external calcium-free solution used in patch-clamp experiments and some calcium imaging experiments contained (in mM) NaCl 140, KCl 4, $MgCl_2$ 2, HEPES 10, glucose 5; the pH was adjusted to 7.4 with NaOH. The pipette solution for patch-clamp recordings contained (in mM) potassium gluconate 135, $MgCl_2$ 3, EGTA 5, Na_2ATP 2, Na_3GTP 0.3, NaCl 4, HEPES 5; the pH was adjusted to 7.25 with KOH. The synthetic interstitial fluid (SIF) used for CGRP experiments contained as follows (in mM): NaCl 108, KCl 3.5, $MgSO_4$ 3.5, $NaHCO_3$ 26, NaH_2PO_4 1.7, $CaCl_2$ 1.5, sodium gluconate 9.6, glucose 5.5, and sucrose 7.6. All reagents were purchased from Sigma-Aldrich. Stock solutions of U73122 (1 mM, DMSO) and U73343 (1 mM, DMSO) were freshly prepared before experiments. Carvacrol (100 mM, DMSO), AITC (1 M in DMSO), capsaicin (1 mM in ethanol), HC-030031 (10 mM in DMSO), BCTC (10 mM in ethanol), and ionomycin (2 mM, DMSO) were kept as stock solution at − 20 °C and diluted to the working concentration before the experiment. In all experiments, the final concentration of DMSO or ethanol was less than 0.1%.

Animals

Breeding and euthanasia and all procedures of animal handling were prospectively approved by the Animal Welfare Authority of the District Government of Unterfranken in Würzburg (Germany) and the local Institutional Animal Care Department (University of Erlangen, Germany) in accordance with the German regulations of animal care and welfare (Tierschutzgesetz). Experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). $TRPA1^{-/-}$ mice were donated by Harvard University (Drs. Kelvin Kwan and David Corey, Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts) and $TRPV1^{-/-}$ mice by Dr. John Davis (formerly GlaxoSmithKline, Harlow, UK); both mouse lines were backcrossed onto C57BL/6 for more than 15 generations and used to breed the respective double knock-out mice. The 2-month-old male mice were maintained in a 12/12-h dark light cycle and food and water was supplied ad libitum. The mice were euthanized by exposure to a rising CO_2 concentration in their breathing air followed by cervical dislocation.

Cell culture and transfection

Adult male mice were used to gather DRGs from all spinal cord levels which were transferred to DMEM containing 50 μg/ml gentamicin. DRGs were mechanically dissociated after 30-min treatment with 1 mg/ml collagenase and 0.1 mg/ml protease and plated onto glass coverslips previously coated with poly-D-lysine (200 μg/ml). DRG neurons were cultured in serum-free TNB 100 cell culture medium supplemented with TNB 100 lipid-protein complex and streptomycin/penicillin (100 μg/ml, Biochrom). Mouse NGF was added at 100 ng/ml (Alomone Labs), and the cells were kept at 37 °C and 5% CO_2 for 24 h.

HEK293t cells were cultured in standard DMEM (D-MEM, Gibco, BRL Life Technologies, Karlsruhe Germany) with 10% FBS (Biochrom, Berlin Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Karlsruhe, Germany). HEK293t cells were transiently transfected with plasmids containing mTRPA1, hTRPA1, hTRPV1, and hTRPA1-C621S/C641S/C665S (hTRPA1-3C) using jetPEI transfection reagent from Polyplus Transfection (Illkirch, France). Cells were cultivated at 37 °C and 5% CO_2, passaged every 3–4 days, plated onto poly-D-lysine-coated glass coverslips, and used for experiments within 24 h.

Calcium imaging

HEK293t cells expressing the mouse or human isoform of TRPA1 or TRPV1 (mTRPA1, hTRPA1, hTRPA1-3C, hTRPV1) and DRG neurons plated on glass coverslips were loaded with Fura-2 AM (3 μM, 30 min at 37 °C, also containing 0.02% Pluronic, both from Invitrogen) and washed for 10 min before recording. Coverslips were then mounted on an Olympus IX71 inverted microscope and imaged using a 20× objective with 0.4 numerical aperture. Cells were permanently superfused with external solution using a software-controlled 7-channel gravity-driven common-outlet system (Dittert et al. 2006). Fura-2 was excited at 340 and 380 nm with a poly-chrome V monochromator (Till Photonics, Planegg, Germany). Fluorescence emission was long-passed at 495 nm, unless otherwise specified, and pairs of images were acquired at a rate of 0.5 or 1 Hz with an exposure time of 4–10 ms with a 12-bit CCD camera (Imago Sensicam QE, Till Photonics). Data were processed off-line using the TILLvisION 4 (Till Photonics). The background intensity was subtracted before calculating the ratio between the fluorescence emitted when the dye was excited at 340 nm and at 380 nm (F340/380). The time course of this ratio was analyzed for regions of interest within individual cells. An average increase in the F340/380 ratio > 0.1 was considered a response. The dose-response curve was fitted using the Hill1 equation from OriginPro 8: $y = Start + (Start{-}End)\frac{x^n}{k^n+x^n}$, where the "End" parameter was fixed. Each calcium imaging trace presented in the figures was obtained by pooling together all cells recorded using the same stimulation protocol (2–5 independent experiments). As there was no qualitative or quantitative difference between individual experiments, all cells were pooled together to generate a mean (thick trace) and SEM values (thin traces bracketing the mean).

Whole-cell patch-clamp recordings

Voltage-clamp experiments were performed on HEK293t cells transiently transfected with an hTRPV1::YFP fusion plasmid or with hTRPA1 equipped with a IRES-based YFP coexpression for selecting successfully transfected cells. Currents were acquired using an Axopatch 200B patch-clamp amplifier (Molecular Devices), low-passed at 1 kHz, and sampled at 2 kHz; pCLAMP 10 was used for voltage control, data acquisition, and off-line analysis. Patch pipettes were pulled from borosilicate glass tubes (TW150F-3, World Precision Instruments) and heat-polished to a final resistance of 2.5–4.0 MΩ. Cells were held at − 60 mV and probed every 4 s by voltage ramps from − 100 to + 100 mV of 400 ms duration.

Neuropeptide release

The experiments measuring release of immunoreactive CGRP were performed as described previously (Averbeck and Reeh 2001; Babes et al. 2010). Briefly, hindpaw skin flaps of adult C57BL/6 control or TRPA1 knockout mice were excised after sacrificing in a rising CO_2 atmosphere. The flaps were washed for 30 min at 32 °C in carbogen-saturated SIF (see Solutions and reagents, above; (Bretag 1969)). Thereafter, the preparations were passed through a series of four consecutive incubation steps each lasting 10 min at 32 °C. In the third incubation step, U73122 was applied at 10 or 30 μM freshly dissolved in SIF. The iCGRP content of the eluates was measured using commercial enzyme immunoassay kits (Bertin Pharma, Montingy le Bretonneux, France) with a detection limit of 5 pg/ml. The enzyme immunoassay plates were analyzed photometrically using a microplate reader (Dynatech, Channel Islands, UK). The data are displayed showing the time course of stimulated iCGRP release as mean ± SEM (Fig. 7a). The column diagrams (Fig. 7b) show the overall stimulated release (AUC). For this, the values of the stimulated and the successive samples were added up, and the sum of the two baseline values was subtracted to gain quasi an area-under-the-curve value (Δ pg/ml/20 min).

Results

U73122 activates mouse and human TRPA1

We investigated whether activation of TRPA1 by U73122 was species-dependent by performing calcium imaging on

recombinant mouse and human TRPA1 expressed in HEK293t cells (HEK293-hTRPA1 and HEK293-mTRPA1).

Application of U73122 (1 μM) induced a robust $[Ca^{2+}]_i$ increase in both HEK293-hTRPA1 and HEK293-mTRPA1 cells. Thus, 93% of carvacrol-sensitive HEK293-mTRPA1 cells and 80% of carvacrol-sensitive HEK293-hTRPA1 cells were activated by U73122 (Fig. 1a). At the same concentration (1 μM), U73122 did not produce any change of the intracellular calcium concentration ($[Ca^{2+}]_i$) in naïve (untransfected) HEK293t cells (Fig. 1a).

U73122 (1 μM) was shown to release intracellular calcium in mouse pancreatic acinar cells (Mogami et al. 1997) and rat sensory neurons (Jin et al. 1994), possibly from the endoplasmic reticulum. However, in our experiments, U73122 (1 μM) failed to evoke any change in $[Ca^{2+}]_i$ when applied in a calcium-free external solution (Fig. 1b), demonstrating that intracellular stores do not contribute to the calcium transients induced by the compound in HEK293-hTRPA1 cells and that the activation of hTRPA1 by U73122 is not secondary to calcium released from stores (Jordt et al. 2004; Zurborg et al. 2007).

hTRPA1 and hTRPA1-3C are activated in a dose-dependent manner by U73122

To explore the mechanism of U73122-induced activation of TRPA1, we employed the TRPA1-3C mutant, in which cysteines in positions 621, 641, and 665 were replaced by serines. This mutant is known to display a markedly reduced sensitivity to electrophilic agents (Eberhardt et al. 2012; Hinman et al. 2006), and we compared its concentration dependence of U73122 activation to that of the wild-type channel. U73122 was able to evoke increases in $[Ca^{2+}]_i$ in cells expressing either wild-type hTRPA1 or the 3C mutant, even at low nanomolar concentrations (the range of concentrations used was 100 pM to 1 μM; Fig. 2a and b). However, when the sensitivity to U73122 was expressed as the concentration dependence of the average amplitude of the calcium transients evoked by the compound, the 3C mutant displayed a diminished sensitivity compared to the wild-type channel with an estimated EC50 of 125 nM compared to 36 nM, respectively (Fig. 2c). The selective TRPA1 antagonist HC-030031 (1,2,3,6-Tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-dioxo-7H-purine-7-acetamide; 1 μM) strongly reduced the $[Ca^{2+}]_i$ increase evoked by U73122 (1 μM), in both hTRPA1- and hTRPA1-3C-expressing HEK293t cells (by approx. 42% and 48%, respectively) (Fig. 2d).

hTRPA1 and hTRPA1-3C are less sensitive to the inactive analogue U73343

We found that U73343 (1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione), an analog of U73122 which is inactive against PLC, was also able to activate both hTRPA1 and hTRPA1-3C, albeit less potently and effectively compared to U73122. At a concentration of 1 μM, U73343 activated 16% (22 of 137) of carvacrol-sensitive HEK293t-hTRPA1 cells and 12% (18 of 149) of carvacrol-sensitive HEK293t-hTRPA1-3C cells (Fig. 3a). Moreover, the amplitudes of the calcium transients evoked by U73343 were substantially smaller than those induced by U73122 at the same concentration (Fig. 3b). Normalized to the carvacrol responses, U73122 1 μM evoked an increase of $[Ca^{2+}]_i$ of

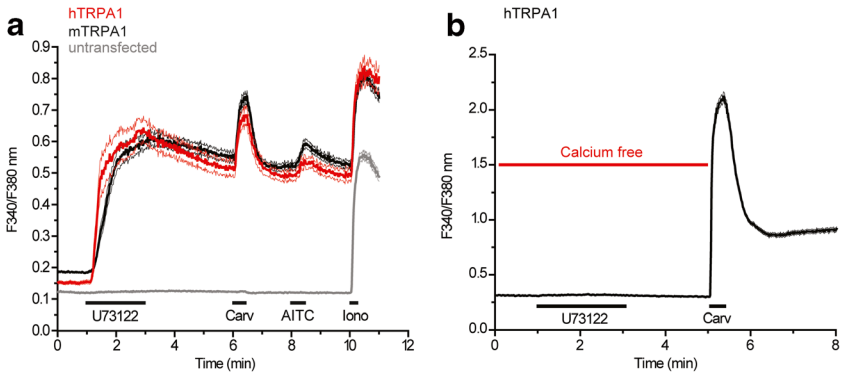

Fig. 1 U73122 evokes calcium entry in HEK293t expressing human TRPA1. **a** U73122 activates recombinant mouse and human TRPA1. Calcium transients in HEK293t cells transiently transfected with human (red traces, $n = 60$ cells) and mouse (black traces, $n = 70$ cells) TRPA1 stimulated with U73122 (1 μM, 2 min). **b** Removal of external calcium abolishes U73122-induced calcium transients in HEK293t cells expressing hTRPA1. U73122 (1 μM) was applied for 150 s ($n = 116$). The red line in Fig. 1b indicates the duration of calcium removal (the first 5 min of the experiment). In both **a** and **b**, the cells were also stimulated with the selective TRPA1 agonist carvacrol (50 μM, 20 s) to confirm functional expression of the channel. In panel **a**, another TRPA1 agonist, allyl isothiocyanate (AITC), was also applied (50 μM, 20 s). Ionomycin (2 μM, 20 s) was applied at the end of the experiment as a positive control. The data are represented as mean (thick lines) ± SEM (thin lines)

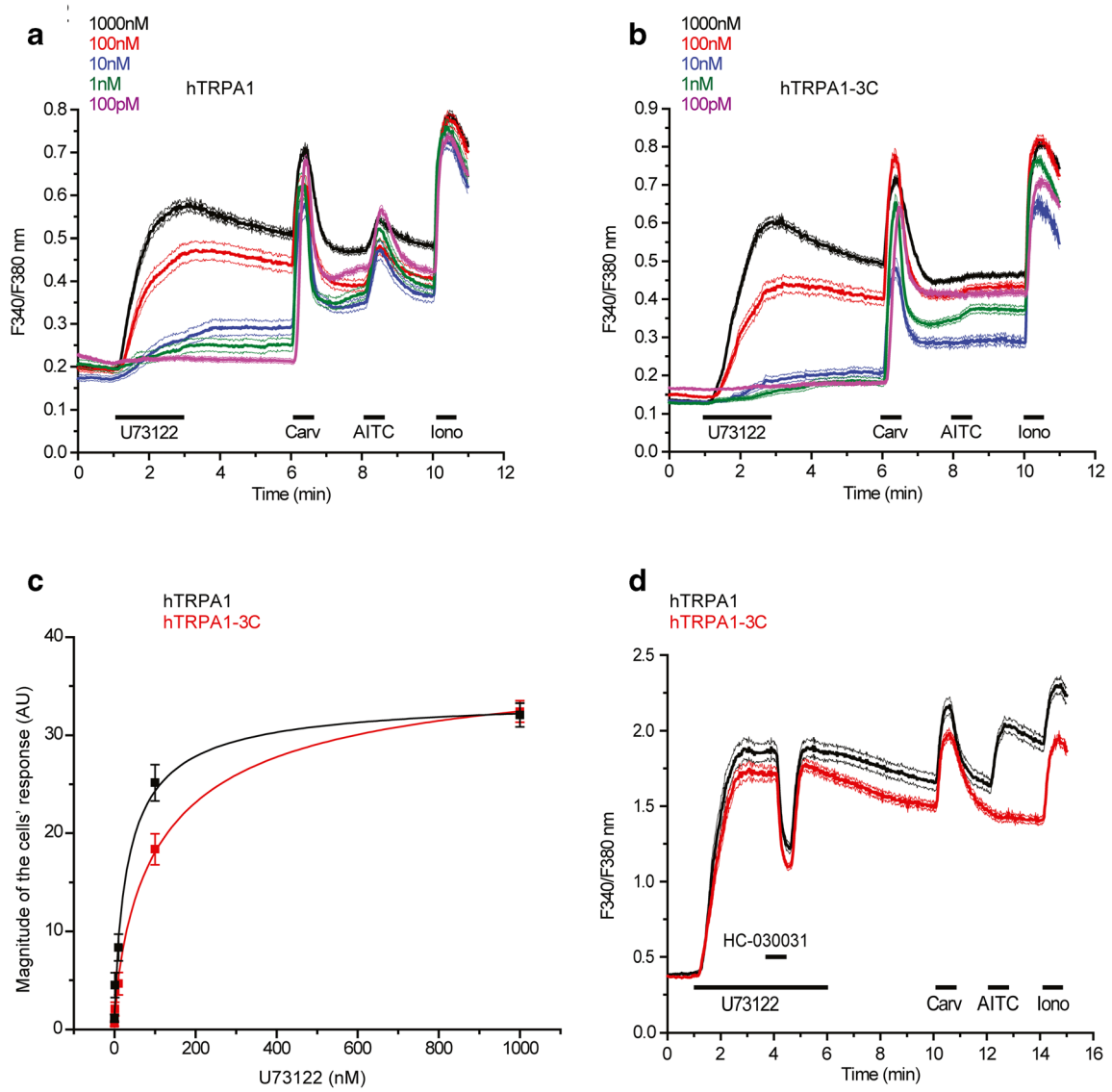

Fig. 2 The activation of hTRPA1 and hTRPA1-3C by U73122 is concentration-dependent and can be blocked by the selective TRPA1 antagonist HC-030031. **a** U73122 activates wild-type hTRPA1 in a concentration-dependent manner. HEK293t-hTRPA1 cells were stimulated with U73122 for 2 min at the following concentrations: 100 pM (purple trace, $n = 330$), 1 nM (green trace, $n = 80$), 10 nM (blue trace, $n = 84$), 100 nM (red trace, $n = 109$), and 1 μM (black trace, $n = 280$). **b** U73122 activates the mutant hTRPA1-3C in a concentration-dependent manner. HEK293t-hTRPA1 cells were stimulated with U73122 for 2 min at the following concentrations: 100 pM (purple trace, $n = 315$), 1 nM (green trace, $n = 159$), 10 nM (blue trace, $n = 81$), 100 nM (red trace, $n = 182$), and 1 μM (black trace, $n = 401$). In both experiments, the cells were also stimulated with the TRPA1 agonists carvacrol (50 μM, 20 s) and AITC (50 μM, 20 s) to confirm functional expression of either wild-type hTRPA1 (**a**) or the hTRPA1-3C mutant (**b**). Note the lack of any response to AITC in HEK293t cells expressing the mutant channel (**b**). **c** Concentration dependence of the mean amplitude of U73122-evoked calcium transients in HEK293t-hTRPA1 cells (black disks) and HEK293t-hTRPA1-3C cells (red squares). Data are collected from the experiments illustrated in parts **a** and **b**, respectively. The data were fitted with a Hill equation (see Materials and methods) and yielded EC50 values of 36 nM and, respectively, 125 nM. **d** The selective TRPA1 antagonist HC-030031 (10 μM) reversibly inhibits the calcium transients evoked by U73122 in HEK293t-hTRPA1 cells (black trace, $n = 90$) and in HEK293t-hTRPA1-3C cells (red trace, $n = 151$). In both experiments, the cells were also stimulated with the TRPA1 agonists carvacrol (50 μM, 20 s) and AITC (50 μM, 20 s) to confirm functional expression of either wild-type hTRPA1 or the hTRPA1-3C mutant. Note the lack of any response to AITC in HEK293t cells expressing the mutant channel (red trace). In all experiments (**a**, **b**, **d**), ionomycin (2 μM, 20 s) was applied at the end of the experiment as a positive control. The data are represented as mean (thick lines) ± SEM (thin lines)

84.0 ± 2.0% ($n = 280$) in TRPA1- and 87.2 ± 2.1% ($n = 401$) in TRPA1-3C-transfected HEK cells, whereas U73343 1 μM induced an increase of 12.6 ± 1.5% ($n = 137$) in TRPA1- and 9.8 ± 1.6% ($n = 149$) in hTRPA1-3C-transfected HEK cells (Fig. 3b).

U73122 does not activate human TRPV1 but modulates the channel via PLC inhibition

We again used Fura-2 calcium microfluorimetry to investigate the effect of U73122 on recombinant human

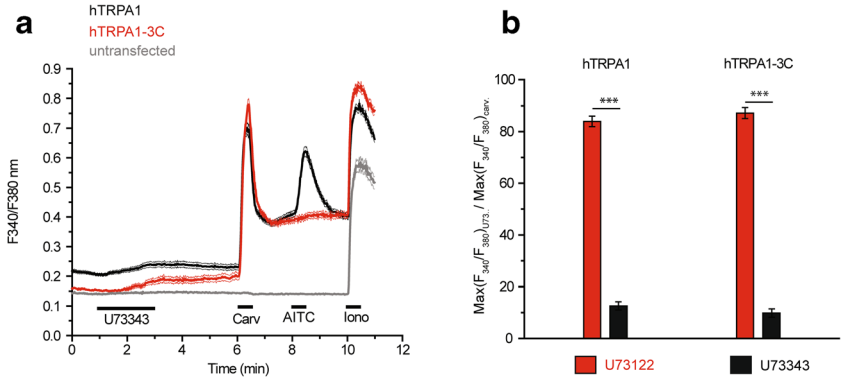

Fig. 3 The inactive analog of U73122, U73343, is a weak TRPA1 agonist. **a** HEK293t-hTRPA1 cells (black traces, $n = 157$) and HEK293t-hTRPA1-3C cells (red traces, $n = 149$) were stimulated with U73343 at 1 μM for 2 min, followed by the TRPA1 agonists carvacrol (50 μM, 20 s) and AITC (50 μM, 20 s). Note the lack of any response to AITC in the case of the hTRPA1-3C-expressing cells. Ionomycin (2 μM, 20 s) was applied at the end of the experiment as a positive control. The data are represented as mean (thick lines) ± SEM (thin lines). **b** Comparison of the activating effects of U73122 (red columns) and U73343 (black columns) on hTRPA1- and hTRPA1-3C-expressing cells (each compound was applied at 1 μM). The columns represent the peak amplitude of the calcium transients (expressed as the fluorescence ratio F340/F380) evoked by each compound, normalized to the peak amplitude of the calcium transients evoked by carvacrol. Statistical comparison of the data was carried out using Student's unpaired *t* test (two-tailed) (***, $p < 0.001$)

TRPV1 expressed in HEK293t cells (HEK293t-hTRPV1 cells). At a concentration of 1 μM, U73122 failed to evoke any change in $[Ca^{2+}]_i$ in HEK293t-hTRPV1 cells. Functional expression of the channel was confirmed by the activation of HEK293t-hTRPV1 cells by capsaicin (Fig. 4a). Horowitz et al. (2005) have reported a weak paradoxical agonistic effect of U73122 on PLC and a similar effect was described on certain PLC isoforms in a cell-free micellar system (Klein et al. 2011). Moreover, certain inflammatory mediators (including bradykinin and PAR2 agonists trypsin and tryptase) sensitize TRPA1 by stimulation of PLC activity (Dai et al. 2007; Wang et al. 2008b). Thus, it could be hypothesized that the U73122-induced activation of TRPA1 may occur through an indirect pathway, involving stimulation of PLC. To investigate whether U73122 produces PLC activation, we monitored the effect of the compound on the amplitudes of capsaicin-induced calcium transients in HEK293t-hTRPV1 cells. Activation of PLC is known to sensitize TRPV1 by degradation of PIP2 and release of the channel from a PIP2-mediated inhibition (Chuang et al. 2001). In our experiments, application of U73122 (at 100 nM and 1 μM) before capsaicin stimulation produced a concentration-dependent inhibition of capsaicin-induced calcium transients in HEK293-hTRPV1 cells (approx. 30% decrease in amplitude following 1 μM U73122 and 14% for 100 nM; Fig. 4b, c), suggesting that U73122 inhibits constitutive PLC activity, leading to increased PIP2 levels and subsequent inhibition of TRPV1. These results are not compatible with a proposed PLC stimulatory action of U73122 in our HEK293t cells, and therefore, it is unlikely that activation of TRPA1 occurs downstream of paradoxical PLC stimulation by the drug.

U73122 activates inward currents in hTRPA1- and hTRPA1-3C-expressing HEK293 cells

Using the patch clamp method, we explored the ability of U73122 to induce whole-cell currents in hTRPA1- and hTRPA1-3C-expressing HEK293t cells. We carried out the initial experiments in a calcium-free extracellular solution, to reduce calcium-induced desensitization of TRPA1. In both HEK293t-hTRPA1 and HEK293t-hTRPA1-3C cells, U73122 (1 μM) induced currents with a peak value of 223 ± 65 pA/pF (for hTRPA1 at + 80 mV; mean ± SD; $n = 3$; Fig. 5a) and, respectively, 186 ± 62 pA/pF (for hTRPA1-3C; $n = 3$; Fig. 5c). The current developed slowly (within ~ 3 min), did not recover upon washout of U73122, and subsequent application of carvacrol and AITC further increased the current. Application of U73122 at a lower concentration (100 nM) resulted in smaller currents (70 ± 20 pA/pF (for hTRPA1; $n = 3$) and 44 ± 9 pA/pF (for hTRPA1-3C; $n = 3$; data not shown). The U73122-evoked current displayed outward rectification and a reversal potential close to 0 mV (Fig. 5b) and was completely abolished by the selective TRPA1 antagonist HC-030031 (1 μM), indicating that the currents were entirely mediated by TRPA1 ($n = 3$; Fig. 5a). In the presence of extracellular calcium, application of U73122 (1 μM) induced a delayed activation of hTRPA1, with a latency of 77 ± 26 s and a maximum current of 83 ± 43 pA/pF (at + 80 mV, $n = 3$; Fig. 5d). The U73122-induced current displayed a sudden increase followed by rapid acute desensitization in the continuous presence of U73122. Following U73122 treatment, responses to both AITC (50 μM) and carvacrol (50 μM) were very small, indicating strong desensitization. This cross-desensitization between U73122 and the other agonists is likely due to calcium entry, as it did not occur in the absence of extracellular calcium (Fig. 5a and c) (Wang et al. 2008b).

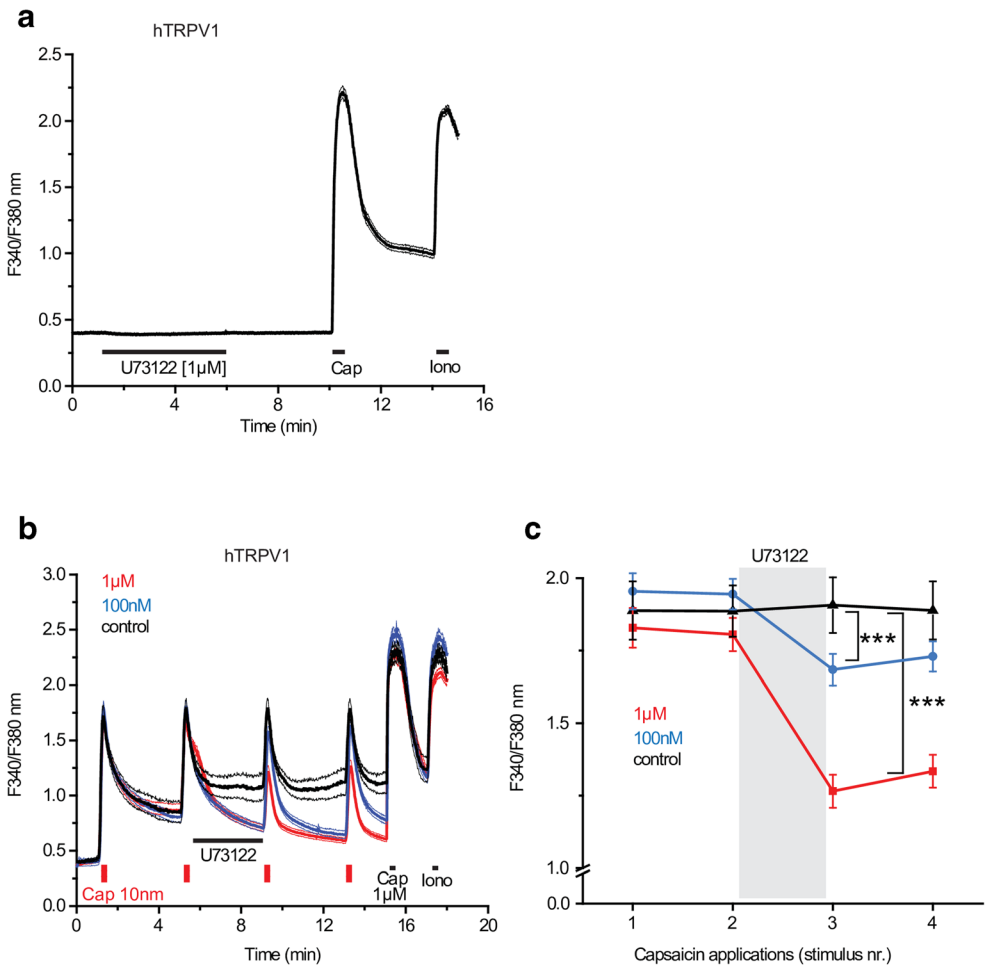

Fig. 4 U73122 desensitizes the human capsaicin receptor, hTRPV1. **a** HEK293t-hTRPV1 cells were not activated when challenged with U73122 (1 μM, 5 min, $n = 130$). The cells were also stimulated with capsaicin (300 nM, 20 s), to confirm functional expression of hTRPV1. **b** HEK293t-hTRPV1 cells were stimulated repeatedly with 4 brief pulses of capsaicin (10 nM, 10 s) at 4 min interval (red bars). Between capsaicin pulses 2 and 3, the cells were exposed for 3 min and 50 s to U73122 at 100 nM (blue trace, $n = 77$) and 1 μM (red trace, $n = 60$) or to extracellular solution as control (black trace, $n = 24$). Capsaicin (1 μM, 20 s) was applied to confirm functional expression of hTRPV1. In both **a** and **b**, ionomycin (2 μM, 20 s) was applied at the end of the experiment as a positive control. The data are represented as mean (thick lines) ± SEM (thin lines). **c** Statistical analysis of the results shown in **b**. Application of U73122 between capsaicin pulses 2 and 3 leads to a concentration-dependent reduction of the capsaicin-evoked calcium transients. The amplitudes of the responses to the 3rd pulse of capsaicin following U73122 application were compared to control values using Student's unpaired *t* test (two-tailed) (***, $p < 0.001$)

U73122 activates mouse dorsal root ganglion (DRG) neurons in a TRPA1-dependent manner

We performed ratiometric Fura-2 calcium imaging on cultured DRG neurons from wild-type C57Bl/6 (WT), TRPV1$^{-/-}$, and TRPA1$^{-/-}$ mice. The cells were challenged with U73122 (1 μM, 3 min), carvacrol (50 μM, 20 s), capsaicin (300 nM, 20 s), and KCl (50 mM, 20 s). About a third (32%, 40 of 126) of all WT DRG neurons responded to U73122 with an increase in $[Ca^{2+}]_i$ (Fig. 6a, b). A large fraction of the U73122-sensitive neurons was also subsequently activated by carvacrol (75%, 30 of 40) and capsaicin (77%, 31 of 40), while about half of U73122-sensitive neurons were activated by both capsaicin and carvacrol (55%, 22 of 40).

DRG neurons from TRPV1$^{-/-}$ and WT mice displayed indistinguishable responses to U73122 (Fig. 6a). Thus, a similar proportion of the total number of neurons (38%, 65 of 173, compared to 32%, 40 of 126 in WT DRG, not significantly different, Chi-square test) was activated by U73122 (Fig. 6b), and the overlap with carvacrol sensitivity was also in the same range as in WT mice (80%, 52 of 64). This indicates that TRPV1 is not involved in the activation of DRG neurons by U73122.

In contrast, genetic ablation of TRPA1 virtually abolished the activation of DRG neurons by U73122, while the fraction of carvacrol-sensitive cells, albeit significantly diminished, remained quite high (24%, 46 of

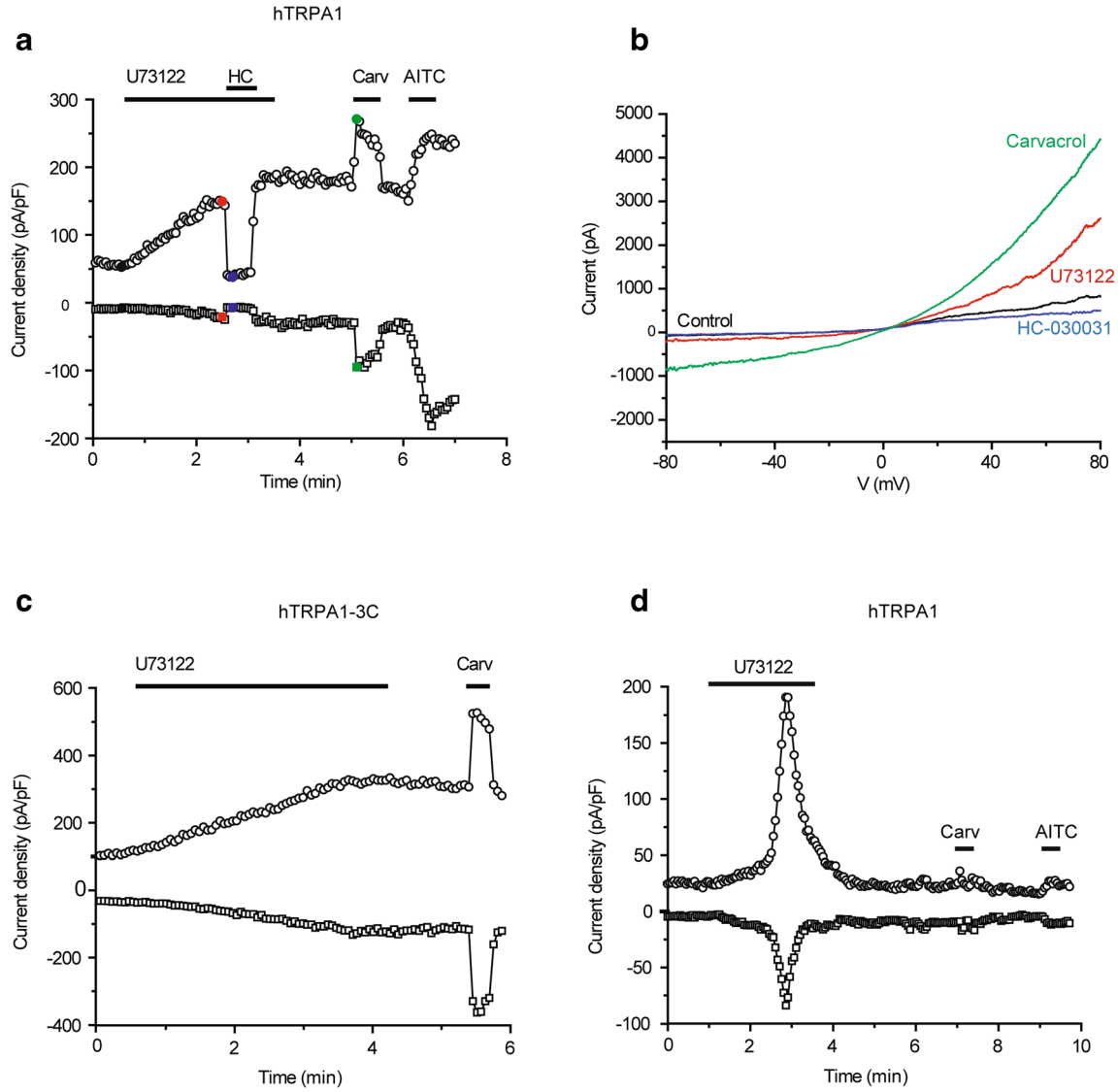

Fig. 5 U73122 evokes outwardly-rectifying membrane currents in HEK293t cells expressing recombinant hTRPA1 or hTRPA1-3C. **a** Membrane currents were recorded in the whole-cell voltage clamp mode in HEK293t-hTRPA1 cells in calcium-free conditions. The cells were challenged with voltage ramps from − 100 to + 100 mV (400 ms) at 4 s interval, from a holding potential of − 60 mV. Illustrative example of membrane currents recorded at + 80 mV (open circles) and − 80 mV (open squares) in a HEK293t-hTRPA1 cell stimulated with U73122 (1 μM), carvacrol (50 μM), and AITC (50 μM). The duration of application for each compound is represented by black bars in the figure. The selective TRPA1 antagonist HC-030031 (10 μM) completely abolished both the outward and the inward currents evoked by U73122. **b** Examples of individual ramp currents corresponding to the filled symbols with the same color in **a**. Note the pronounced outward rectification of the U73122-induced current, and its reversal potential close to 0 mV. Also note the complete block of the U73122-induced current by HC-030031. **c** Example of a U73122 (1 μM)-induced outwardly-rectifying current in calcium-free conditions in a HEK293t cells expressing the mutant hTRPA1-3C. The cell is also activated by the TRPA1 agonist carvacrol (50 μM). The same voltage protocol as described in **a** was used. **d** Illustrative example of a membrane current induced by U73122 in a HEK293t-hTRPA1 cell, in the presence of extracellular calcium. The cell was challenged with U73122 (1 μM), carvacrol (50 μM), and AITC (50 μM). Note the almost complete desensitization of the response to carvacrol and AITC, most likely due to calcium entry. The same voltage protocol as described in **a** was used

188 in$TRPA1^{-/-}$ DRGs vs. 40%, 50 of 126 in WT DRGs, $p < 0.01$, Chi-square test) (Fig. 6a, b). This indicates that while carvacrol (at 50 μM) activates other targets in addition to TRPA1 in mouse DRG neurons, this channel is solely responsible for the effect of U73122, at least at the concentration tested in our study (1 μM).

U73122 desensitizes TRPA1 expressing mouse DRG neurons to carvacrol

It has recently been shown that TRPA1 activation followed by profound desensitization may produce long-lasting hypoalgesia in mice (Kistner et al. 2016). In order to

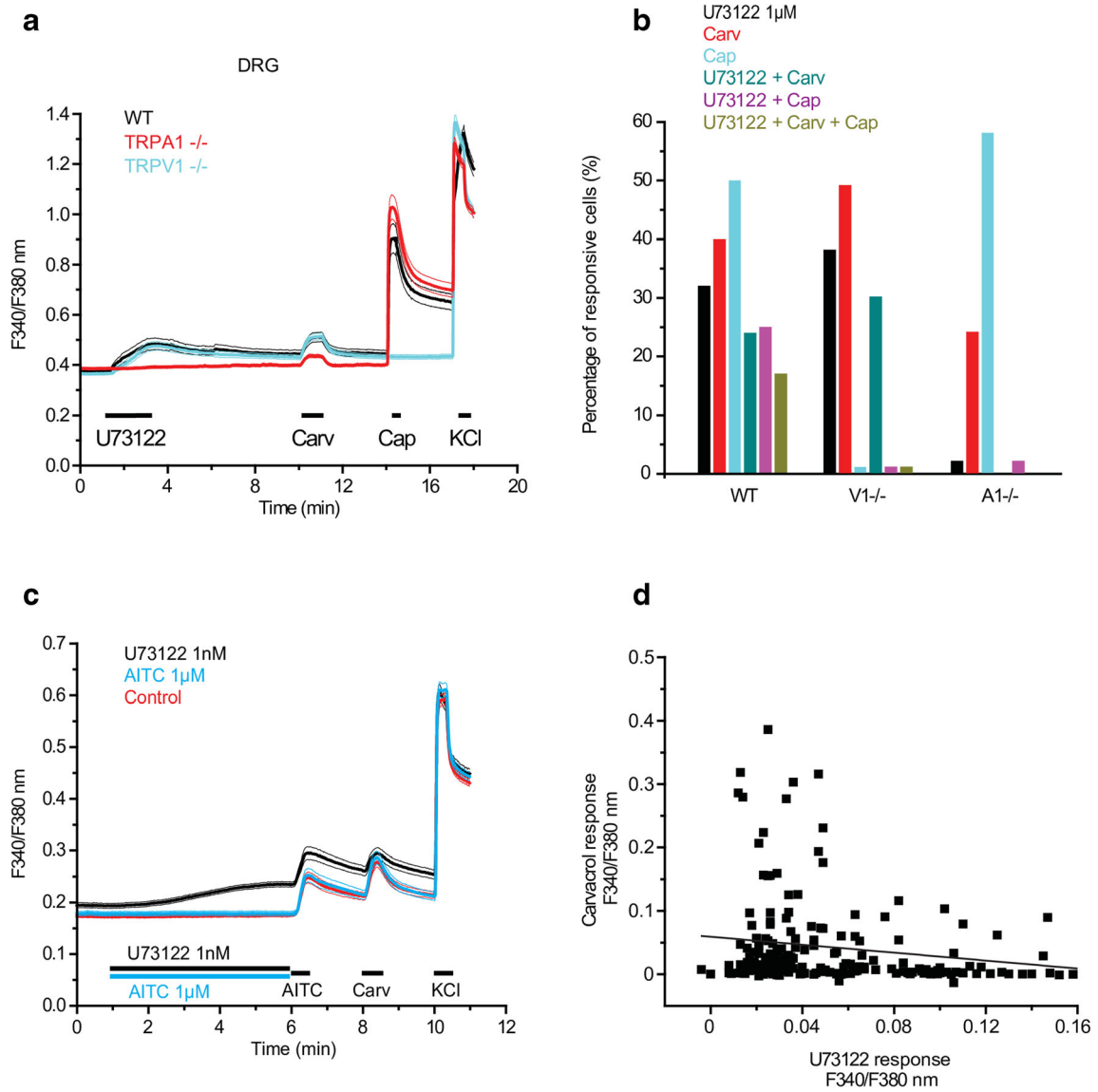

Fig. 6 U73122 induces calcium transients in a subpopulation of cultured mouse dorsal root ganglion (DRG) neurons. **a** Cultured DRG neurons from wild-type C57BL/6 mice (black traces), TRPA1$^{-/-}$ mice (red trace) and TRPV1$^{-/-}$ mice (light blue trace) were challenged with U73122 (1 μM, 180 s), followed by carvacrol (Carv 50 μM, 20 s), capsaicin (Cap 300 nM, 20 s), and KCl (50 mM, 20 s). The continuous traces represent the averaged response of all neurons for each genotype: C57/Bl6 ($n = 126$), TRPA$^{1-/-}$ ($n = 188$) and TRPV1$^{-/-}$ ($n = 173$). The data are represented as mean (thick lines) ± SEM (thin lines). **b** The percentages of responding neurons for each agonist or combination of agonists identified in the experiment shown in part **a** are represented with columns of matching color. **c** Prolonged exposure to a low concentration of U73122 induced marked desensitization to the non-electrophyilic TRPA1 agonist carvacrol. Cultured neurons from wild-type C57BL/6 mice were stimulated for 5 min with U73122 (1 nM; black traces, $n = 191$), AITC (1 μM; blue traces, $n = 268$), or standard extracellular solution (control; red traces, $n = 168$). This stimulus was followed by the application of AITC (100 μM, 20 s), carvacrol (Carv, 100 μM, 20 s), and KCl (50 mM, 20 s). The data are represented as mean (thick lines) ± SEM (thin lines). **d** The amplitudes of the responses to carvacrol are represented against the amplitudes of the responses to U73122, for the neurons stimulated with U73122 in the experiment shown in part **c**. A linear fit for the data is also shown (Pearson's correlation coefficient $r = -0.16$, $p = 0.02$)

determine the desensitization potential of U73122, we exposed cultured mouse DRG neurons to a low concentration of U73122 (1 nM) or AITC (1 μM) for 5 min and then stimulated the cells with AITC (100 μM) and carvacrol (100 μM) (Fig. 6c). In a separate control experiment, DRG neurons were just superfused with extracellular solution (ES), followed by a similar stimulation protocol with AITC and carvacrol. During exposure to U73122, 62% of all neurons responded with a slow increase in $[Ca^{2+}]_i$, while exposure to AITC or ES only led to increases in $[Ca^{2+}]_i$ in 6 and, respectively, 8% of neurons, likely corresponding to spontaneous neuronal activity. Following this 5-min pretreatment with U73122, AITC, or ES, stimulation with AITC (100 μM) led to very similar responses in all three conditions, activating approx. 30% of all neurons. However, responses to carvacrol were strongly desensitized following pretreatment

with U73122, resulting in only 33% carvacrol-responsive neurons compared to DRG neurons pretreated with AITC or ES (53% and 56% carvacrol-sensitive neurons, respectively). Moreover, there was an inverse correlation between $[Ca^{2+}]_i$ increases during U73122 pretreatment and the subsequent responses to carvacrol (100 μM) (Fig. 6d). This points to a U73122-induced desensitization of carvacrol responses, most likely due to calcium entry (Hinman et al. 2006; Ibarra and Blair 2013; Wang et al. 2008b).

U73122 induces TRPA1-dependent CGRP release from mouse hindpaw skin

In isolated skin from control wild-type C57BL/6 mice U73122 caused a concentration dependent and reversible release of CGRP into the eluate. The application of U73122 at 30 μM induced significant CGRP release (ANOVA repeated measures, $p < 0.05$, LSD post hoc test $p = 0.01$, $n = 4$ Fig. 7a); U73122 at 10 μM ($n = 4$) induced only a minor and not significant increase of CGRP release, that was significantly smaller than the response to 30 μM U73122 (one-way ANOVA, $p < 0.01$, Fig. 7b). The response to U73122 at 30 μM was largely dependent on the expression of TRPA1 receptors. In TRPA1-deficient mice, the amount of CGRP released by U73122 at 30 μM was significantly lower compared to control wild-type mice (one-way ANOVA $p = 0.01$, $n = 4$). Nevertheless, 30 μM U73122 still caused a small but significant increase of CGRP compared to baseline levels in TRPA1-deficient mice (ANOVA repeated measures $p < 0.01$, LSD post hoc test $p < 0.01$), suggesting that this high concentration activates other receptors contributing to neuropeptide release.

Discussion

Our results demonstrate that the PLC inhibitor U73122 (Bleasdale et al. 1990; Smith et al. 1990) is a potent TRPA1 agonist with a threshold concentration in the picomolar range. This compound evoked calcium transients in HEK293t cells transiently transfected with hTRPA1 but not in un-transfected HEK293t cells or TRPV1-expressing HEK293t cells, and these responses were inhibited by the selective TRPA1 antagonist HC-030031. U73122-induced calcium transients were completely abolished upon removal of extracellular calcium, indicating that the source of these signals is calcium entry through TRPA1 channels, rather than calcium release from intracellular stores. Moreover, U73122 induced whole-cell currents in hTRPA1-expressing HEK293 cells which were completely blocked by HC-030031. Interestingly, the U73122-evoked currents through TRPA1 displayed strong desensitization in the presence of extracellular calcium, while in calcium-free conditions, the currents were activated in a sustained manner, and did not return to baseline following washout of the agonist. This is suggestive of slowly reversible covalent interaction between U73122 and TRPA1 and resembles activation of TRPA1 by electrophilic agents and UVA light (Babes et al. 2016; Hinman et al. 2006).

Interestingly, the triple cysteine TRPA1 mutant (hTRPA1-3C), in which the intracellular N-terminal cysteines in positions 621, 641, and 665 are substituted by serines, was also activated by U73122, albeit with lower potency, as demonstrated by our calcium imaging results. This is similar to the activation of TRPA1 by the endogenous metabolite methylglyoxal (Eberhardt et al. 2012). Replacement of these three critical cysteines does not render the channel insensitive to electrophilic compounds, but produces a rightward shift in the concentration dependence of activation without a loss of maximal efficiency at saturating concentrations, 1 μM in case of U73122.

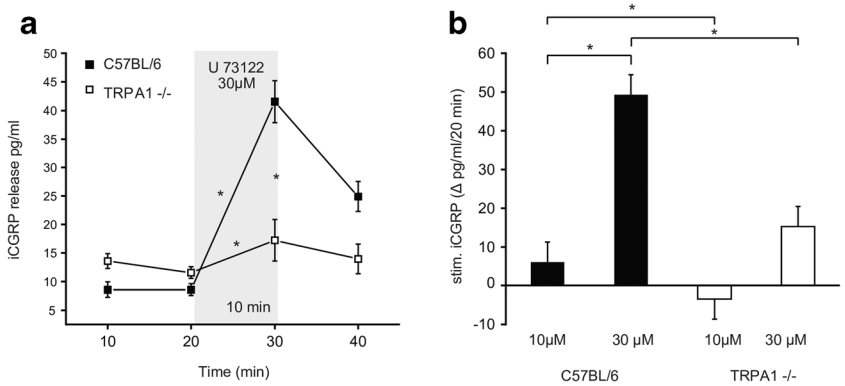

Fig. 7 U73122 induces neuropeptide release from the skin of wild type C57BL/6 but not TRPA1$^{-/-}$ mice. **a** Time course of CGRP release from the hindpaw skin of C57BL/6 mice (filled squares, $n = 4$) and TRPA1$^{-/-}$ mice (open squares, $n = 4$). The gray rectangle indicates the duration of application of U73122 (30 μM, 10 min). The data are displayed showing the time course of stimulated iCGRP release as mean ± SEM. **b** Concentration dependence of U73122-stimulated CGRP release in skin from wild-type C57BL/6 mice (filled columns) and TRPA1$^{-/-}$ mice (open columns). Error bars represent the SEM. The concentration of U73122 used is indicated below each column. Statistical significance was determined using one-way ANOVA (*$p < 0.01$)

To further explore whether the electrophilic properties of U73122 are required for hTRPA1 and hTRPA1-3C activation, we used U73343, a non-electrophilic analog of U73122, which is inactive against PLC (Bleasdale et al. 1990; Klose et al. 2008). The electrophilic character is lost in U73343 through the replacement of a pyrroledione with a pyrrolidinedione. The very minor activation of TRPA1 and TRPA1-3C by U73343 indicates that the electrophilic nature of U73122 plays a significant role in its agonistic action on the TRPA1 channel.

U73122 and NMM are both N-substituted maleimides. NMM is an electrophilic TRPA1 agonist, and eliminating the same three cysteine residues (621, 641, and 665) renders the resulting mutant (hTRPA1-3C) insensitive to concentrations up to 50 μM NMM in patch-clamp experiments (Hinman et al. 2006). In our calcium imaging experiments, 100 pM U73122 was sufficient to activate a small fraction of hTRPA1-expressing cells and also activated, albeit to a lesser extent, hTRPA1-3C expressing cells. Increasing the concentration of U73122 recruited larger fractions of hTRPA1 and hTRPA1-3C expressing cells in a concentration-dependent manner (Fig. 2a–c). Moreover, U73122 elicited inward currents in both hTRPA1 and hTRPA1-3C expressing cells, both in the presence and in the absence of extracellular calcium (Fig. 5). Considering the chemical structure and electrophilic nature of U73122, the sensitivity of the hTRPA1-3C mutant to this compound was unexpected. One can only speculate that other cysteine residues than C621, C641, and C665 are involved in the process, or a completely new mechanism becomes prominent.

The very high potency of the water insoluble U73122, in contrast to the slightly water soluble NMM, may result from its high lipophilicity that is associated with its amonisteroid moiety, conveying membrane permeability or even membrane anchorage. The flexible hexyl chain linking the steroid with the terminal maleimide group may allow the electrophilic carbons of this reactive group to approach any one of the eight cytosolic cysteine thiols of TRPA1 and to form a covalent bond (Hinman et al. 2006). The responsiveness of TRPA1 to concentrations of U73122 in the low nanomolar range comes close to the most potent morpanthridine analogs, derivatives of the tear gas CR (dibenzoxazepine), that have been synthesized as TRPA1 activators (Gijsen et al. 2010).

Acute application of U73122 evokes calcium transients in a subpopulation of wild-type (WT) mouse DRG neurons in primary culture. Of these U73122-sensitive neurons, 75% were subsequently also weakly activated by the TRPA1 agonist carvacrol, while the TRPV1-selective agonist capsaicin activated a similar fraction (Fig. 6). This may be explained by strong cross-desensitization of TRPA1 between U73122 and carvacrol, similar to what we describe using patch clamp in hTRPA1-expressing HEK293t cells between U73122 and carvacrol or AITC in the presence of external calcium (Fig. 6a). However, strong confirmation of the involvement of TRPA1 in the U73122-induced activation of DRG neurons is provided by our experiments with cultured DRG neurons from TRPA1$^{-/-}$ mice, in which U73122 failed to produce a significant level of activity. In contrast, U73122 evoked calcium transients in a large subpopulation of DRG neurons from TRPV1$^{-/-}$ mice, ruling out a significant role of TRPV1 in mediating the effects of U73122. Interestingly, carvacrol evoked calcium transients in a significant proportion of DRG neurons from TRPA1-null mice (approx. 24%, compared to 40% in WT mice), most likely due to an off-target effect. Carvacrol is known to activate TRPV3 (Earley et al. 2010); however, this channel is not expressed in mouse DRG neurons. Carvacrol was also reported to trigger calcium release from the ER in human glioblastoma cells in a PLC-dependent manner, as well as to generate ROS (Liang and Lu 2012). Both effects may contribute to the residual carvacrol sensitivity in spite of the genetic ablation of TRPA1.

Recent work from our lab has shown that long lasting agonist-induced desensitization of TRPA1 may lead to prolonged anti-nociception in mice (Kistner et al. 2016). U73122 at very low concentration (1 nM) was able to evoke stronger desensitization of carvacrol-induced calcium transients compared to a thousand-fold higher concentration of AITC (1 μM). This effect most likely involves a calcium-dependent process, as there was an inverse correlation between the amplitude of U73122- and carvacrol-induced calcium transients. Interestingly, in contrast to carvacrol, the response to the electrophilic agonist AITC, at a concentration not evoking calcium influx, did not display U73122-induced cross-desensitization; this may suggest that U73122 and AITC interact with different cysteine residues of TRPA1.

Finally, our cutaneous CGRP release experiments provide confirmation of the activation of TRPA1 by U73122 in a more physiological preparation containing intact nociceptive nerve endings in the skin. U73122 evoked strong CGRP release in the hindpaw skin of wild-type mice, while it was substantially less effective when applied to skin flaps from TRPA1$^{-/-}$ animals (Fig. 7).

Taken together, our data demonstrate that the PLC inhibitor U73122 also acts as a potent agonist of human and mouse TRPA1, both in the native system (DRG neurons) and in a heterologous expression system (HEK293t cells transiently transfected with recombinant TRPA1), while in ex vivo skin flaps, U73122 induces TRPA1-mediated neuropeptide release. In this context, we believe that careful consideration is required when interpreting putative effects of U73122 (perhaps the most frequently used PLC inhibitor), particularly on sensory neurons or other cell types which express the polymodal receptor-channel TRPA1. In addition, the molecular structure of U73122 may provide some insights into structure-activity relationship of TRPA1 ligands that convey high affinity, covalent binding, and strong desensitization, perhaps not only of TRPA1 but of the nociceptive nerve ending as a whole.

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Authors' contribution C.N. and S.K.S conducted experiments; C.N., S.K.S., and A.B. analyzed data; A.B., C.N., S.K.S., and P.W.R. wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Breeding and euthanasia and all procedures of animal handling were prospectively approved by the Animal Welfare Authority of the District Government of Unterfranken in Würzburg (Germany) and the Institutional Animal Care Department (University of Erlangen, Germany) in accordance with the German regulations of animal care and welfare (Tierschutzgesetz). Experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

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