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Acetylsalicylic acid enhances tachyphylaxis of repetitive capsaicin responses in TRPV1-GFP expressing HEK293 cells

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HIGHLIGHTS

- Acetylsalicylic acid (ASA) inhibits cyclooxygenase (COX) by irreversible acetylation.
- The capsaicin receptor TRPV1 was suggested as a putative additional target of ASA.
- ASA (1 μM) enhanced tachyphylaxis of TRPV1 during repeated capsaicin stimulation.
- Our data suggest inhibition of the cloned TRPV1 by low ASA doses independent of COX.

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ABSTRACT

Since many years acetylsalicylic acid (ASA) is known for its antithrombotic, antiphlogistic and analgesic effects caused by irreversible acetylation of cyclooxygenase. ASA also inhibits capsaicin- and heat-induced responses in cultured dorsal root ganglia (DRG) neurons, suggesting TRPV1 (transient receptor potential channel of the vanilloid receptor family, subtype 1) to be an additional target of ASA. We now studied the effect of ASA on heterologously expressed rat TRPV1 using calcium microfluorimetry. Capsaicin dose-dependently increased intracellular calcium with an EC_{50} of 0.29 μM in rTRPV1 expressing HEK293 cells. During repetitive stimulation the second response to capsaicin was reduced (53.4 ± 8.3% compared to vehicle control; $p < 0.005$; Student's unpaired *t*-test) by 1 μM ASA, a concentration much below the one needed to inhibit cyclooxygenase (IC_{50} of 35 μM in thromboxane B2 production assay). In contrast, calcium transients induced by a single stimulus of 0.3 or 1 μM capsaicin were not significantly reduced by 0.3 or 1 μM ASA. These data suggest that ASA increases the tachyphylaxis of rTRPV1 channel activation. Mechanisms are unknown and may be direct by e.g. stabilization of the desensitized state or indirect via inhibition of intracellular signaling pathways e.g. of the mitogen-activated protein kinase family (MAPK/ERK).

1. Introduction

The analgesic potential of acetylsalicylic acid (ASA)-like drugs has already been known hundreds of years ago [1] and nowadays ASA has become one of the most popular drugs. Irreversible acetylation of the cyclooxygenase (COX) has already been identified as the mechanism of action of ASA in 1971 [2]. Unfortunately, ASA shows a higher affinity for the constitutively expressed COX-1 than for inducible COX-2 isoforms – causing a wide range of side effects [3]. However, COX-1 and COX-2 deficient mice still show sensitivity to the analgesic action of NSAIDs, suggesting an additional target for pain modulation apart from COX [4].

The involvement of ASA in a COX-independent, peripheral mechanism of pain modulation was first observed in rat DRGs [5,6].

Abbreviations: $[Ca^{2+}]_i$, free intracellular calcium; ASA, acetylsalicylic acid; COX, cyclooxygenase; CPZ, capsazepine; DAG, 1,2-diacylglycerol; DRG, dorsal root ganglia; EC_{50}/IC_{50}, half maximal excitatory/inhibitory concentration; GFP, green fluorescent protein; HEK293 cells, human embryonic kidney (293) cells; IP3, inositol-1,4,5-trisphosphat; MAPK/ERK, mitogen-activated protein kinase/extracellular-signal regulated kinase; NSAIDS, non-steroidal anti-inflammatory drugs; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; (r)TRPV1, (rat) transient receptor potential channel of the vanilloid receptor family, subtype 1.

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Capsaicin leads to activation of cells expressing TRPV1 [7]. ASA reversibly inhibits capsaicin-induced currents in isolated DRG neurons [5] and inhibits the activation of small DRG neurons when activated by noxious heat [6], suggesting TRPV1 as a new target for ASA-like drugs. Pharmacological actions of ASA at TRPV1 may include direct interaction with the receptor and/or indirect modulation via intracellular targets [8]. We now investigated the direct interaction of ASA and the TRPV1 receptor in a rTRPV1 expression system and compared the results to ASA-induced COX-1 inhibition in platelets.

2. Materials and methods

2.1. Cloning and cell culture

Rat TRPV1was amplified with forward (5′-GAA TTC GAA AGG ATG GAA CAA CGG-3′) and reverse primer (5′-GGT ACC TTC TCC CCT GGG ACC AT-3′) using pcDNA3-TRPV1 as template and High Fidelity Expand PCR system (Roche Diagnostics, Mannheim, Germany). Artificial restriction enzyme sites for cloning in the pTagGFP2-N vector (Evrogen, Moscow, Russia) were inserted and GFP expressed at the C-terminus of TRPV1.

Human embryonic kidney (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (PAA, Pasching; Austria) supplemented with 10% fetal calf serum (FCS Gold, PAA), 100 U/l penicillin (PAA) and 100 μg/ml streptomycin (PAA) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Cells (with a density of 10–40,000/well) were plated on poly-L-lysine-covered (10 μg/ml, Sigma–Aldrich, Steinheim; Germany) coverslips (Ø15 mm), transfected using 6.4 μl nanofectamine (PAA) and 2 μg pTagGFP2-N-rTRPV1 per coverslip and used for functional calcium imaging within 24–48 h after transfection.

2.2. Calcium Imaging

For measurements of $[Ca^{2+}]_i$ transiently transfected HEK293 cells were loaded with 1 μM FURA-2AM (Calbiochem; Darmstadt; Germany) and same amount (in μl) of Pluronic F-127 (Calbiochem) for 45–60 min in Tyrode's solution (137.6 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES; 5 mM glucose; adjusted to pH 7.3 with NaOH). After washing with Tyrode's solution for 30–60 min, cells were mounted on the stage of an inverted microscope (Olympus IX81 equipped with cellR Imagingsystem; Olympus, Tokyo, Japan) in an open bath chamber (Series 40 Chamber; Warner Instruments, Hamden, USA) and superfused by Tyrode's solution (1–3 ml/min) at room temperature (22–24 °C). Cells were illuminated every 2 s (340/380 nm wavelength) and respective fluorescent signals (510 nm) were detected by an ORCA-R2 camera (Hamamatsu Photonics, Hamamatsu, Japan). The ratio of emission for 340/380 nm excitation was used as relative change in $[Ca^{2+}]_i$.

2.3. Thromboxane B2 quantification assay

Whole blood samples of 4 healthy donors were taken in hirudin-supplemented blood tubes (Sarstedt, Nümbrecht, Germany) and pre-incubated with 0.1–100 μM ASA for 30 min. Thromboxane generation in platelets was induced using ASPI-Test 0.5 mM (Multiplate®analysis; Cobas/Roche, Mannheim, Germany) and subsequent aggregation measured by impedance aggregometry (Multiplate®Analyzer, Verum Diagnostica GmbH, Munich, Germany). Thromboxane quantification measurements were performed using the supernatant of aggregated whole blood samples in a multiplate reader (VictorTM X4, Perkin Elmer, Rodgau, Germany). Procedures were done as described in the assay protocol (Thromboxan B2 EIA Kit, Cayman Chemicals, Ann Arbor, USA) and results calculated using tables provided by the manufacturer.

2.4. Chemicals

Capsaicin and capsazepine (Roth, Karlsruhe, Germany; Sigma) were dissolved in ethanol and DMSO at a concentration of 100 mM and 10 mM respectively as stock solutions and stored at 4 °C. ASA (Sigma) was dissolved in Tyrode's solution (10 mM). Dilutions were prepared from stock solutions shortly before each experiment. Drugs did not change the pH of the solutions by more than 0.1; maximum ethanol concentration was 0.1‰.

2.5. Data analysis

After background subtraction and calculation of 340/380 nm ratio 50–150 regions of interest (ROIs, capsaicin-sensitive cells) were defined. Mean value of 50–150 cells/experiment was defined as one experiment ($n = 1$). Results are demonstrated as mean ± standard error of the mean (SEM) or as standard deviation (SD). For comparison between experiments responses were normalized to the first capsaicin response to account for differences in expression rates. Results were tested for significance by paired/unpaired Student's *t*-test, one-way or mixed-model two-way repeated measures ANOVA followed by Least Significant Difference test (LSD; Statistica 4.5, StatSoft Inc., Tulsa, OK, USA). *p*-Values ≤ 0.05 were considered significant ($^* = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$). 50% excitatory/inhibitory concentrations (EC_{50}, IC_{50}) were calculated using ORIGIN PRO Software 5.0 (OriginLab Corp., USA).

3. Results

3.1. Capsaicin induced $[Ca^{2+}]_i$-responses display tachyphylaxis in a rTRPV1-GFP expression system

Application of 0.3 μM capsaicin for 30 s to rTRPV1-GFP expressing HEK293 cells induced a transient rise of $[Ca^{2+}]_i$ significantly exceeding the effect of vehicle ($p < 0.001$, Student's unpaired *t*-test; 826 cells from nine independent experiments; Fig. 1). With a dose-dependent activation (one-way ANOVA $F_{(5,54)} = 93.5$, $p < 0.001$) and EC_{50} of about 0.29 μM (log_{10}-concentration: -6.54 ± 0.13; mean ± SD, Fig. 1b; $n = 2$–9 experiments including 2372 separate cells) our newly generated rTRPV1expression system displays typical response patterns to capsaicin already described by others [7,9], allowing reliable pharmacological investigation of TRPV1. No significant responses were seen in non-transfected HEK293 cells (0.3 μM induced an increase in ratio by 0.02 ± 0.03; $n = 3$ experiments), in vector-transfected cells (GFP-alone; 0.13 ± 0.06; $n = 4$ experiments), and in TRPV1-GFP-transfected cells challenged with vehicle solution (0.01 ± 0.02; $n = 9$ experiments; Fig. 1c, d). All effects in those experiments did not differ from each other (all $p > 0.07$, LSD post hoc test) and were significantly lower than effects of 0.3 μM capsaicin on TRPV1-transfected cells (0.69 ± 0.17; $n = 9$; one-way ANOVA $F_{(3,21)} = 70.0$, $p < 0.001$; $p < 0.001$ versus all remaining groups, LSD post hoc test).

When 0.3 μM capsaicin was applied repetitively to rTRPV1 cells with an interstimulus interval of 5 min, the second response was reduced to $53.4 \pm 6.2\%$, the third to $50.8 \pm 8.2\%$ of the first, i.e., capsaicin responses displayed marked tachyphylaxis (one-way repeated measures ANOVA $F_{(3,24)} = 101.3$, $p < 0.001$; see Fig. 1c, d, Fig. 2).

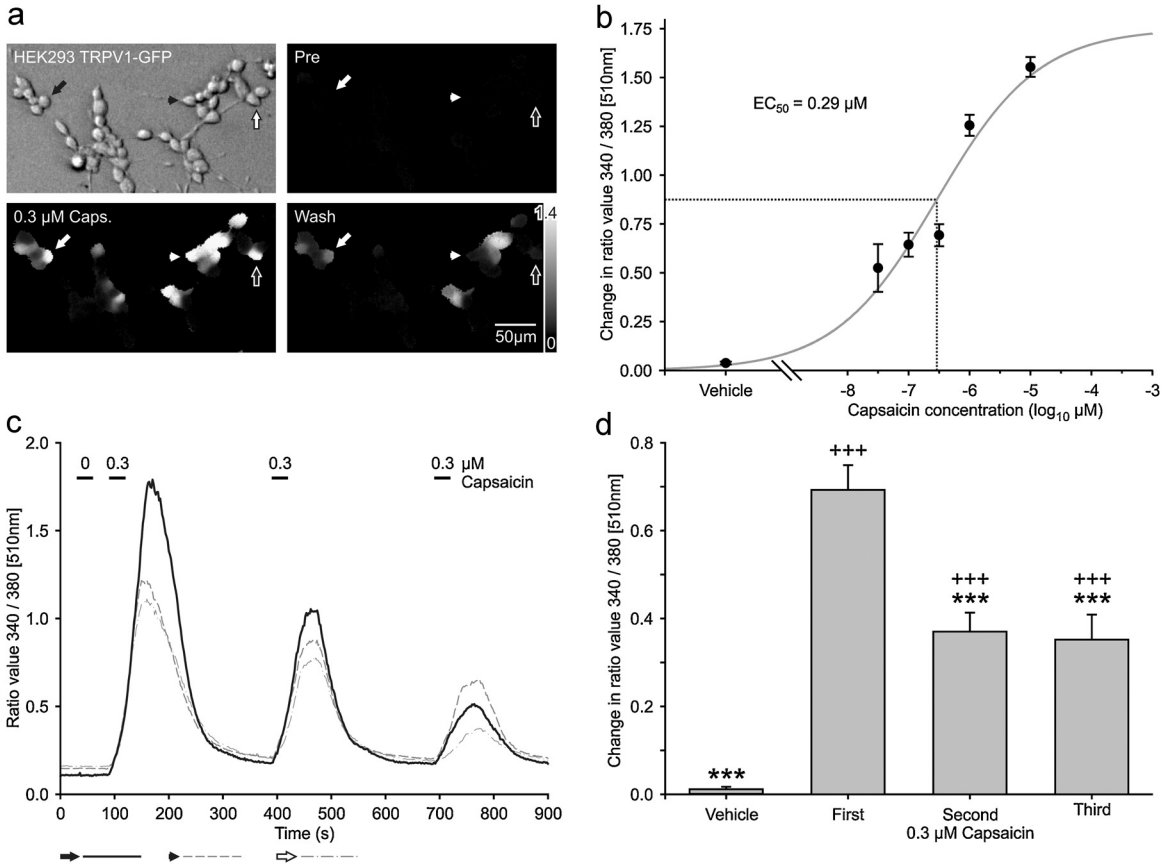

Fig. 1. Capsaicin increases $[Ca^{2+}]_i$ in TRPV1-GFP expressing HEK293 cells and displays marked tachyphylaxis when applied repetitively. (a) Representative brightfield and pseudocoloured images of calcium microfluorimetry during stimulation with 0.3 μM capsaicin. (b) Application of capsaicin evokes a concentration-dependent change in 340/380 nm fluorescence ratio at 510 nm indicating an increase in $[Ca^{2+}]_i$ (EC_{50} = 0.29 μM; n = 2–9 experiments). (c) Change in fluorescence ratio of representative single cells (shown in a, marked by arrows) induced by repetitive stimulation with 0.3 μM capsaicin (dotted, dashed and continuous lines). (d) Mean change in fluorescence ratio in cells challenged with vehicle or repetitive capsaicin (0.3 μM) stimulation displaying marked tachyphylaxis of the TRPV1 receptor (n = 9 experiments). $^{***}p < 0.001$ versus first capsaicin, $^{+++}p < 0.001$ versus vehicle, ANOVA with post hoc LSD.

3.2. Acetylsalicylic acid reduces responses to capsaicin during repeated stimulation of the capsaicin receptor rTRPV1

Application of ASA alone as high as 1 mM did not affect $[Ca^{2+}]_i$ (data not shown). When ASA (1 μM) was applied 30 s before and during the second capsaicin stimulus (0.3 μM) it further reduced capsaicin responses as compared to tachyphylaxis (main effect of treatment: $F_{(1,10)} = 16.5$, $p < 0.005$; main effect of repetition: $F_{(2,20)} = 164.6$, $p < 0.001$; interaction: $F_{(2,20)} = 5.6$, $p < 0.05$; two-way ANOVA; $n = 6$ experiments with ASA and vehicle, respectively, total numbers of 433 and 139 cells). With co-application of ASA the second capsaicin response was reduced to 53.4 ± 8.3% of the vehicle group (Fig. 2; $p < 0.001$ LSD-test). This reduction even persisted after a wash-out period of 5 min, as well as until the fourth response induced by a higher capsaicin stimulus (10 μM).

3.3. Considerably higher concentrations of ASA are needed for inhibition of COX-mediated thromboxane B2 production than for reducing TRPV1 activation

Measurement of thromboxane B2 production was used as an indirect method to determine COX activity in human blood. Incubation of whole blood samples with 0.1–100 μM ASA for 30 min followed by arachidonic acid-induced aggregation caused a significant dose-dependent suppression of thromboxane synthesis (one-way ANOVA $F_{(4,15)} = 23.8$, $p < 0.001$) with an IC_{50} of 35.5 μM ($\log_{10}$-concentration: −4.45 ± 1.25; mean ± SD, Fig. 3), a value that is in accordance with those given in the literature [3]. Thus, also in our hands ASA at a low concentration of 1 μM was fully ineffective in affecting COX activity.

3.4. Acetylsalicylic acid does not affect $[Ca^{2+}]_i$-responses induced by a single capsaicin stimulus

When ASA (0.3 μM) was applied 30 s before and during a single application of capsaicin (0.3 μM, 30 s) a slight but insignificant decrease in $[Ca^{2+}]_i$ was observed as compared to pre-incubation of vehicle (Fig. 4). Combinations of increased capsaicin concentration and/or ASA concentration (1 μM) never affected the capsaicin response significantly (main effect of treatment: $F_{(1,42)} = 0.41$, $p > 0.5$; main effect of concentrations: $F_{(3,42)} = 0.22$, $p > 0.8$; two-way ANOVA; 59–103 cells/experiment in 4–7 experiments). In contrast, the competitive TRPV1 antagonist capsazepine (CPZ; 1 μM) significantly reduced also the initial responses to 0.3 μM capsaicin (Fig. 4b; $p < 0.001$ versus vehicle, Student's unpaired t-test, $n = 4$ experiments with CPZ and vehicle, respectively). Inhibition of repetitive rTRPV1-responses without affection of single responses indicates that the mechanisms of TRPV1 inhibition by ASA differ from those of a direct competitive receptor antagonist.

4. Discussion

This study has shown that ASA – when shortly applied (30 s) at a dose much below (by a factor of 35) effective blocking

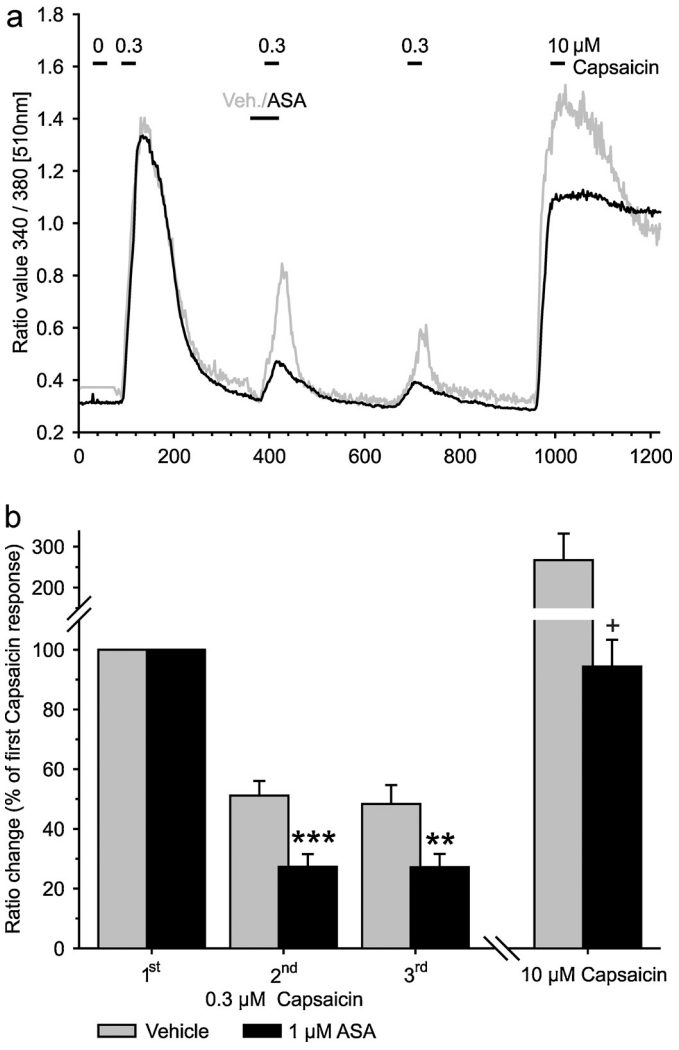

Fig. 2. ASA reduces the response of TRPV1 to repetitive capsaicin stimulation. (a) Kinetics of representative single cells challenged repetitively with capsaicin (0.3 µM) with (black trace) and without (gray trace) 1 µM ASA 30 s before and during second capsaicin application. Values are given as change in 340/380 nm fluorescence ratio. (b) Averaged peak values of capsaicin stimulations (± ASA/vehicle) corresponding to a, normalized to the respective first capsaicin application ($n = 6$ experiments for vehicle, $n = 6$ experiments for ASA), indicating a long lasting inhibitory effect of ASA ($^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle, two-way ANOVA with LSD-test; $^{+}p < 0.05$ versus vehicle, Student's unpaired t-test).

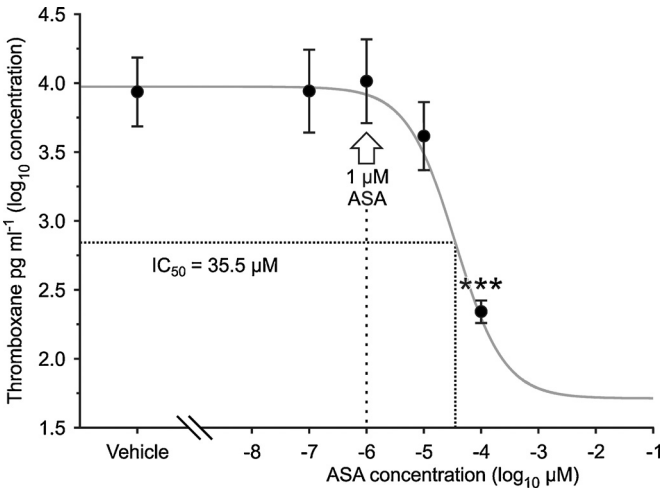

Fig. 3. ASA dose-dependently reduces COX-mediated thromboxane B2 synthesis in human blood. Thromboxane B2 concentration was determined after incubation of human blood with 0.1–100 µM ASA for 30 min and arachidonic acid-induced aggregation. Logarithmized thromboxane B2 values are shown as average of $n = 4$ different samples. ASA inhibits thromboxane B2 synthesis with an IC_{50} of about 35 µM. The concentration effectively antagonizing TRPV1 (indicated as dashed line) was completely ineffective in the COX assay ($^{***}p < 0.001$ versus vehicle, one-way ANOVA with LSD-test).

concentrations of COX 1 – significantly increased the degree of tachyphylaxis during repeated stimulation of cells expressing TRPV1 without affecting its primary activation.

Capsaicin- and heat-evoked currents in native nociceptive DRG neurons were previously reduced by ASA with an IC_{50} of about 1 µM and 0.375 µM, respectively [5,6]. It was suggested that ASA may act on TRPV1, which has now been confirmed using rTRPV1 expressing HEK cells devoid of modulation by prostanoid receptor pathways [10]. In our study, inhibition by ASA was long lasting and even effective for a late stimulus of 10 µM capsaicin (10 min after washout of ASA) at room temperature, whereas blockade of heat response was rapidly reversible in native neurons [6]. Differences between capsaicin and heat might be explained by reduced ligand binding at higher temperatures [11]. The effects of ASA on tachyphylaxis to other adequate stimuli of TRPV1 (such as heat, protons, endovanilloids) still remain to be investigated.

ASA irreversibly blocks the active site of the cyclooxygenase enzyme, causing reduced production of pro-inflammatory mediators [2]. However, several facts suggest that our results are independent of COX pathways: Firstly, inhibition occurred within 30 s of pre-incubation, whereas reduction of prostaglandine synthesis takes several minutes [12]. Additionally, the concentration causing inhibition of TRPV1 responses (1 µM) is far less than required for irreversible COX inhibition reported in the literature ($IC_{50} = 50$ µM; [12]) and verified in our experiments ($IC_{50} = 35.5$ µM). Our observations might be explained by interaction of ASA with other targets within the cell. In recent studies, ASA activates heat shock proteins [13] and prevents NFκB activation [14]. However, effective concentrations of ASA for these actions were in the range of 1–10 mM [13,14], hence all of these pathways are unlikely to contribute to the effects of 1 µM ASA in our study.

We observed that acetylsalicylic acid does not inhibit capsaicin-induced responses in rTRPV1 expressing HEK 293 cells when co-applied with a single capsaicin stimulus, but reduces responses to repetitive capsaicin stimulations, suggesting that ASA might act on the process of desensitization. Repetitive excitations of nociceptive neurons by capsaicin or other stimuli show decreasing response patterns [7] that have been named "acute desensitization" or "tachyphylaxis" [15]. ASA may directly facilitate tachyphylaxis by stabilizing the desensitized state of the rTRPV1 channel. Such a mechanism has been identified for inhibition of voltage gated sodium channels by lacosamide [16] and gating of TRPV1 exhibits similarities to gating of voltage gated channels [17].

Tachyphylaxis of TRPV1 channel responses is decreased by phosphorylation via cAMP-dependent PKA at TRPV1 sites Ser116 and Thr370, calcium/calmodulin dependent kinase II (CaMKII) and PKC [18–20], whereas dephosphorylation of TRPV1 by calcineurin increases capsaicin- and proton-induced tachyphylaxis [9]. The extent of tachyphylaxis is also decreased by phosphatidylinositol-4,5-bisphosphate (PIP_2) at TRPV1 whereas depletion of PIP_2 in the plasma membrane via hydrolysis of PIP_2 to DAG and IP3 caused by the phospholipase C (PLC) increases tachyphylaxis of TRPV1 responses [21]. ASA might therefore also indirectly enhance tachyphylaxis of capsaicin-induced responses via inhibition of PKA or activation of PLC/calcineurin-mediated pathways.

Sensitization is another typical feature of the nociceptive system [8,22] and according to the dual-process theory, desensitization and sensitization in the same system may occur simultaneously

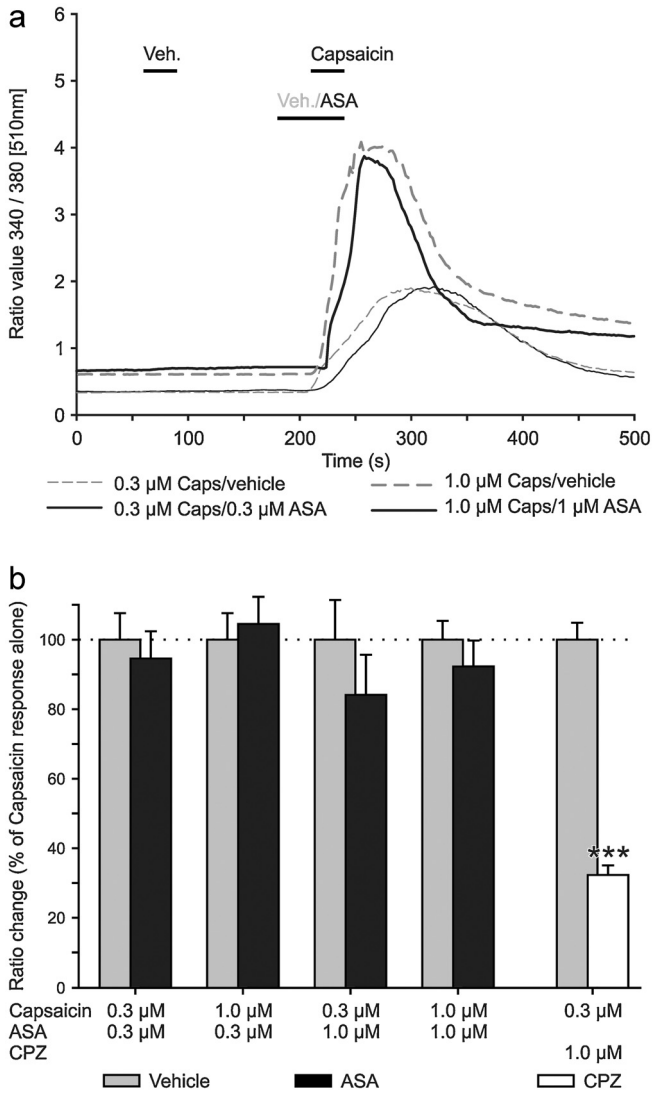

Fig. 4. ASA does not affect TRPV1 responses to single capsaicin stimuli. (a) Kinetics of representative single cells challenged with 0.3 or 1 μM capsaicin with (dark traces) or without (bright traces) application of 0.3 or 1 μM ASA 30 s before and during the capsaicin stimulus. (b) Mean change in fluorescence ratio for different concentrations of capsaicin and ASA, as well as the positive control using the competitive TRPV1 antagonist capsazepine (CPZ; $n = 4$ experiments; $^{***}p < 0.001$ versus vehicle, Student's unpaired t-test). Values are normalized to the mean of capsaicin with vehicle (only) obtained from the same batch of HEK cells (mean ± SEM, $n = 4$–7 experiments).

and provide multiple targets of modulation [23,24]. Thus, an alternative explanation for what looks like an increased tachyphylaxis is a decrease of the parallel sensitization process.

Sensitization of capsaicin-induced TRPV1 transients in primary sensory neurons is caused by calcium-dependent phosphorylation of a subgroup of mitogen-activated protein kinases (MAPK), the extracellular signal-related protein kinase (ERK) via the mitogen-activated protein kinase/extracellular signal-related protein kinase (MEK) [25,26]. In assays investigating neuroprotection and apoptosis ASA as low as 1 nM prevented phosphorylation of MAPK p38, p44/42 MAPK and ERK [27,28]. These observations suggest that ASA at the low dose used in our study (0.3–1 μM) most probably decreases sensitization – and hereby increases tachyphylaxis – of capsaicin-induced TRPV1 responses by interaction with the MAPK-pathway.

In summary, additionally to irreversible blockade of COX, ASA may influence nociception by increasing tachyphylaxis of the repeatedly activated capsaicin receptor TRPV1, possibly directly via stabilization of the desensitized state or indirectly via inhibition of intracellular protein kinase pathways such as MAPK/ERK. The findings of our preliminary study implicate that ASA might be more effective against prolonged pain in clinical use as compared to experimental pain, but further investigation with rat and human TRPV1 will be necessary to prove this hypothesis and to clarify the underlying mechanism of action of ASA.

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