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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: $-6.54 \pm 0.13$; 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 $0.02 \pm 0.03$; $n = 3$ experiments), in vector-transfected cells (GFP-alone; $0.13 \pm 0.06$; $n = 4$ experiments), and in TRPV1-GFP-transfected cells challenged with vehicle solution ($0.01 \pm 0.02$; $n = 9$ 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 ($0.69 \pm 0.17$; $n = 9$; 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).