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Taurolidine and congeners activate hTRPA1 but not hTRPV1 channels and stimulate CGRP release from mouse tracheal sensory nerves

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Abstract

Taurolidine has long been in clinical use as an antimicrobial irrigation that does not impede wound healing. It can even be administered intravenously (30 g/day) to treat sepsis or to exert newly recognized antineoplastic actions. Only one irritant effect is reported, that is, to temporarily induce burning pain of unknown origin when applied to body cavities or peripheral veins. The structure of the molecule suggested the chemoreceptor channel TRPA1 as a potential target, which was verified measuring stimulated CGRP release from sensory nerves of the isolated mouse trachea and calcium influx in hTRPA1-transfected HEK293 cells. With both methods, the concentration–response relationship of taurolidine exceeded the threshold value below 500 μmol/L and 100 μmol/L, respectively, and reached saturation at 1 mmol/L. The clinical 2% taurolidine solution did not evoke greater or longer lasting responses. The reversible tracheal response was abolished in TRPA1$^{-/-}$ but retained in TRPV1$^{-/-}$ mice. Consistently, hTRPV1-HEK showed no calcium influx as a response, likewise native HEK293 cells and hTRPA1-HEK deprived of extracellular calcium did not respond to taurolidine 1 mmol/L. The metabolite taurultam and its oxathiazine derivative, expected to cause less burning pain, showed weak tracheal irritancy only at 10 mmol/L, acting also through hTRPA1 but not hTRPV1. In conclusion, taurolidine, its metabolite, and a novel derivative showed no unspecific cellular effects but selectively, concentration-dependently and reversibly activated the irritant receptor TRPA1 in CGRP-expressing, thus nociceptive, neurons. The clinical solution of 2% taurolidine (~70 mmol/L) can, thus, rightly be expected to cause transient burning pain and neurogenic inflammation.

Abbreviations

CGRP, calcitonin gene-related peptide; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid type 1.

Introduction

Taurolidine gained first approval to market in 1981 and proved itself as a broad spectrum antimicrobial irrigation and instillation in the treatment of severe traumatic or surgical infections, abdominal sepsis and pleura empyema. In addition, taurolidine neutralizes bacterial endotoxins and exotoxins as well as it inhibits proinflammatory cytokines. More recently antineoplastic activities of taurolidine were recognized which led to clinical studies in which up to 30 g/day were intravenously infused over days without serious adverse effects (Neary et al. 2010; Möhler et al. 2014). The only concern is that instillation of taurolidine in body cavities causes burning pain and infusion into peripheral veins discomfort and irritation (Gong et al. 2007). This transient side effect awaited clarification.

We applied taurolidine, the active metabolite taurultam and an oxothiazine derivative of it to an isolated mouse trachea preparation, measuring stimulated release of the neuropeptide calcitonin gene-related peptide (CGRP) as a lump index of nociceptive neuron activation. This preparation, taken from null mutant and wild-type mice, has previously proved to be sensitive to reveal various irritant drug effects, effects of nicotine and real cigarette smoke, of lipopolysaccharides, bradykinin, tissue acidosis, and noxious heat (Kichko and Reeh 2009; Kichko et al. 2013, 2015a,b; Meseguer et al. 2014; Held et al. 2015). In addition, to elucidate the mechanism of taurolidine′s irritancy, we exposed cultured human embryonic kidney cells (HEK293t) that were transfected with the transient receptor potential channels A1 (hTRPA1) or V1 (hTRPV1), the mustard oil receptor or the capsaicin receptor, respectively, both known to serve as irritant sensors in nociceptive nerves potentially causing pain (Julius 2013).

Materials and methods

Animals

The husbandry and usage of the animals were carried out in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann 1983). Adult C57BL/6, congenic $TRPV1^{-/-}$ and $TRPA1^{-/-}$ knockout mice were used. Breeding pairs of heterozygous TRPV1 and TRPA1 mutants were obtained from Dr John Davis (Davis et al. 2000) and Dr David Corey (Kwan et al. 2006) and continuously backcrossed to C57BL/6 in our animal facility. The mice were housed in group cages in a temperature-controlled environment on a 12 h light/dark cycle and were supplied with water and food ad libitum. Mice of either sex (body weight 15–25 g) were sacrificed by exposure to a rising CO_2 concentration (approved by the Animal Protection Authority, District Government of Mittelfranken, Ansbach, Germany).

Trachea preparation and incubation

The trachea was excised together with the two main bronchi, and hemisected along the sagittal midline. One half of the bronchotracheal preparation was used as the control and the other half for chemical treatments (Kichko et al. 2013). Samples were placed in carbogen-gassed (95% O_2, 5% CO_2, obtaining pH 7.4) synthetic interstitial fluid (SIF) inside a shaking bath set at 37°C for 30 min. SIF was containing (in mmol/L) 107.8 NaCl, 3.5 KCl, 1.53 $CaCl_2$, 0.69 $MgSO_4$, 26.2 $NaHCO_3$, 1.67 NaH_2PO_4, 9.64 sodium gluconate. After the initial rest period, the trachea preparations were consecutively incubated for 5 minutes in each of four tubes containing 125 μl SIF $\pm$ stimulating chemicals and mounted in a shaking bath at 37°C The first two incubations were performed to determine the basal CGRP release and variations at 37°C. The third tube contained the stimulating chemicals (taurolidine, taurultam or the oxathiazine derivative) diluted in SIF. The fourth and final tube was for washout and to check for reversal of the response after stimulation.

CGRP enzyme immunoassay (EIA)

The CGRP content of the incubation fluid was measured using commercial enzyme immunoassay (EIA) kits with a detection threshold of 5 pg/ml (Bertin Pharma, Montigny-le-Bretonneux, France). For this purpose, 100μl of the sample fluid was stored on ice and immediately after the trachea incubation period mixed with 25 μl of fivefold concentrated commercial CGRP enzyme immunoassay buffer that contained a proprietary cocktail of peptidase inhibitors.

The further CGRP-EIA procedures were run after the end of the experiment; the antibody reactions took place overnight. The EIA plates were measured photometrically using a microplate reader (Dynatech, Channel Islands, UK).

All results are presented as measured by the enzyme immunoassay in pg CGRP /ml SIF. Reducing the interindividual variability and day-to-day baseline variability, the data were baseline-corrected by the second individual baseline value (before stimulation). This value was subtracted from all four data points of a typical experiment so that only the absolute change in CGRP release (Δ picogram per milliliter) is displayed in the figures.

Cell culture and transfection

HEK293 cells were transiently transfected with plasmids containing hTRPA1 and hTRPV1 using JetPEI transfection reagent from Polyplus Transfection (Illkirch, France) according to the manufacturer's protocol. Generously, human TRPV1 was provided by David Julius (San Francisco, CA) and human TRPA1 by Ardem Patapoutian (La Jolla, CA).

Ratiometric $[Ca^{2+}]_i$ Measurements

HumanTRPA1, humanTRPV1-expressing HEK 293 cells and untransfected HEK 293 cells were stained by 3 μmol/L Fura-2 acetoxymethyl ester (Fura-2 AM) and 0.02% pluronic (both from Invitrogen, Darmstadt, Germany) dissolved in TNB100 medium for about 30 minutes. After a 30-minute washout period to allow for Fura-2-AM deesterification, the coverslips were mounted on an

Olympus IX71 inverse microscope with a 10x objective (Kichko et al. 2013).

The cultured cells were constantly superfused with extracellular fluid (in mmol/L: 145 NaCl, 5 KCl, 1.25 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES) using a software controlled 7-channel gravity-driven common-outlet superfusion system (Dittert et al. 2006). Fura-2 was excited at 340 nm and 380 nm with a Polychrome V monochromator (Till Photonics, Graefelfing, Germany). The images were exposed for 200 μs and were acquired at a rate of 1 Hz with a 12-bit CCD camera (Imago Sensicam QE; Till Photonics). Data were recorded and further analyzed using TILLvisION 4.0.1.3 software (Till Photonics). The background was subtracted before the calculation of ratios. All experimental protocols were preprogrammed. During experiments, the cells on the coverslip were exposed to different stimulations for 20 seconds each (capsaicin, ionomycin, or carvacrol at the end of protocols for 30 seconds). Between stimuli, the cells were washed with fresh extracellular buffer for 4 minutes to allow the cells to recover before the next stimulus. Capsaicin, ionomycin, and carvacrol were used for control of successful transfection and as a reference for comparison between the different stimulations. Ratio (F340/F380) increases reflecting calcium concentration were calculated (Poenie and Tsien 1986). An increase in the intracellular calcium concentration of at least 50 nM, corresponding to a ratio increase by 0.06 in our calibration, during the application period was considered as activation. Stimulated responses were quantified as the mean area under the curve (±SEM) of the fluorescence ratio during the application period plus 20 sec; the period 10 seconds before application was used as reference.

Chemicals

Taurolidine [bis(1,1-dioxoperhydro-1,2,4-thiadiazinyl-4)-methane], taurultam [1,2,4-thiadiazinane 1,1-dioxide] and its oxathiazine derivative [tetrahydro-1,4,5-oxathiazine-dioxyde-4,4] were obtained from Geistlich Pharma AG (Wolhusen/ Switzerland); the chemical structures are shown as insets in the figures. Initial stock solutions were made in 100% DMSO (dimethylsulfoxid) and were stored at -24°C. Each solution for stimulation of trachea or cells in concentrations from 10 μmol/L up to 10 mmol/L contained less than 1% DMSO, which DMSO concentration did not have any influence on tracheal CGRP release (data not shown). The final solutions ready to use were freshly diluted in SIF before each experiment.

The solubilizer polyvinylpyrrolidone (Kollidon 17 PF [PVP_{K17}]) obtained from Geistlich Pharma AG (Wolhusen/ Switzerland) was used for preparing solutions of taurolidine 2% and of the oxathiazine derivative 2%.

Statistical analysis

Statistical comparisons were performed using Statistica 7 software (Statsoft, Tulsa, USA). All time series of experimental values were first analyzed for the effect of stimulation (by taurolidine, oxathiazine derivative, taurultam) as compared to baseline, using the nonparametric Wilcoxon matched pairs signed–rank test to ensure that eventual increases in CGRP release were significant, that is, $p<0.05$. Then the baseline-corrected (i.e., Δ pg/ml) CGRP values were entered into a one-way analysis of variance (ANOVA) followed, if F was significant, by Fisher's least significant difference (LSD) test, focusing on the peak values of stimulated CGRP release in order to compare the different chemically stimulated responses in WT, $TRPA1^{-/-}$ and $TRPV1^{-/-}$ knockout mice. P values < 0.05 were considered statistically significant and are presented in the figures. Data points and vertical bars represent means ± SEM of the given number (n) of experiments on different animals or of cells, respectively.

Results

Taurolidine-activated tracheal CGRP release is concentration- and TRPA1 dependent

The basal neurosecretion of the isolated mouse trachea preparation was 15.11 ± 7.4 pg/ml (mean ± SD, $n = 28$) during the first 5 min incubation period and 14.69 ± 6.65 pg/ml during the second, i.e. just before chemical stimulation – similar values as previously reported and clearly above detection limit (5pg/ml) of the EIA (Kichko and Reeh 2009). The tracheal taurolidine concentration response was investigated from 10 μmol/L to 10 mmol/L in wild-type (C57Bl/6) mice. The threshold of the taurolidine effect was between 100 μmol/L and 500 μmol/L and the response at 1 mmol/L was maximal and saturating (Figs.1A, B). The taurolidine 1 mmol/L-evoked tracheal CGRP release was not significantly different in WT and $TRPV1^{-/-}$ mice (59.8 ± 7.2 pg/ml, $n = 6$ and 52.7 ± 10.8 pg/ml, $n = 4$; respectively), excluding the capsaicin receptor as a mediator of the irritant response. In contrast, the TRPA1 knockouts did not show any response at maximal (1 mmol/L) taurolidine concentration.

In clinical use, taurolidine solution is recommended to be warmed up to at least body temperature in order to reduce the pain from, for example, washing a wound. TRPA1, on the other hand, is known to be inhibited and desensitized by warming to 39°C (Wang et al. 2012). Thus, we tried if further warming to just tolerable 44°C would reduce the submaximal effect of taurolidine 500 μmol/L (Fig. 1C). Although the reduction in taurolidine response from 37°C to 44°C was not signifi-

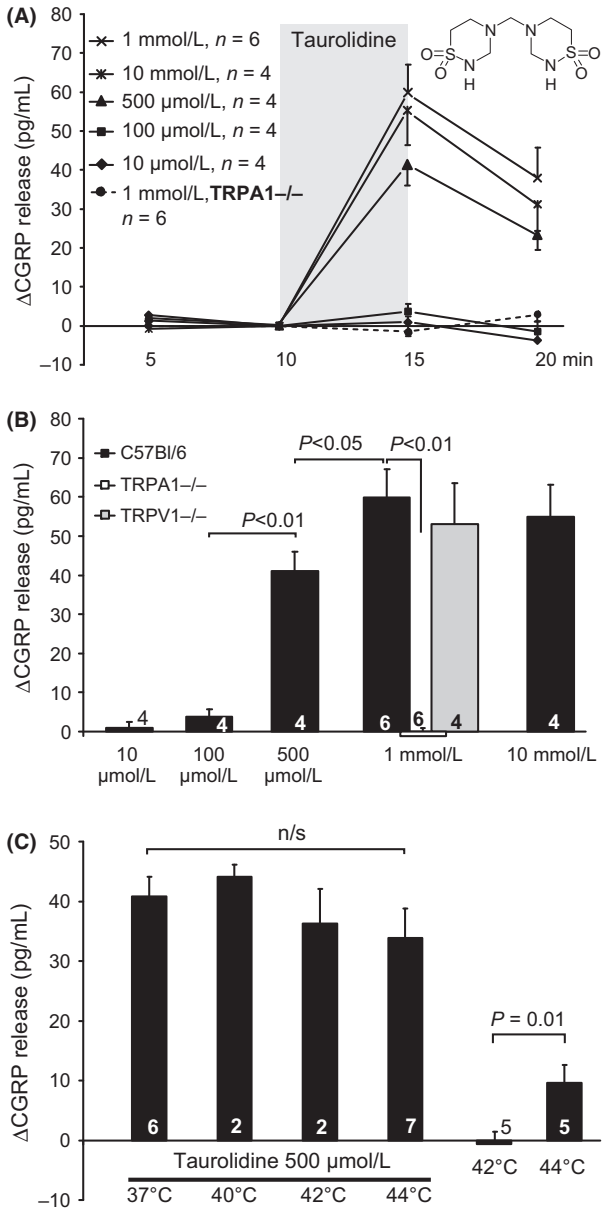

Figure 1. Taurolidine-induced CGRP release from the incubated mouse trachea is concentration and TRPA1 dependent. (A) Time course of taurolidine-induced CGRP release from isolated trachea of wildtype and TRPA1$^{-/-}$ mice. (B) Peak values of stimulated tracheal CGRP release at various taurolidine concentrations; the maximal response is equal in WTs and TRPV1$^{-/-}$ mice but totally abolished in TRPA1$^{-/-}$ mice. (C) Submaximal taurolidine responses are insignificantly reduced at higher than body temperatures; 44°C causes significant CGRP release by itself which would add to the taurolidine effect.

cant, one must consider that the temperature step by itself evoked a small but significant increase in tracheal CGRP release which should have been additive to the remaining taurolidine effect. From this perspective, heating the solution to 44°C could indeed be recommended, at least, to be tried in clinical practice.

Taurolidine activates Ca^{2+} influx in hTRPA1-expressing HEK293 cells concentration dependently and only in the presence of Ca^{2+}

The cellular models of TRP channel function are mostly more sensitive to agonists than isolated tissues.

Because more and more species differences between human and mouse TRP channels have become evident, we investigated HEK 293 cells expressing hTRPA1 and hTRPV1 channels (Fig. 2, A-F). The hTRPA1 cells showed taurolidine-stimulated calcium influx that was concentration dependent with a threshold below 100 μmol/L, which concentration activated 36% of the cells (Figs.2A, B). Almost all cells responded to the saturating concentrations of taurolidine 500 μmol/L (89%) and 1 mmol/L (93%). With TRP channels, an adequate concentration–response relationship cannot be established with sequential agonist applications, due to the marked tachyphylaxis (desensitisation) resulting from foregoing stimulations. This explains the relatively smaller response to 1 mmol/L taurolidine following upon 500 μmol/L (Fig. 2A) as compared to the response when 1 mmol/L taurolidine is the very first chemical stimulus to the cell population (in Fig. 2C). Carvacrol 100 μmol/L at the end of the protocol was the positive control for the hTRPA1-expressing HEK 293 cells. Such expression control responses (also to capsaicin or ionomycin) are not fully depicted in the figures and were not further evaluated. Taurolidine 2% (70 mmol/L) with PVP 5% corresponds to the clinically used solution and did not activate more cells or greater or longer lasting responses than 1 mmol/L (Fig. 2C). Even this high taurolidine concentration did not decelerate the reversal of the calcium transient, indicating that the clearance mechanisms (calcium pumps) were not compromised.

In the absence of extracellular calcium and presence of 10 mmol/L EGTA taurolidine did not activate any intracellular calcium rise, arguing against a release from the endoplasmic reticulum of the cells (Fig. 2D). However, returning calcium to the superfusate evoked a marked off response, indicating an outlasting activation of hTRPA1 and/or possibly of store-operated calcium channels.

Human TRPV1-expressing HEK293 cells and untransfected native HEK293 cells did not respond to taurolidine up to 1 mmol/L, but capsaicin and the calcium ionophore ionomycin as positive controls demonstrate the viability of the cells, respectively (Fig. 2E,F).

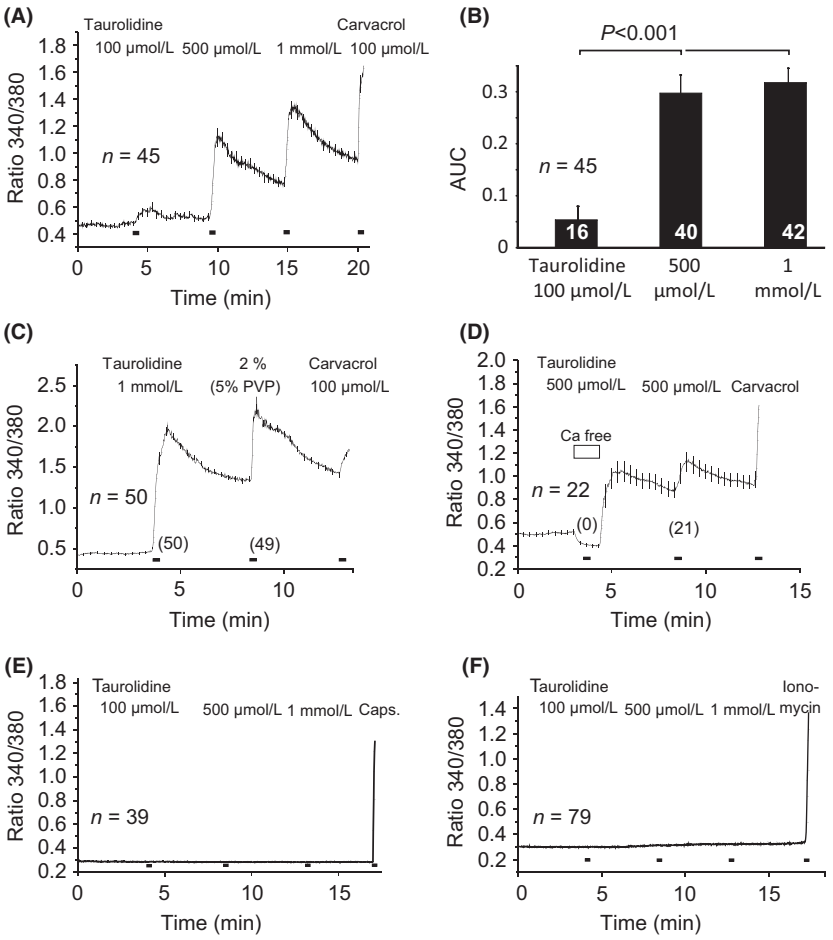

Figure 2. Taurolidine activates calcium influx in hTRPA1-expressing HEK293 cells. (A) Concentration dependence of taurolidine and control stimulation with the TRPA1 agonist carvacrol. (B) Areas under the curve (AUC) show the response magnitudes and the number of neurons responding. (C) Supramaximal 1 mmol/L and 2% taurolidine responses are reversible and activate the same numbers of cells (in brackets). (D) Taurolidine activates calcium influx only in the presence of extracellular Ca^{2+}, note the rebound effect upon returning calcium. (E) Human TRPV1-expressing cells do not respond to taurolidine but to capsaicin. (F) Untransfected HEK293 cells do not respond to taurolidine but to the calcium ionophore ionomycin.

The oxathiazine derivative is less potent than taurolidine in the tracheal and cellular models

The oxathiazine derivative (MW 137.01 g/mol) of taurultam, expected to cause less burning pain in wounds, showed a significant activation of tracheal CGRP release (12.2 ± 3.1 pg/ml, $n = 4$) only at 10 mmol/L concentration (Fig. 3A). Even the 2% solution (~146 mmol/L in 5% PVP) evoked a significantly smaller response than 1 mmol/L taurolidine. The PVP used to solubilize taurolidine as in the clinical formulation, did not stimulate any CGRP release from the trachea ($n = 4$, data not shown).

In calcium imaging experiments on HEK293 cells expressing hTRPA1, the oxathiazine derivative 1 mmol/L did not evoke any calcium transients in the absence of extracellular calcium, but in the presence of calcium 6 of 12 cells responded weakly and subsequently showed normal responses to carvacrol 100 µmol/L (Fig. 3B). A concentration–response relationship of oxathiazine derivative was to be observed (Figs.3C, D), but even 1 mmol/L activated only a minor calcium influx in just 37% of cultured cells, as compared to taurolidine.

The hTRPV1-expressing HEK293 cells did not respond to the oxathiazine derivative but to capsaicin, and the untransfected native HEK293 cells only responded to ionomycin as a positive control (Figs.3 E, F).

Taurultam is a weak tracheal irritant

Taurultam (MW 136.18 g/mol) is a product of taurolidine hydrolysis in aqueous solution. As the oxathiazine

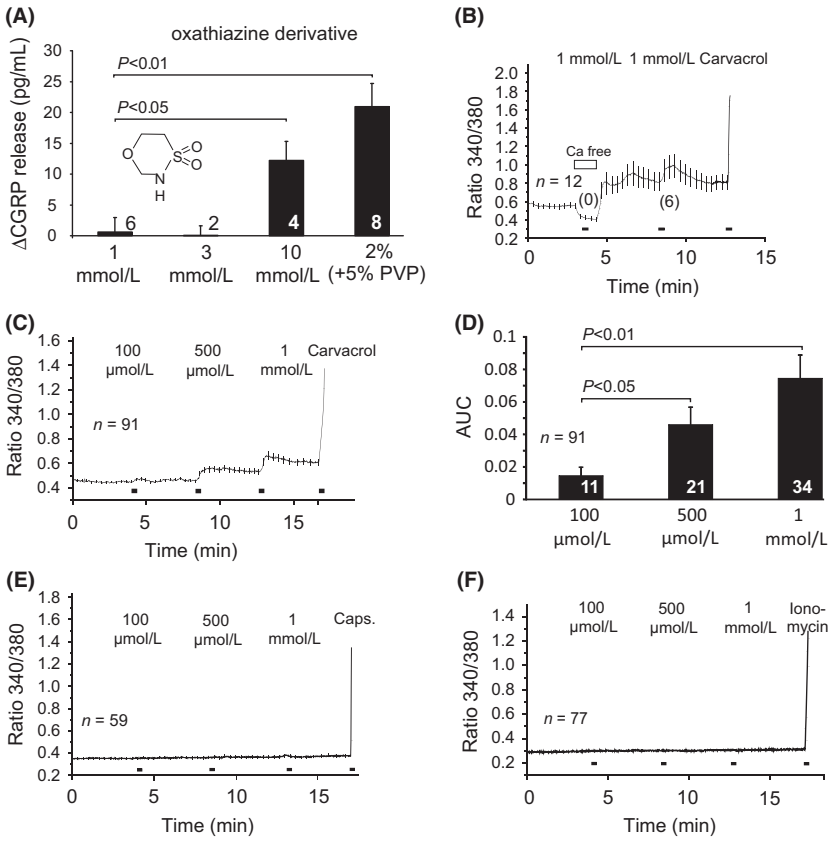

Figure 3. The oxathiazine derivative of taurultam is a weak irritant inducing tracheal CGRP release and calcium influx in hTRPA1-expressing HEK293 cells. (A) Concentration dependence of stimulated CGRP release; the 2% solution of the compound corresponds to a 146 mmol/L concentration; the chemical structure is shown as an inset. (B) The oxathiazine derivative activates little calcium influx only in the presence of extracellular Ca^{2+}. (C) Minor concentration-dependent calcium influx. (D) Areas under the curve (AUC) show the response magnitudes and the numbers of neurons responding. (E) Human TRPV1-expressing cells do not respond but to capsaicin. (F) The untransfected HEK cells do not respond but to ionomycin.

derivative, taurultam induced significant CGRP release from the mouse trachea only at a high concentration of 10 mmol/L (28.5 ± 11.3, $n = 6$; Fig. 4), but the response was more than twice as large, however only half the magnitude of the taurolidine response. Thus, at equimolar (10 mmol/L) concentration taurultam seems to be a weaker tracheal irritant than taurolidine but a stronger one than its oxathiazine derivative.

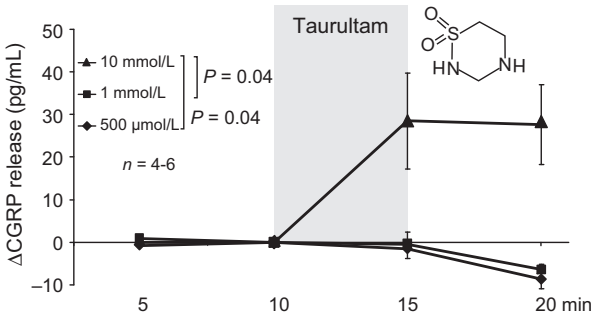

Figure 4. Taurultam activates tracheal CGRP release only at 10 mmol/L concentration.

Discussion

We set out to clarify why instillation of taurolidine in body cavities or infusion into peripheral veins is transiently painful and found that it selectively activates the universal chemoreceptor channel TRPA1 which enables calcium entry into primary nociceptive neurons and, thereby, release of pro-inflammatory neuropeptides, measured as CGRP release. In our sensitive *ex vivo* preparation of the mouse trachea and in the heterologous expression system all these effects were dependent on relatively high drug concentrations and reversible as well as completely absent, if extracellular calcium was missing or TRPA1 not expressed. Even the clinically used 2% concentration of taurolidine, 70-fold supramaximal and

hyperosmolar (in 5% PVP), did not induce calcium entry outlasting the saturating effect of 1 mmol/L isotonic taurolidine solution. These results do not provide an indication for cytotoxic effects, while they obviously account for the local pain evoked by taurolidine.

In the same surrogate model of the isolated mouse trachea *in vitro*, other irritants were much more potent than taurolidine and effective in inducing CGRP release. For example, at equal and saturating concentration of 1 mmol/L the TRPA1 agonist formaldehyde evoked three-times more CGRP release, and exposure of the trachea to the smoke of one cigarette released almost five times more CGRP (Kichko et al. 2015a). The prototypic pain inducer and selective TRPV1 agonist capsaicin at more than 3000-fold lower concentration (0.31 μmol/L) liberated still 50% more CGRP than taurolidine 1 mmol/L (Kichko and Reeh 2009). This finding is not only due to a greater potency of capsaicin than taurolidine but also to the greater prevalence of TRPV1 than TRPA1-expressing sensory neurons, because the latter are a (~ 60%) subpopulation of the former in the nodose/jugular ganglia that innervate the trachea (Kichko et al. 2013). In view of this, it is relevant that taurolidine did neither activate recombinant hTRPV1, nor had it lost its irritancy in TRPV1 null mutants. The low potency and efficacy of taurolidine and congeners to activate TRPA1 and release neuropeptides is sufficient reason why even mega doses systemically administered are well tolerated by cancer patients.

Taurolidine hydrolyzes in aqueous solution to form taurultam, N-methyloltaurultam and N-methyloltaurinamide. The N-methylol derivatives are considered as the active metabolites that exert the antimicrobial actions, reacting with proteins of the bacterial cell wall and cross-linking the endotoxins and exotoxins (Neary et al. 2010). Thus, we assume that it is these N-methyolol metabolites that preferentially react with the TRPA1 protein rather than the taurultam, which would match the lower potency and efficacy of taurultam and its closely related oxathiazine derivative. The most potent TRPA1 agonists such as the tear gas dibenzoxazepine (CR, EC_{50} 0.3 nM) have an electrophilic carbon (double bound to nitrogen as an imine) in common that invites nucleophilic attack by thiols (cysteines) or amine (lysine) present in the intracellular N-terminal tail of TRPA1 and forming a covalent bond that activates the channel (Gijsen et al. 2010). Lipophilicity is another determinant of TRPA1 agonist potency due to the most reactive binding sites being intracellular. Taurolidine and its congeners are lacking an electrophilic carbon as well as they are water soluble, which properties together may account for the relatively low potency and efficacy of the compounds in TRPA1 activation. However, also non-electrophilic agents such as the flavor compounds menthol (mint), thymol (thyme), and its isomer carvacrol (oregano) specifically activate human TRPA1, although at relatively high concentrations (1 mmol/L and 100 μmol/L, respectively), acting through a glycine-binding site in the pore region (TM 5) of the channel (Xiao et al. 2008). To determine the allosteric site where taurolidine is activating TRPA1 was beyond the scope of this study. There is a plethora of exogenous and endogenous small molecules, agonists, and antagonists that modulate TRPA1 function most probably by allosteric mechanisms and binding sites are rarely known.

In conclucion, taurolidine is a weak activator of hTRPA1, selective versus hTRPV1 and reversible in effect even at clinically used supramaximal concentration. Transient pain induction and irritation due to neuropeptide release are a cogent consequence of these properties.

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Authorship Contributions

Kichko and Reeh participated in research design and performed data analysis, while Kichko also conducted experiments. Kichko, Pfirrmann, and Reeh contributed new reagents or analytic tools as well as wrote or contributed to the writing of the manuscript.

Disclosures

None declared.

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