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TRPA1, substance P, histamine and 5-hydroxytryptamine interact in an interdependent way to induce nociception

Luana Fischer[1] · Maria Isabel Lavoranti[1] · Mariana de Oliveira Borges[1] · Alana Farias Miksza[1] · Natalia Fantin Sardi[1] · Bruno Jacson Martynhak[1] · Claudia H. Tambeli[2] · Carlos Amílcar Parada[2]

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Abstract
Background Although TRPA1, SP, histamine and 5-hydroxytryptamine (5-HT) have recognized contribution to nociceptive mechanisms, little is known about how they interact with each other to mediate inflammatory pain in vivo. In this study we evaluated whether TRPA1, SP, histamine and 5-HT interact, in an interdependent way, to induce nociception in vivo.
Methods and results The subcutaneous injection of the TRPA1 agonist allyl isothiocyanate (AITC) into the rat's hind paw induced a dose-dependent and short lasting behavioral nociceptive response that was blocked by the co-administration of the TRPA1 antagonist, HC030031, or by the pretreatment with antisense ODN against TRPA1. AITC-induced nociception was significantly decreased by the co-administration of selective antagonists for the NK1 receptor for substance P, the H1 receptor for histamine and the 5-$HT_{1A\ or\ 3}$ receptors for 5-HT. Histamine- or 5-HT-induced nociception was decreased by the pretreatment with antisense ODN against TRPA1. These findings suggest that AITC-induced nociception depends on substance P, histamine and 5-HT, while histamine- or 5-HT-induced nociception depends on TRPA1. Most important, AITC interact in a synergistic way with histamine, 5-HT or substance P, since their combination at non-nociceptive doses induced a nociceptive response much higher than that expected by the sum of the effect of each one alone. This synergistic effect is dependent on the H1, 5-$HT_{1A\ or\ 3}$ receptors.
Conclusion Together, these findings suggest a self-sustainable cycle around TRPA1, no matter where the cycle is initiated each step is achieved and even subeffective activation of more than one step results in a synergistic activation of the overall cycle.

Keywords TRPA1 · Substance P · Histamine · 5-Hydroxytryptamine (5-HT) · Nociception

Introduction

Acute inflammation and its resultant pain are biological responses to tissue injury essential to promote healing. However, inflammation may outlive its usefulness, exacerbating tissue damage and pain. How to prevent this exacerbation remains a challenge in clinical practice. Certainly, understanding the complex interactions among the several interdependent pathways co-activated in inflammatory pain would contribute to overcome such challenge.

Transient receptor potential channels ankiryn 1 (TRPA1) and vanilloid 1 (TRPV1) have been considered central players in inflammatory nociceptive mechanisms, since they are activated by numerous inflammatory mediators and also contribute to indirectly enhance their release [1]. For example, the activation of TRPA1 expressed on nociceptive C-fibers, induces nociception and releases neuropeptides [1, 2]. Among these neuropeptides, substance P (SP) mediates a well-known neuroimmune response by activating NK1 receptors on mast cells to induce their degranulation with the consequent release of histamine and 5-hydroxytryptamine

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✉ Luana Fischer
fischer@ufpr.br

[1] Division of Biological Sciences, Department of Physiology, Federal University of Parana, Curitiba, Parana, Brazil

[2] Department Functional and Structural Biology, Institute of Biology, State University of Campinas-UNICAMP, Campinas, São Paulo, Brazil

(5-HT) [2]. However, although TRPA1, SP, histamine and 5-HT have recognized contribution to nociceptive mechanisms, little is known about how they interact with each other to mediate inflammatory pain in vivo. For example, it was already demonstrated that histamine contributes to the nociception [3] and hyperalgesia [4] induced by TRPA1 agonists and the ability of histamine to activate TRPA1 downstream its receptors was demonstrated in vitro [5]. However, whether the nociceptive response induced by histamine is dependent on TRPA1 is not known. We have previously demonstrated that 5-HT contributes to the hyperalgesia induced by a TRPA1 agonist [4], TRPA1 activation induces the release of 5-HT in different models and tissues [6–8] and a 5-HT agonist activates TRPA1 in vitro [9]. However, it is not known whether TRPA1-mediated nociception depends on 5-HT or whether 5-HT-induced nociception depends on TRPA1. Most important, considering that TRPA1 may be gated by histamine and 5-HT and also contribute to their release, through SP, they could act synergistically with each other, i.e., intense pain could emerge from the subthreshold activation of TRPA1 and histamine, 5-HT or SP receptors. This has never been tested before.

Therefore, we aimed to evaluate whether TRPA1, SP, histamine and 5-HT could interact, in an interdependent way, to induce nociception in vivo. To this end we evaluated whether (1) SP, histamine or 5-HT contribute to the nociception induced by a TRPA1 agonist; (2) TRPA1 contributes to histamine- or 5-HT-induced nociception and (3) a TRPA1 agonist act synergistically with histamine, 5-HT or SP to induce nociception.

Materials and methods

Animals

This study was carried out in male Wistar rats (150–250 g), housed five per cage, in a temperature-controlled room (±23 °C) with a 12/12-h light/dark cycle with rat chow and water available ad libitium. They were handled for at least one week prior to the experiments, which were in accordance with IASP guidelines for the study of pain in animals [10]. All animal experimental procedures and protocols were approved by the Ethics Committee on Animal Research of the Federal University of Parana.

Drugs

Allyl isothiocyanate (AITC, TRPA1 agonist, 1–600 μg/paw; [4]; HC 030031 (TRPA1 antagonist, 300; 600; 1200 μg/paw [11, 12]; L-703,606 (NK1 receptor antagonist, 76 μg/paw, [13]); compound 48/80 (mast cell degranulator, four consecutive daily injections of 1, 3, 10, 10 μg/paw, [3]); pyrilamine (H_1 receptor antagonist, 400 μg/paw, [14]); tropisetron (5-HT_3 receptor antagonist, 450 μg/paw, [14]); WAY 100, 135 (5-HT_{1A} receptor antagonist 450 μg/paw, [14]); histamine (1–25 μg/paw, [15]); 5-HT (0.1–25 μg/paw, [15]); substance P (0.5–100 μg/paw, [16]); m-3m3FBS (a phospholipase C activator, 5 μg/paw). All drugs were dissolved in 0.9% NaCl, except AITC dissolved in propylene glycol and HC 030031 dissolved in pure dimethylsulfoxide (DMSO). HC 030031 and WAY 100, 135 were obtained from Tocris Bioscience, Avonmouth, Bristol, UK; L-703,606 was obtained from Enzo Life Sciences AG, Lausen, Switzerland, the other drugs were obtained from Sigma-Aldrich, São Paulo, SP, Brazil.

All drugs were subcutaneously injected into the plantar surface of the rat's hind paw and the dose of each drug was based on previous literature data. Total volume injection was always 30 μl. Compound 48/80 was used to degranulate local mast cells before experiments. To this end, a daily administration of crescent doses (1, 3, 10, 10 μg/paw) of compound 48/80 was performed for four consecutive days before AITC injection, as described previously [3, 4].

Antisense oligodeoxynucleotides (ODNS)

The oligodeoxynucleotide (ODN) antisense (AS) sequence for TRPA1 mRNA, 5′-TATCGCTCCACATTGCTAC-3′ and the mismatch-ODN 5′-ATTCGCCTCACATTGTCAC-3′, which corresponded to the antisense sequence except that six bases were changed, were synthesized by Erviegas (São Paulo, SP, Brazil). Before use, the lyophilized ODNs were reconstituted in 0.9% NaCl.

Antisense- or mismatch-ODN was intrathecally injected as previously described [17]. Briefly, for each injection, rats were anesthetized with 1/3 O_2 to 2/3 N_2O and halothane at 5 to 1.5%. A 26-gauge needle was inserted in the subarachnoid space on the midline between L5 and L6 vertebrae. A dose of 5 nmol of TRPA1 antisense- or mismatch-ODN [11, 12] was intrathecally administered in a volume of 10 μl (1 μl/s) once daily for 4 days. The animals regained consciousness approximately 1 min after discontinuing the anesthetic. Experiments were initiated 1 h after the fourth injection.

By using the exact same protocol, we have previously shown that this ODN AS sequence is able to down regulate the expression of TRPA1 in L5–6 dorsal root ganglia neurons [11, 12].

Nociceptive test

Testing sessions were carried out during the light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23 °C. Rats did not have access to food or water

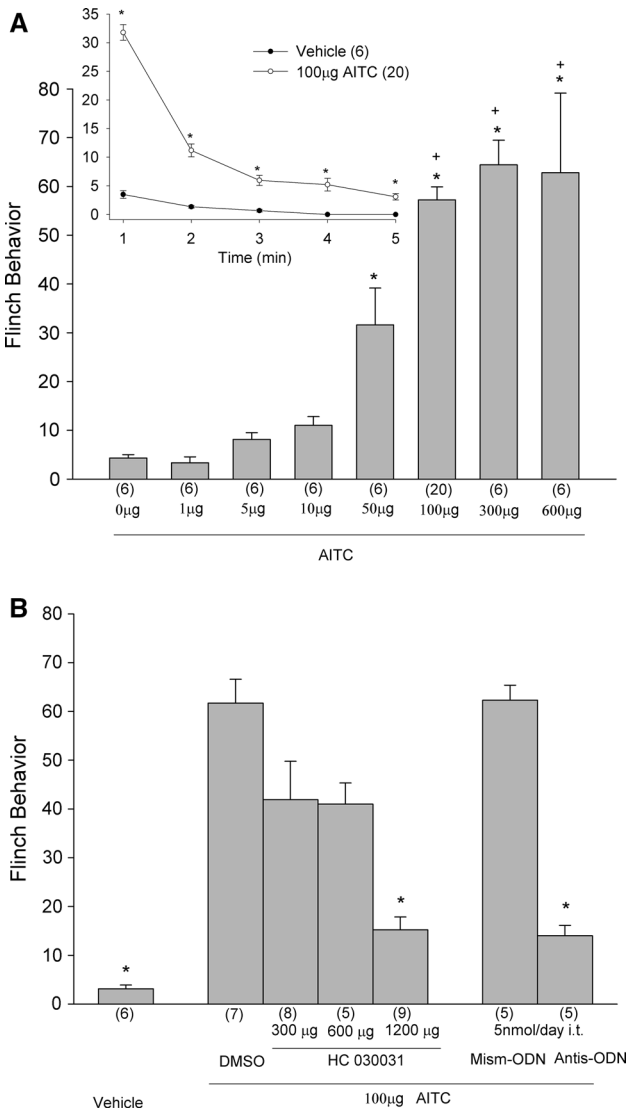

Fig. 1 AITC-induced nociception. **a** The injection of AITC into the hind paw induced a dose-dependent behavioral nociceptive response with a ceiling effect. The symbol "*" indicates a response significantly higher than that induced by vehicle, the symbol "+" indicates a response significantly higher than that induced by AITC at 50 µg (one-way ANOVA and Tukey test, $p < 0.05$). The nociceptive response induced by AITC lasts for 5 min, there is a statistically significant interaction between time and treatment (*small panel*, Two Way Repeated Measure ANOVA and Tukey test, $f = 2.994$; $p = 0.026$). **b** The nociceptive response induced by AITC depends on TRPA1. AITC-induced nociception was dose-dependently blocked by the co-administration of the selective TRPA1 antagonist, HC030031, or by the previous intrathecal treatment (four daily intrathecal injections of 5 nmol/10 µl between L5 and 6, see "Materials and methods") with antisense-ODN against TRPA1. The symbol "*" indicates a response significantly lower than that of the other groups (one-way ANOVA and Tukey test, $p < 0.05$). The administration of HC030031 at the contralateral hind paw had no effect (see "Results"). In these and in subsequent figures, data are expressed as means ± EPM. *Numbers in parentheses* indicate the number of animals in each group. See "Materials and methods" for additional details regarding data presentation and analysis. *AITC* allyl isothiocyanate, *Vehicle* propylene glycol, *DMSO* dimethylsulfoxide, *Mism-ODN* mismatched-oligodeoxynucleotide, *Antis-ODN* antisense-oligodeoxynucleotide

during the test and each animal was used once. For subcutaneous injection into the plantar surface of the rat′s hind paw, a 30 gauge needle connected to a of polyethylene tube (PE-50; Intramedic, Clay Adams, Becton–Dickinson, Franklin Lakes, NJ, USA) and to a 50 µl syringe (Hamilton, Reno, NV, USA) was used. Animals were briefly restrained and the total injection volume was 30 µl.

The test chamber consists of a mirrored-wood chamber (30 × 30 × 30 cm) with a glass at the front side. Each animal was individually placed into the test chamber 15 min before the beginning of the tests, to minimize stress. The behavioral nociceptive response was quantified by counting the number of spontaneous flinches of the injected hind paw, during two different time frames, depending on drug's pharmacokinetic. AITC induces a short lasting nociceptive response that typically lasts 5 min; therefore AITC-induced flinching behavior was assessed during 5 min. In contrast, histamine, SP and 5-HT induce a behavioral response that decreases progressively through 30 min; therefore flinching behavior was assessed during 30 min whenever they were injected. These time frames are in accordance with previous literature data [1–3, 14, 18]. The number of flinches during the entire duration of the experiment was used as a measure of nociception.

Data presentation and analysis

Data are expressed in figures as number of flinches during the entire duration of the experiment and are presented as mean ± SEM. Two-way repeated measures ANOVA with one between-subjects factor (treatment) and one within-subjects factor (time) was used to determine if there were significant time/treatment interactions in data plotted in the small panel in Fig. 1a. One-way ANOVA was used to determine if there were significant differences between treatment groups in remaining figures. Post hoc contrasts using the Tukey test were performed to determine the basis of the significant difference. The level of significance was set at $p < 0.05$.

Isobolographic analyses were performed to investigate whether the TRPA1 agonist, AICT, act in synergism with histamine, 5-HT, and substance P to induce nociception. An isobologram provides a graphical representation of the nature of the interaction between two drugs A and B [19]. A line of additivity, in a two-coordinate plot, is constructed by connecting two points, one in the *x*-axis and the other in the *y*-axis (e.g., 1;0 and 0;1 for *x*;*y*). The *x* and *y* values are the doses of the two drugs (A and B) that induce, alone, the

same effect induced by their combination. The doses of the combinations are then plotted as a point in the same graph. Synergy, additivity, or antagonism is indicated when the point is located below, on, or above the line of additivity, respectively [19].

The effect induced by combining different non-nociceptive doses of AITC and histamine, 5-HT, or substance P was evaluated. The combination that induced a nociceptive effect with a magnitude within the dose–response curves of each agent alone was included in the isobolographic analyses. A second order polynomial fitting was used to calculate the theoretical individual doses of histamine, substance P, 5-HT and AICT necessary to induce the same effect of the combination (EDc) ($R^2 > 0.99$ for all regressions, with exception for 5-HT, which $R^2 = 0.95$). The obtained values were plotted in the *x*- and *y*-axis of the isobologram and used for calculating the combination index (CI). The CI provides a quantitative measure of the drug interaction at a given effect level. CI is calculated as: (dose of A in combination/dose of A alone) + (dose of B in combination/dose of B alone) with all doses having the same effect (equi-nociceptive doses). Synergy, additivity, or antagonism is indicated by CI values lower, equal or higher than 1.

Results

AITC-induced nociception

To characterize AITC-induced nociception, we injected it at different doses into the rat′s hind paw and quantified the behavioral nociceptive response (hind paw flinching). As shown in Fig. 1a, AITC induced a dose-dependent behavioral nociceptive response (one-way ANOVA and Tukey test, $p < 0.05$). AITC at 100 μg induced a nociceptive response significantly higher than that induced at 50 μg and similar to that induced at 300 or 600 μg. These findings demonstrated that a ceiling nociceptive effect is reached at 100 μg. As shown in the small panel in Fig. 1a, the nociceptive response induced by AITC lasts for 5 min, it is maximal at the first minute and declines thereafter (two-way repeated measures ANOVA and Tukey test, $f = 28.23$; $p < 0.001$).

To determine whether AITC induced-nociception depends on TRPA1 activation, we used a selective TRPA1 antagonist HC 030031 (300; 600 or 1200 μg/paw, co-administered with AITC) or an antisense-ODN sequence against TRPA1 (intrathecally administered, between L5 and 6, at 5 nmol/10 μl per day, during four days, a protocol that significantly decreases TRPA1 expression DRG cells, see "Materials and methods"). As shown in Fig. 1b, the nociception induced by AITC (100 μg) was blocked by the selective TRPA1 antagonist HC 030031 (HC, one-way ANOVA and Tukey test, $p < 0.05$). When administered at the contralateral hind paw, HC at the dose of 1200 μg (49.5 ± 3.8, data not shown) did not affect AITC-induced nociception (57.9 ± 2.2, t test, $p > 0.05$). This finding demonstrates that AITC-induced nociception depends on local activation of TRPA1. The nociception induced by AITC (100 μg) was also blocked by pretreatment with antisense-ODN, but not with mismatch-ODN (one-way ANOVA and Tukey test, $p < 0.05$), suggesting that AITC-induced nociception depends on the activation of neuronal TRPA1.

The contribution of mast cells; the NK1 receptor for substance P; the H1 receptor for histamine and the 5-$HT_{1A\ and\ 3}$ receptors for 5-hydroxytryptamine to AITC-induced nociception

To evaluate whether mast cell degranulation and substance P contribute to AITC-induced nociception, we tested the ability of a mast cell degranulator, compound 48/80 (four daily intraplantar injections of increasing doses, see "Materials and methods"), or of a NK1 receptor antagonist L-703,606 (76 μg/paw) to decrease it. As shown in Fig. 2a, the nociceptive response induced by AITC was significantly decreased by pretreatment with compound 48/80 or by the co-administration of L-703,606 into the ipsilateral but not contralateral (52.0 ± 3.9, data not shown) hind paw. These findings suggest that SP and mast cells contribute to AITC-induced nociception.

To ensure that compound 48/80 effectively induced local mast cell degranulation, we compared the nociceptive response (assessed during 5 min) induced by a single injection of 10 μg of compound 48/80 (65.7 ± 4.4, data not shown), with that induced by the same treatment in a group that had received previously four daily intraplantar injections. The absence of nociceptive response in the latter group (7.0 ± 2.7, a response similar to that induced by injection of 0.9% NaCl, 4.8 ± 1.1, data not shown) indicates that this protocol effectively depletes mast cell granule contents (one-way ANOVA, and Tukey test, $p < 0.05$).

To evaluate whether histamine and 5-HT contribute to AITC-induced nociception through their respective H1 and 5-$HT_{1A\ and\ 3}$ receptors, we evaluated the ability of the H1 receptor antagonist pyrilamine (400 μg), of the 5-HT_{1A} receptor antagonist WAY 100,135 (450 μg) or of the 5-HT_3 receptor antagonist tropisetron (450 μg) to decrease AITC-induced nociception. As shown in Fig. 2b, the nociceptive response induced by AITC was significantly decreased by the co-administration of pyrilamine, WAY 100,135 or tropisetron (one-way ANOVA and Tukey test, $p < 0.05$). The administration of each one of these antagonists into the

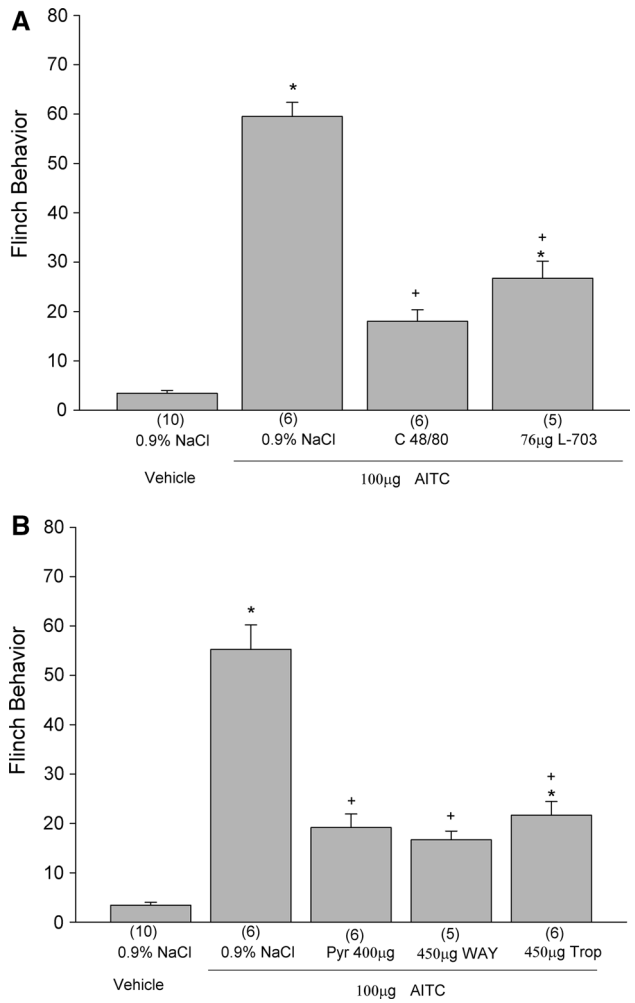

Fig. 2 AITC-induced nociception is significantly decreased by a mast cell degranulator and antagonists for NK1, H1, 5-HT_{1A} and 5-HT_3 receptors. **a** The nociceptive response induced by the injection of AITC into the hind paw was significantly decreased by the previous treatment (see "Materials and methods") with the mast cell degranulator, compound 48/80, or by the co-administration of the selective NK1 receptor antagonist L-703,606, as indicated by the symbol "+". The symbol "*" indicates a response significantly higher than that induced by vehicle (propylene glycol + 0.9% NaCl) (one-way ANOVA and Tukey test, $p < 0.05$). The administration of L-703,606 into the contralateral hind paw had no effect (see "Results"). **b** The nociceptive response induced by the injection of AITC into the hind paw was significantly decreased by the co-administration of the selective H1, 5-HT_{1A} or 5-HT_3 receptor antagonists, pyrilamine (Pyr); WAY 100, 135 (WAY) or tropisetron (trop), respectively, as indicated by the symbol "+". The symbol "*" indicates a response significantly higher than that induced by vehicle (propylene glycol + 0.9% NaCl) (one-way ANOVA and Tukey test, $p < 0.05$). The administration of each one of the antagonists into the contralateral hind paw had no effect (see "Results")

contralateral hind paw (52.0 ± 2.9; 53.2 ± 1.6; 56.6 ± 1.4, respectively, data not shown) did not affect AITC-induced nociception (57.9 ± 2.9, one-way ANOVA, $p > 0.05$), showing that, at these doses, the effect of each antagonist is local, rather than systemic. These findings suggest that the activation of local H1, 5-HT_{1A} and 3 receptors contributes to AITC-induced nociception.

Interaction between histamine and TRPA1

The next step was to evaluate whether TRPA1 contributes to histamine-induced nociception. First, we characterized histamine-induced nociception by injecting it at different doses into the hind paw. As shown in Fig. 3a, histamine induced a dose-dependent behavioral nociceptive response (one-way ANOVA and Tukey test, $p < 0.05$). Then, we tested the ability of the antisense-ODN against TRPA1 to decrease histamine-induced nociception. As shown in Fig. 3b, the nociceptive response induced by histamine was blocked by the pretreatment with antisense-ODN (one-way ANOVA and Tukey test, $p < 0.05$). This finding suggests that TRPA1 is a downstream key effector of the nociceptive response induced by histamine.

To evaluate whether AITC and histamine act synergistically to induce nociception, we combined them at different non-nociceptive doses. As shown in Fig. 3c, the combination of the highest non-nociceptive doses of AITC (10 μg) and histamine (2.5 μg) induced a nociceptive effect similar in magnitude to the ceiling nociceptive effect induced by AITC. This effect was blocked by the co-administration of the H1 receptor antagonist pyrilamine (400 μg, One-way ANOVA and Tukey test, $p < 0.05$). Combination of lower doses induced weaker nociceptive responses (one-way ANOVA and Tukey test, $p < 0.05$), but even these responses were higher than that expected by the sum of the effect of each drug alone. To provide a graphical representation of the nature of the interaction between histamine and AITC we construct an isobologram (see "Materials and methods") where the line of additivity connects the doses of histamine (x-axis) and AITC (y-axis) necessary to induce the same effect induced by their combination at 1 μg each. The point represents the doses combined (1 μg each), the greater its distance bellow the line of additivity, the stronger the synergism. To provide a quantitative measure of histamine and AITC interaction at this effect level, we calculated the combination index (see "Materials and methods"). A combination index <1 indicates synergy, and the lower the value, the stronger the synergism (Fig. 3d).

Interaction between 5-hydroxytryptamine and TRPA1

To evaluate whether TRPA1 also contributes to 5-HT-induced nociception, we first characterized 5-HT-induced nociception by injecting it at different doses into the hind paw. As shown in Fig. 4a, 5-HT induced a dose-dependent behavioral nociceptive response (one-way ANOVA and

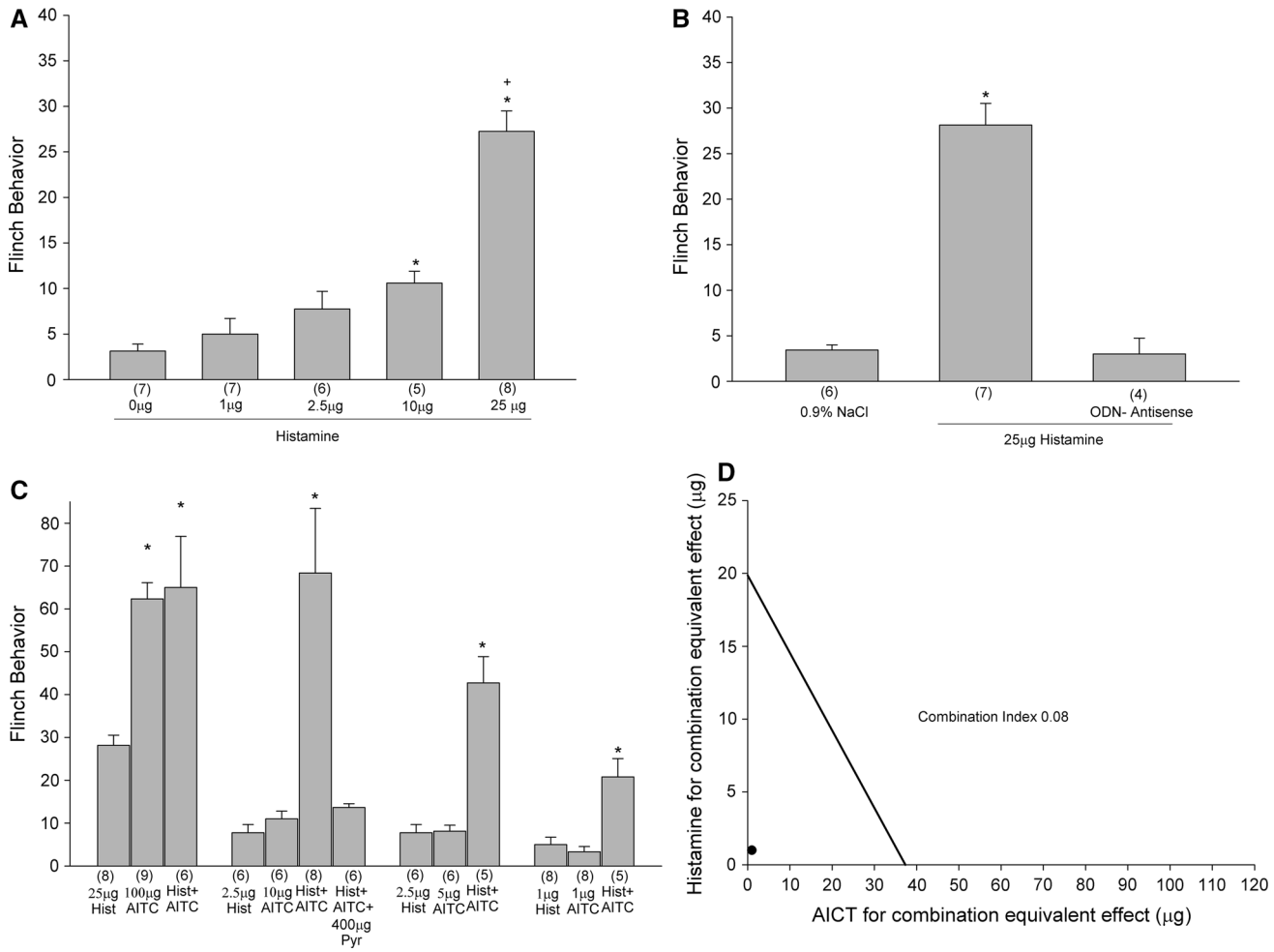

Fig. 3 Histamine-induced nociception and its interaction with TRPA1. **a** The injection of histamine into the hind paw induced a dose-dependent behavioral nociceptive response. The symbol "*" indicates a response significantly higher than that induced by vehicle, the symbol "+" indicates a response significantly higher than that induced by histamine at 10 μg (one-way ANOVA and Tukey test, $p < 0.05$). **b** The nociceptive response induced by histamine was blocked by the previous intrathecal treatment with antisense-ODN against TRPA1. The symbol "*" indicates a response significantly higher than that of the other groups (one-way ANOVA and Tukey test, $p < 0.05$). **c** The combination of the highest tested dose of histamine (25 μg) did not increase the ceiling nociceptive effect induced by AITC (100 μg, one-way ANOVA and Tukey test, $p < 0.05$). Combination of different non-nociceptive doses of AITC and histamine induced a nociceptive response higher than that expected by the sum of the effect of each one alone (one-way ANOVA and Tukey test, $p < 0.05$, performed for each combination analysis). The potentiated effect induced by the combination of histamine at 2.5 μg and AITC at 10 μg was blocked by the co-administration of the H1 receptor antagonist pyrilamine (400 μg). The symbols "*" indicate a response significantly higher than that of the other group(s) in the respective combination analysis. **d** The combination of histamine at 1 μg and AITC at 1 μg induced a potentiated nociceptive effect with a magnitude within the dose–response curve to histamine and was used in the isobolographic analysis (see "Materials and methods"). A second order polynomial fitting was used to calculate the theoretical individual doses of histamine and AICT necessary to induce the same effect of the combination (EDc) ($R^2 > 0.99$). The obtained values, plotted in the *x*-and *y*-axis of the isobologram, originated the line of additivity. The *point* representing the doses of the combinations is located far below this line, indicating synergism. The combination index [CI = (dose of A in combination/dose of A alone) + (dose of B in combination/dose of B alone)] of 0.08 provided a quantitative measure of synergism (the lower the value <1, the stronger the synergism)

Tukey test, $p < 0.05$). Then, we evaluated the ability of the antisense-ODN against TRPA1 to decrease 5-HT-induced nociception. As shown in Fig. 4b, the nociceptive response induced by 5-HT was significantly decreased by the pre-treatment with antisense-ODN (one-way ANOVA and Tukey test, $p < 0.05$), supporting the contribution of TRPA1 to the nociceptive response induced by 5-HT.

To evaluate whether AITC and 5-HT act synergistically to induce nociception, we combined them at different non-nociceptive doses. As shown in Fig. 4c, the combination of AITC at 10 μg and 5-HT at 0.1 μg induced a nociceptive effect similar in magnitude to the ceiling nociceptive effect induced by AITC. This effect was blocked by the co-administration of the 5-HT_{1A} receptor antagonist WAY

100,135 (450 μg) or the 5-HT_3 receptor antagonist tropisetron (400 μg). Combinations of lower doses of AITC and 5-HT induced weaker nociceptive responses, but even these responses were higher than that expected by the sum of the effect of each drug alone (one-way ANOVA and Tukey test, $p < 0.05$). The synergistic interaction between 5-HT and AITC is graphically represented by the isobologram (see "Materials and methods") and quantitatively determined by the combination index showed in Fig. 4d.

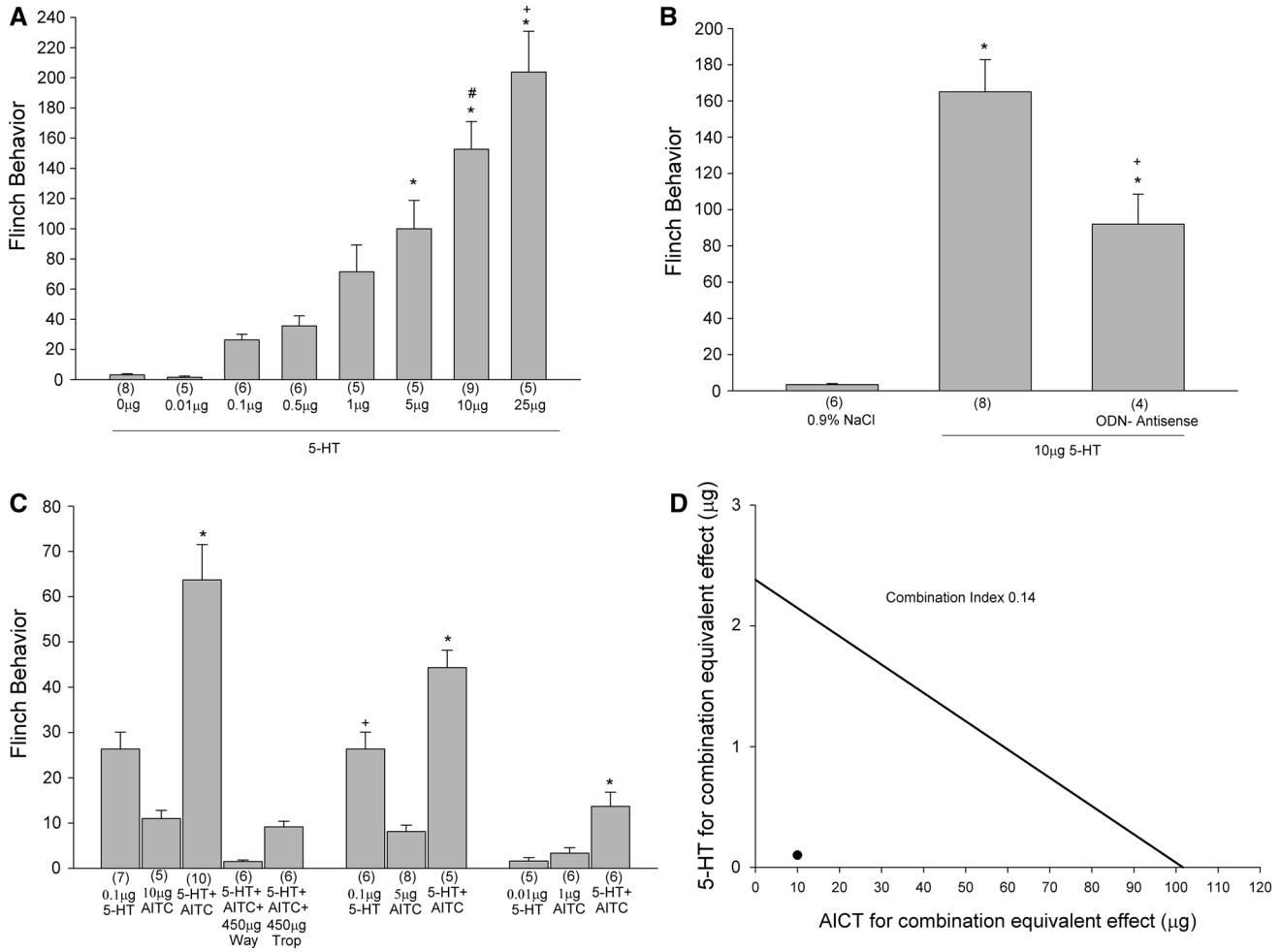

Fig. 4 5-HT-induced nociception and its interaction with TRPA1. **a** The injection of 5-HT into the hind paw induced a dose-dependent behavioral nociceptive response. The symbol "*" indicates a response significantly higher than that induced by vehicle, the symbol "#" indicates a response significantly higher than that induced by 5-HT at lower doses, the symbol "+" indicates a response significantly higher than that induced by 5-HT at 10 μg (one-way ANOVA and Tukey test, $p < 0.05$). **B-** The nociceptive response induced by 5-HT was significantly decreased by the previous intrathecal treatment with antisense-ODN against TRPA1. The symbol "*" indicates a response significantly higher than that induced by vehicle, the symbol "+" indicates a response significantly lower than that induced by 5-HT at 10 μg (one-way ANOVA and Tukey test, $p < 0.05$). **c** The combination of different non-nociceptive doses of AITC and 5-HT induced a nociceptive response higher than that expected by the sum of the effect of each one alone (one-way ANOVA and Tukey test, $p < 0.05$, performed for each combination analysis). The potentiated effect induced by the combination of 5-HT at 0.1 μg and AITC at 10 μg was blocked by the co-administration of the 5- $HT_{1A\ or\ 3}$ receptor antagonists WAY 100,135 (Way, 450 μg) and tropisetron (Trop, 450 μg), respectively. The symbols "*" indicate a response significantly higher than that of the other group(s) in the respective combination analysis, the symbol "+" indicates a response higher than that induced by AITC at 10 μg in the respective combination analysis. **d** The combination of 5-HT at 0.1 μg and AITC at 1 μg induced a potentiated nociceptive effect with a magnitude within the dose–response curve to 5-HT or AITC and was used in the isobolographic analysis (see "Materials and methods"). A second order polynomial fitting was used to calculate the theoretical individual doses of 5-HT and AICT necessary to induce the same effect of the combination (EDc) ($R^2 > 0.95$). The obtained values, plotted in the *x*- and *y*-axis of the isobologram, originated the line of additivity. The *point* representing the doses of the combinations is located far below this line, indicating synergism. The combination index [CI = (dose of A in combination/dose of A alone) + (dose of B in combination/dose of B alone)] of 0.14 provided a quantitative measure of synergism (the lower the value <1, the stronger the synergism)

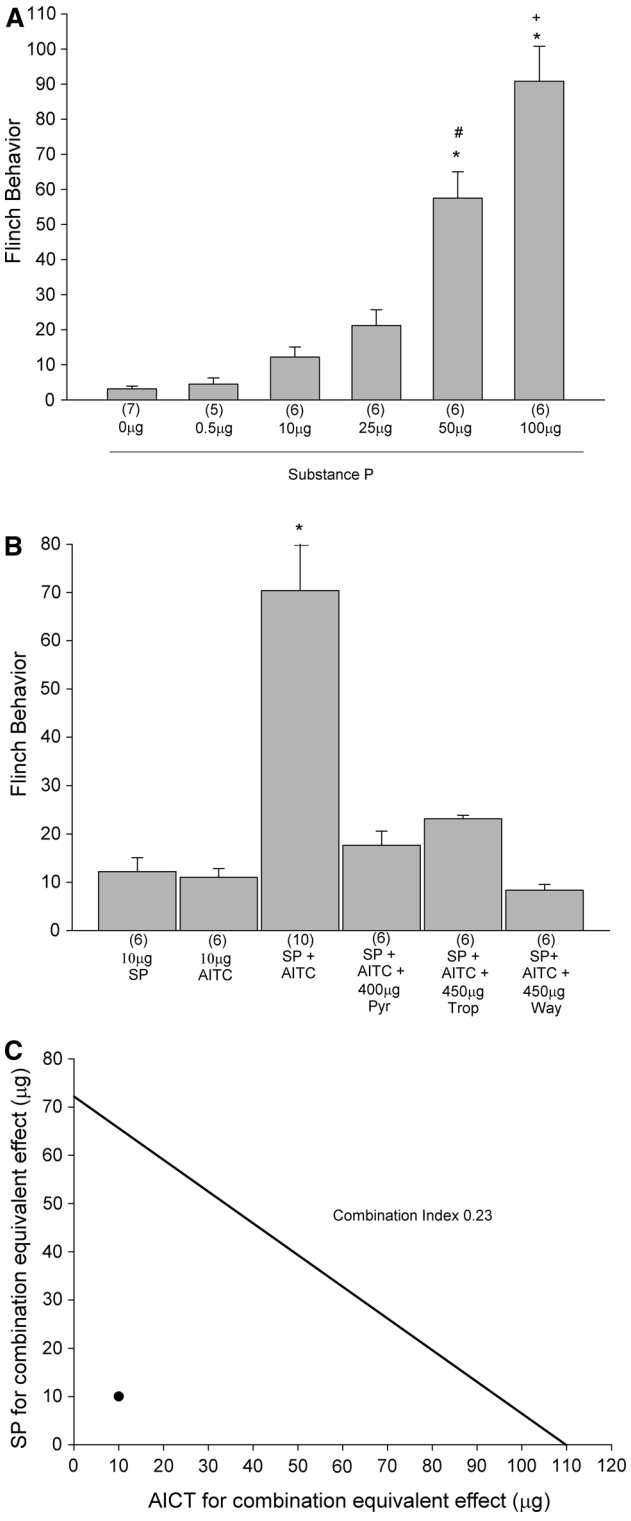

Fig. 5 Substance P-induced nociception and its interaction with TRPA1. **a** The injection of substance P (SP) into the hind paw induced a dose-dependent behavioral nociceptive response. The symbol "*" indicates a response significantly higher than that induced by vehicle, the symbol "#" indicates a response significantly higher than that induced by SP at lower doses, the symbol "+" indicates a response significantly higher than that induced by SP at 50 μg (one-way ANOVA and Tukey test, $p < 0.05$). **b** The combination of non-nociceptive doses of AITC (10 μg) and SP (10 μg) induced a nociceptive response higher than that expected by the sum of the effect of each one alone. This potentiated effect was blocked by the co-administration of the H1, 5-$HT_{1A\ or\ 3}$ receptor antagonists pyrilamine (Pyr 400 μg), WAY 100,135 (Way, 450 μg) and tropisetron (Trop, 450 μg), respectively. The symbols "*" indicate a response significantly higher than that of the other groups (one-way ANOVA and Tukey test, $p < 0.05$). **d** For this isobolographic analysis (see "Materials and methods"), a second order polynomial fitting was used to calculate the theoretical individual doses of SP and AICT necessary to induce the same effect of the combination (EDc) ($R^2 > 0.99$). The obtained values, plotted in the *x*- and *y*-axis of the isobologram, originated the line of additivity. The *point* representing the doses of the combinations is located far below this *line*, indicating synergism. The combination index [CI = (dose of A in combination/dose of A alone) + (dose of B in combination/dose of B alone)] of 0.23 provided a quantitative measure of synergism (the lower the value <1, the stronger the synergism)

Interaction between TRPA1 and substance P and its dependence on the H1 and 5-$HT_{1A\ and\ 3}$ receptors

To characterize SP-induced nociception we injected it at different doses into the hind paw. As shown in Fig. 5a, SP induced a dose-dependent behavioral nociceptive response (one-way ANOVA and Tukey test, $p < 0.05$). To determine whether AITC and SP act synergistically to induce nociception, we combined them at non-nociceptive doses. As shown in Fig. 5b, the combination of AITC (10 μg) and SP (10 μg) induced a nociceptive response similar in magnitude to the ceiling nociceptive effect induced by AITC (one-way ANOVA and Tukey test, $p < 0.05$). This effect was blocked by the co-administration of the H1 receptor antagonist pyrilamine (400 μg); of the 5-HT_{1A} receptor antagonist WAY 100,135 (450 μg) or of the 5-HT $_3$ receptor antagonist tropisetron (450 μg). These findings suggest that the synergistic interaction between AITC and SP is largely dependent on the histamine and 5-HT acting on their respective receptors. The synergistic interaction between SP and AITC (see "Materials and methods") is graphically represented by the isobologram and quantitatively determined by the combination index showed in Fig. 5c.

Interaction between TRPA1 and a phospholipase C activator

To evaluate whether the nociceptive response induced by AITC is potentiated by the PLC activator 3-m3 m-FSB we combined them at non-nociceptive doses. As shown in Fig. 5a, the combination of AITC and 3-m3 m-FSB induced a nociceptive response higher than that expected by the sum of the effect of each drug alone, suggesting that AITC-induced nociceptive response is potentiated by PLC activation (one-way ANOVA and Tukey test, $p < 0.05$).

Discussion

This study demonstrates that AITC-induced nociception in vivo depends on TRPA1 and is decreased by the blockade of the NK1 receptor for SP, the H1 receptor for histamine and the 5-$HT_{1A\ and\ 3}$ receptors for 5-HT. In a complementary way, TRPA1 contributes to the nociceptive response induced by histamine or 5-HT. Importantly, AITC act synergistically with histamine, 5-HT or SP to induce nociception, which further supports the interdependence of the nociceptive mechanisms mediated by them.

At an injured site, TRPA1 may be activated by numerous inflammatory mediators triggering nociception and releasing neuropeptides that further contribute to inflammatory mediators release [2]. These complementary functions of TRPA1 as a detector, transducer and amplifier of inflammatory mechanisms ensure its pivotal role in inflammatory pain [2]. Here, we demonstrated that AITC induced a dose-dependent and short lasting nociceptive behavioral response (Fig. 1a), which was decreased in 75% by a selective TRPA1 antagonist or by antisense ODN against TRPA1 (Fig. 1b). This last finding shows that AITC-induced nociception is largely dependent on TRPA1. However, although numerous studies have demonstrated that AITC-induced responses are dependent on TRPA1 and independent on TRPV1 [3, 20–24], others have suggested that AITC can also act on TRPV1 in in vitro assays and/or in TRPA1 deficient mice [25–30]. It is important to point that AITC activates TRPV1 at high, but not low concentrations [30] and these studies have used high concentrations of AITC (millimolar concentrations in vitro and concentrations higher than 1% in TRPA1 deficient mice). However, a contribution of TRPV1 to AITC-induced nociception, especially to the residual response that persists after blocking TRPA1, cannot be ruled out in the present study.

SP released in response to TRPA1 activation [18, 31] induces mast cell degranulation [2, 32], in a well-known example of neuroimmune integration. In fact, it has been demonstrated that TRPA1-mediated nociception depends on SP [3, 18, 31] and mast cells [3]. This study reinforced these findings by showing that AITC-induced nociception in vivo is significantly decreased by a NK1 receptor antagonist or by previous treatment with a mast cell degranulator (Fig. 2a). However, although SP is the highest affinity ligand for the NK1 receptor [33], the contribution of other tachykinins, such as neurokinin A, B and hemokinin-1 [34], cannot be fully excluded.

Among the main products of mast cell degranulation are histamine and 5-HT [32] and among their receptors, the H1 receptor for histamine and the 5-$HT_{1A\ and\ 3}$ receptors for 5-HT appear to have a pivotal role in nociception. For example, these receptors [14], in addition to TRPA1 [35, 36], are essential to the nociception induced by formalin, one of the most used models of inflammatory pain. In this study, we showed that the blockade of the H1 receptor for histamine or of the 5-$HT_{1A\ or\ 3}$ receptors for 5-HT significantly decreases AITC-induced nociception (Fig. 2b). Although controversies exist [37], the contribution of the H1 receptor to AITC-induced nociception has already been showed [3], while that of 5-$HT_{1A\ or\ 3}$ is being shown here for the first time. These findings suggest that histamine and 5-HT are resealed in response to TRPA1 activation and, therefore, act on their respective receptors to contribute to AITC-induced nociception in vivo. Although the release of histamine in response to TRPA1 activation has never been directly demonstrated, that of 5-HT has been demonstrated in different models and tissues [6, 7] including the skin, where it levels are strongly reduced in TRPA1-deficient mice [8].

In addition to contribute to AITC-induced nociception, histamine and 5-HT depends on TRPA1 to induce nociception, since pretreatment with antisense-ODN against TRPA1 prevented histamine- (Fig. 3b) and decreased 5-HT-induced nociception (Fig. 4b). Considering that histamine, in contrast to 5-HT, has no ionotropic receptors, this finding suggests that TRPA1 may be a downstream key effector of the nociceptive response induced by histamine. In fact, the finding that even a high dose of histamine (25 μg) is not able to increase the ceiling nociceptive effect mediated by AITC (100 μg) support this idea (Fig. 3c). Although the contribution of TRPA1 to histamine- and 5-HT-induced nociception in vivo has never been directly demonstrated before, in vitro studies have provided evidences that TRPA1 is gated in response to the activation of histamine [5] and 5-HT [9] receptors. This is in accordance with TRPA1 properties, since its gating promiscuity allow it to be gated by intracellular Ca^{2+} [5, 38] or in response to inflammatory mediators that activate phospholipase C (PLC) [21, 39, 40] or protein kinase A [41] signaling pathways. The histamine H1 receptor activates PLC [42], the ionotropic receptor 5-HT_3 increases intracellular Ca^{2+} levels [43] and although the primary function of 5-HT_{1A} receptors is to inhibit adenylyl cyclase, it is also involved in Gβγ-mediated stimulation of PLC [43]. Therefore, theoretically, TRPA1 could be activated downstream either the H1, 5-HT_{1A} or 5-HT_3 receptors.

Our results so far discussed demonstrate that AITC-induced nociception depends on histamine and 5-HT (Fig. 2b) and that histamine- or 5-HT-induced nociception depends on TRPA1 (Figs. 3b, 4b), suggesting an interdependence of the nociceptive mechanisms mediated by TRPA1, histamine and 5-HT. Based on this findings we hypothesized that enhanced nociceptive response could

emerge from non-nociceptive activation of TRPA1 and histamine or 5-HT receptors. In fact, the combination of non-nociceptive doses of AITC and histamine (Fig. 3c) or of AITC and 5-HT (Fig. 4c) induced a nociceptive response much higher than that expected by the sum of the effects induced by each drug alone. These findings indicate that AITC and histamine (Fig. 3d) or 5-HT (Fig. 4d) interact in a synergistic way to induce nociception. Actually, this interaction can be described as the ability of a small amount of histamine or 5-HT to potentiate and extend the nociceptive response induced by AITC in such a way that its ceiling nociceptive effect is achieved at a dose 10 times lower. This probably results from a broad TRPA1 activation in response to the combination of small doses of AITC and histamine or 5-HT. Of note is the ability of the H1 receptor antagonist to prevent the potentiation induced by histamine and of the 5-HT_{1A} or of the 5-HT_3 receptor antagonists to prevent the potentiation induced by 5-HT (Figs. 3c, 4c, respectively). These last findings demonstrate that the synergistic interaction between AITC and histamine or 5-HT is dependent on the activation of the H1, 5-$HT_{1A\ or\ 3}$ receptors. Although there is no data showing that the blockade of TRPA1 is also able to prevent the synergistic effect of AITC and histamine or 5-HT, this is quite possible, since AITC's effect, at a 10 times higher dose (100 µg), was blocked by the selective TRPA1 antagonist.

An important link between AITC and histamine and 5-HT is SP released in response to neuronal TRPA1 activation and known to induce mast cell degranulation [32, 33]. Therefore, we hypothesized that SP could also potentiate, in a synergistic way, the nociceptive effect induced by AITC and that this potentiation could be dependent on histamine and 5-HT. In fact, the addition of a non-nociceptive dose of SP potentiated and extended the nociceptive response induced by AITC, decreasing 10 times the dose needed to induce its ceiling nociceptive effect (Fig. 5b), in a clearly synergistic interaction (Fig. 5c). This finding fits well with evidences showing that TRPA1 contributes not only to the neurogenic release of SP, but also to the neuronal responses induced by SP [8]. SP, in turn, is released not only by nociceptive fibers, but also by mast cells [32]. Therefore, TRPA1 and SP reinforce each other's actions. The synergistic interaction between AITC and SP is largely dependent on the H1, 5-HT_{1A} and 5-HT_3 receptors, since it was blocked by the pharmacological blockade of each one of these receptors (Fig. 5b).

Since PLC pathway is the main signaling mechanism downstream the NK1 and the H1 receptor and it has also been associated to 5-HT_{1A} signaling, we hypothesized that a PLC activator should also potentiate AITC-induced nociception. In fact, 3-m3 m-FSB potentiated the nociceptive response induced by AITC, decreasing 10 times the dose needed to induce a nociceptive effect similar in magnitude to its ceiling nociceptive effect (Fig. 6). Since TRPA1 is gated downstream to PLC activation [21, 39, 40], the combination of small doses of AITC and PLC activator could induce a broad TRPA1 activation.

Based on the current knowledge about the mechanisms underlying TRPA1-mediated nociception, which mainly derives from in vitro studies, this study gathered functional data to support TRPA1 as key initiator and amplifier of inflammatory pain in vivo (Fig. 7). In an inflammatory site, numerous compounds are able to directly or indirectly activate TRPA1 [2]. In nociceptive C-fibers, TRPA1 activation induces nociception (Fig. 7, #1) and releases SP (Fig. 7, #2). SP activates NK1 receptors in mast cells to induce their degranulation (Fig. 7, #3). The consequent release of histamine, 5-HT and SP [32] amplifies the response. On nociceptive C-fibers, the activation of 5-HT_3 receptors also induces nociception (Fig. 7, #1) and increases intracellular calcium (Fig. 7, #4). The activation of H1, NK1 and, perhaps, 5-HT_{1A} receptors activates PLC (Fig. 7, #5) originating its downstream signaling molecules and further increasing intracellular calcium. Either increased calcium or PLC activation are known to activate intracellularly TRPA1 (Fig. 7, #6) [2]. Therefore, a self-sustainable cycle is generated around TRPA1, no matter where the cycle is initiated each step is achieved and the blockade of just one step could be able to block the overall pain response. Accordingly to the present data, even subeffective activation of more than one step results in a synergistic activation of the overall cycle.

In summary, this study provides functional data to support interdependence in the nociceptive mechanisms

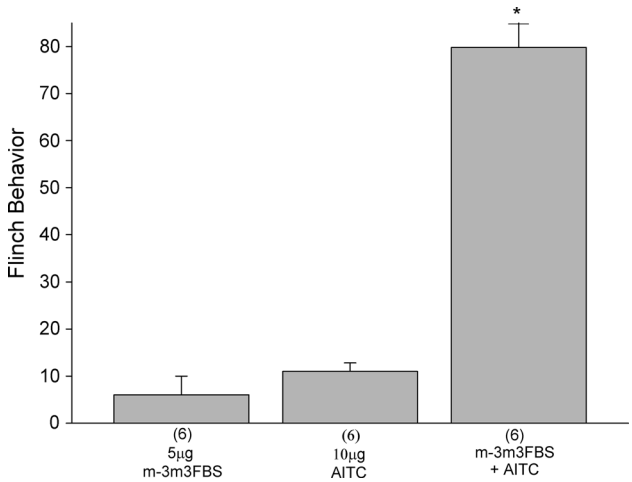

Fig. 6 A phospholipase C activator potentiates AITC-induced nociception. **a** The combination of non-nociceptive doses of the phospholipase C activator, m-3m3FBS (5 µg) and AITC (10 µg) induced a nociceptive response higher than that expected by the sum of the effect of each one alone. The symbol "*" indicates a nociceptive response significantly higher than that of the other groups (one-way ANOVA and Tukey test, $p < 0.05$)

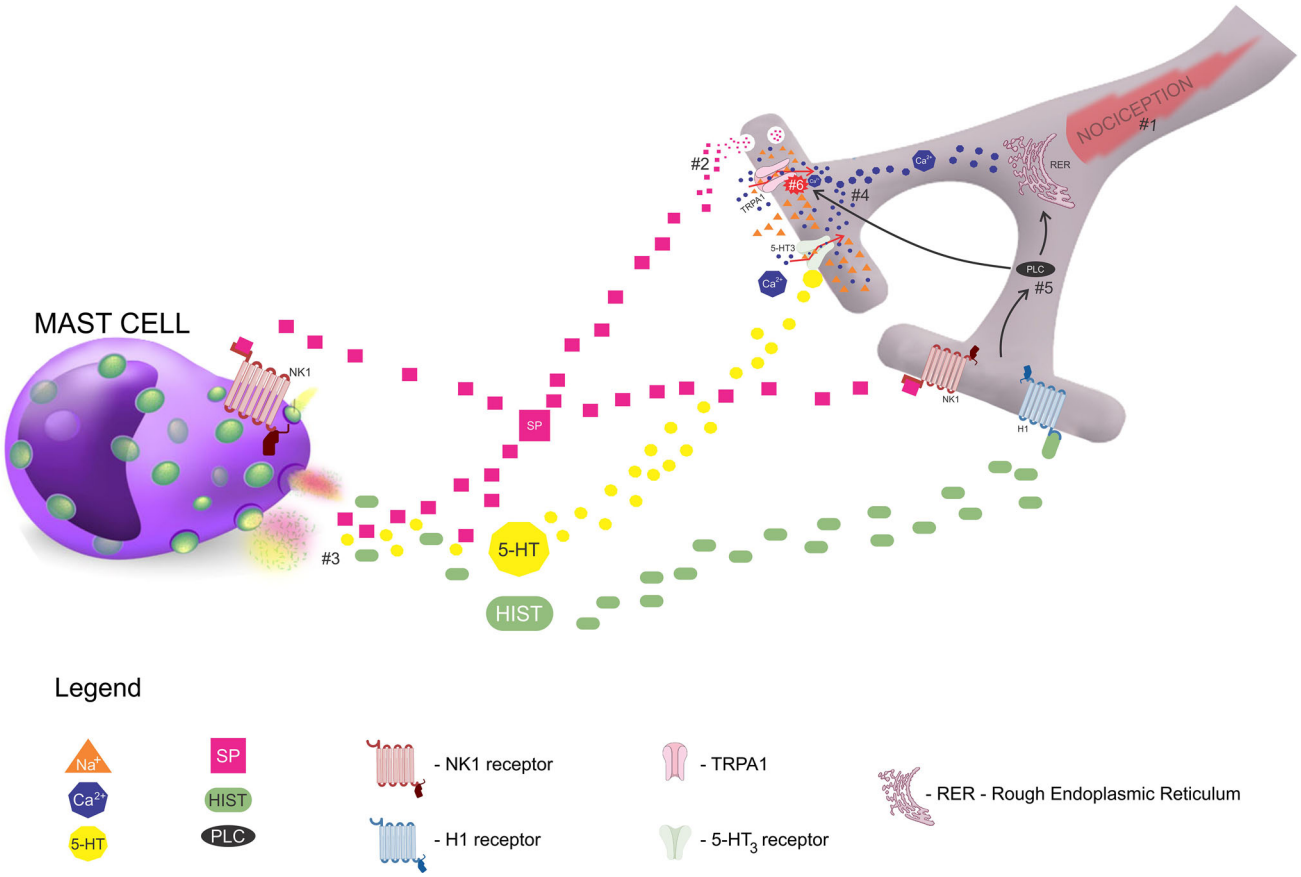

Fig. 7 A self-sustainable cycle around TRPA1. TRPA1 activation induces nociception (*#1*) and releases SP (*#2*), which activates NK1 receptors in mast cells to induce their degranulation (*#3*). Histamine, 5-HT and SP contribute to increase intracellular calcium (*#4*) and activate PLC (*#5*), further contributing to TRPA1 activation (*#6*)

mediated by TRPA1, SP, histamine and 5-HT in vivo. The potent synergism emerging from their in vivo interaction certainly contributes to exacerbate inflammatory pain. Therefore, strategies to limit this interaction, based on the concomitant blockade of two or more resulting mechanisms, are of potential therapeutic interest.

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References

1. Julius D. TRP channels and pain. Annu Rev Cell Dev Biol. 2013;29:355–84.
2. Bautista DM, Pellegrino M, Tsunozaki M. TRPA1: a gatekeeper for inflammation. Annu Rev Physiol. 2013;75:181–200.
3. Andrade EL, Luiz AP, Ferreira J, Calixto JB. Pronociceptive response elicited by TRPA1 receptor activation in mice. Neuroscience. 2008;152:511–20.
4. Perin-Martins A, Teixeira JM, Tambeli CH, Parada CA, Fischer L. Mechanisms underlying transient receptor potential ankyrin 1 (TRPA1)-mediated hyperalgesia and edema. J Peripher Nerv Syst. 2013;18:62–74.
5. Cavanaugh EJ, Simkin D, Kim D. Activation of transient receptor potential A1 channels by mustard oil, tetrahydrocannabinol and Ca^{2+} reveals different functional channel states. Neuroscience. 2008;154:1467–76.
6. Delgermurun D, Yamaguchi S, Ichii O, Kon Y, Ito S, Otsuguro K. Hydrogen sulfide activates TRPA1 and releases 5-HT from epithelioid cells of the chicken thoracic aorta. Comp Biochem Physiol C Toxicol Pharmacol. 2016;187:43–9.
7. Doihara H, Nozawa K, Kojima R, Kawabata-Shoda E, Yokoyama T, Ito H. QGP-1 cells release 5-HT via TRPA1 activation; a model of human enterochromaffin cells. Mol Cell Biochem. 2009;331:239–45.
8. Liu B, Escalera J, Balakrishna S, Fan L, Caceres AI, Robinson E, et al. TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. FASEB J. 2013;27:3549–63.
9. Morita T, McClain SP, Batia LM, Pellegrino M, Wilson SR, Kienzler MA, et al. HTR7 mediates serotonergic acute and chronic itch. Neuron. 2015;87:124–38.
10. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983;16:109–10.
11. Bonet IJ, Fischer L, Parada CA, Tambeli CH. The role of transient receptor potential A 1 (TRPA1) in the development and maintenance of carrageenan-induced hyperalgesia. Neuropharmacology. 2013;65C:206–12.
12. Dall'Acqua MC, Bonet IJ, Zampronio AR, Tambeli CH, Parada CA, Fischer L. The contribution of transient receptor potential

ankyrin 1 (TRPA1) to the in vivo nociceptive effects of prostaglandin E(2). Life Sci. 2014;105:7–13.
13. Naono-Nakayama R, Sunakawa N, Ikeda T, Matsushima O, Nishimori T. Subcutaneous injection of endokinin C/D attenuates carrageenan-induced inflammation. Peptides. 2010;31:1767–71.
14. Parada CA, Tambeli CH, Cunha FQ, Ferreira SH. The major role of peripheral release of histamine and 5-hydroxytryptamine in formalin-induced nociception. Neuroscience. 2001;102:937–44.
15. Mello GC, Desouza IA, Marangoni S, Novello JC, Antunes E, Macedo ML. Oedematogenic activity induced by Kunitz-type inhibitors from *Dimorphandra mollis* seeds. Toxicon. 2006;47:150–5.
16. Gadotti VM, Schmeling LO, Machado C, Liz FH, Filho VC, Meyre-Silva C, et al. Antinociceptive action of the extract and the flavonoid quercitrin isolated from *Bauhinia microstachya* leaves. J Pharm Pharmacol. 2005;57:1345–51.
17. Papir-Kricheli D, Frey J, Laufer R, Gilon C, Chorev M, Selinger Z, et al. Behavioural effects of receptor-specific substance P agonists. Pain. 1987;31:263–76.
18. Nakamura Y, Une Y, Miyano K, Abe H, Hisaoka K, Morioka N, et al. Activation of transient receptor potential ankyrin 1 evokes nociception through substance P release from primary sensory neurons. J Neurochem. 2012;120:1036–47.
19. Zhao L, Au JL, Wientjes MG. Comparison of methods for evaluating drug–drug interaction. Front Biosci (Elite Ed). 2010;2:241–9.
20. Andre E, Gatti R, Trevisani M, Preti D, Baraldi PG, Patacchini R, et al. Transient receptor potential ankyrin receptor 1 is a novel target for pro-tussive agents. Br J Pharmacol. 2009;158:1621–8.
21. Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, et al. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell. 2006;124:1269–82.
22. Brozmanova M, Mazurova L, Ru F, Tatar M, Kollarik M. Comparison of TRPA1-versus TRPV1-mediated cough in guinea pigs. Eur J Pharmacol. 2012;689:211–8.
23. Sculptoreanu A, Kullmann FA, Artim DE, Bazley FA, Schopfer F, Woodcock S, et al. Nitro-oleic acid inhibits firing and activates TRPV1- and TRPA1-mediated inward currents in dorsal root ganglion neurons from adult male rats. J Pharmacol Exp Ther. 2010;333:883–95.
24. Someya S, Nagao M, Shibata C, Tanaka N, Sasaki H, Kikuchi D, et al. Intracolonic administration of the TRPA1 agonist allyl isothiocyanate stimulates colonic motility and defecation in conscious dogs. J Gastrointest Surg. 2016;19:1342–9.
25. Eckert WA 3rd, Julius D, Basbaum AI. Differential contribution of TRPV1 to thermal responses and tissue injury-induced sensitization of dorsal horn neurons in laminae I and V in the mouse. Pain. 2006;126:184–97.
26. Everaerts W, Gees M, Alpizar YA, Farre R, Leten C, Apetrei A, et al. The capsaicin receptor TRPV1 is a crucial mediator of the noxious effects of mustard oil. Curr Biol. 2011;21:316–21.
27. Gees M, Alpizar YA, Boonen B, Sanchez A, Everaerts W, Segal A, et al. Mechanisms of transient receptor potential vanilloid 1 activation and sensitization by allyl isothiocyanate. Mol Pharmacol. 2013;84:325–34.
28. Ohta T, Imagawa T, Ito S. Novel agonistic action of mustard oil on recombinant and endogenous porcine transient receptor potential V1 (pTRPV1) channels. Biochem Pharmacol. 2007;73:1646–56.
29. Tekus V, Horvath A, Hajna Z, Borbely E, Bolcskei K, Boros M, et al. Noxious heat threshold temperature and pronociceptive effects of allyl isothiocyanate (mustard oil) in TRPV1 or TRPA1 gene-deleted mice. Life Sci. 2016;154:66–74.
30. Weller K, Reeh PW, Sauer SK. TRPV1, TRPA1, and CB1 in the isolated vagus nerve–axonal chemosensitivity and control of neuropeptide release. Neuropeptides. 2011;45:391–400.
31. Trevisani M, Siemens J, Materazzi S, Bautista DM, Nassini R, Campi B, et al. 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. Proc Natl Acad Sci USA. 2007;104:13519–24.
32. Chatterjea D, Martinov T. Mast cells: versatile gatekeepers of pain. Mol Immunol. 2015;63:38–44.
33. Mantyh PW. Neurobiology of substance P and the NK1 receptor. J Clin Psychiatry. 2002;63(Suppl 11):6–10.
34. Page NM. Hemokinins and endokinins. Cell Mol Life Sci. 2004;61:1652–63.
35. Macpherson LJ, Xiao B, Kwan KY, Petrus MJ, Dubin AE, Hwang S, et al. An ion channel essential for sensing chemical damage. J Neurosci. 2007;27:11412–5.
36. McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, et al. TRPA1 mediates formalin-induced pain. Proc Natl Acad Sci USA. 2007;104:13525–30.
37. Trevisan G, Rossato MF, Hoffmeister C, Oliveira SM, Silva CR, Matheus FC, et al. Mechanisms involved in abdominal nociception induced by either TRPV1 or TRPA1 stimulation of rat peritoneum. Eur J Pharmacol. 2013;714:332–44.
38. Sura L, Zima V, Marsakova L, Hynkova A, Barvik I, Vlachova V. C-terminal acidic cluster is involved in Ca^{2+}-induced regulation of human transient receptor potential ankyrin 1 channel. J Biol Chem. 2012;287:18067–77.
39. Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, et al. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron. 2004;41:849–57.
40. Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Hogestatt ED, et al. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature. 2004;427:260–5.
41. Wang S, Dai Y, Fukuoka T, Yamanaka H, Kobayashi K, Obata K, et al. Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a molecular mechanism of inflammatory pain. Brain. 2008;131:1241–51.
42. Arrang JM. Pharmacological properties of histamine receptor subtypes. Cell Mol Biol (Noisy-le-grand). 1994;40:275–81.
43. Pucadyil TJ, Kalipatnapu S, Chattopadhyay A. The serotonin 1A receptor: a representative member of the serotonin receptor family. Cell Mol Neurobiol. 2005;25:553–80.