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Unexpected Ca^{2+}-mobilization of oxaliplatin via H1 histamine receptors

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ABSTRACT

Oxaliplatin is a widely used chemotherapeutic drug and represents the cornerstone of colorectal cancer therapy, in combination with 5-fluorouracil and folinic acid. As with many chemotherapeutic agents, its use is associated with a number of side effects, ranging from hypersensitivity reactions to haematological dyscrasias. Oxaliplatin also induces acute and chronic peripheral neuropathy.

While it is likely that the haematological side effects are associated with its anti-proliferative effects and with the ability to form DNA adducts, the molecular mechanisms underlying peripheral neuropathy and hypersensitivity reactions are poorly understood, and therefore the choice of adequate supportive therapies is largely empirical.

Here we show that an acute low dose oxaliplatin application on DRG neurons is able to induce an increase in intracellular calcium that is dependent on the Histamine 1 receptor (H1). Oxaliplatin-induced intracellular calcium rises are blocked by two selective H1 antagonist, as well as by U73122, a PLC inhibitor, and by 2-APB, a non-specific IP_3 receptor blocker. Moreover, expression of the H1 receptor on HEK293 t cells unmasks an oxaliplatin-induced Ca^{2+}-rise. Last, activation of H1 *via* either histamine or oxaliplatin activates TRPV1 receptors, a mechanism that has been associated with itch. These data, together with literature data that has shown that anti-histamine agents reduce the incidence of oxaliplatin-induced hypersensitivity, may provide a molecular mechanism of this side effect in oncological patients.

1. Introduction

Oxaliplatin (OHP) is a pillar chemotherapeutic agent in colorectal cancer therapy, mainly in combination with 5-fluorouracil and folinic acid [1]. Its primary mechanism of action can be re-conducted to the formation of inter-strand and intrastrand adducts with DNA.

While the formation of DNA adducts underlies most likely the haematological effects of OHP, *e.g.* anemia, thrombocytopenia and neutropenia, it is less likely to be responsible for other side effects, including peripheral neuropathy and hypersensitivity reactions [2,3]. OHP-induced peripheral neuropathy is thought to arise from sensitization of TRP channels in dorsal root ganglia (DRG) neurons [4]. Indeed, either pharmacological antagonism or genetic ablation of TRPA1 and TRPV1 channels renders rodents less susceptible to OHP-induced cold allodynia [5,6].

Modification of the Ca^{2+}-toolkit by OHP has been shown in DRG neurons, in which prolonged exposure to OHP (24 h) leads to a sensitization of ATP-induced Ca^{2+}-release [7]. We have recently hypothesized that these modifications are most likely a result of pH acidification of the cytosol [8]. In other words, OHP affects indirectly calcium signalling by modifying intracellular pH and therefore modulating pH-sensitive channels, including TRPA1 and TRPV1, or by triggering transcriptional rearrangements [9]. Indeed, a change in the qualitative and quantitative components of the calcium toolkit has been shown to affect Ca^{2+}-signals in a number of conditions [10].

Yet, these mechanisms are unlikely to explain acute side effects, including hypersensitivity reactions. In the present manuscript, we set to investigate the acute effects of OHP on DRG neurons, as contrasting effects have been reported. Shultze et al. reported that low micromolar concentrations of OHP do not induce any Ca^{2+}-signal in DRG cells or in the SH-SY5Y neuroblastoma cells [7], while Kawashiri et al. showed that OHP, at non-therapeutic concentrations induced an increase in intracellular Ca^{2+} secondary to voltage-operated Ca^{2+}-channel opening [11]. Such contrasting reports may well be attributed to the concentrations used, given that it is highly likely that the higher the concentration, the more non-specific protein adducts will be formed.

We now report that acute treatment of DRG neurons with therapeutic concentrations of OHP (0.1 μg/ml) leads to Ca^{2+}-release that is dependent on type 1 histamine receptors (H1). How this occurs is at present unknown, although the fact that the effect is antagonized by a

Abbreviations: OHP, Oxaliplatin; DRG, dorsal root ganglia

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competitive antagonist of H1 would lead to speculate that OHP binds close to the histamine site. Furthermore, activation of H1 receptors with OHP, as it has been previously reported for histamine [12], leads to opening and desensitization of TRPV1 receptors. The interaction between TRPV1 and H1 receptors in DRG neurons has been associated with histamine-induced itch [12]. These data, together with the knowledge that histamine H1 antagonists reduce the percentage of patients hypersensitivity reactions and the itch induced by OHP [13], might therefore provide a molecular mechanism for this side effect and a rationale for a supportive therapy.

2. Materials and methods

2.1. Animals and husbandry

BALB/C male mice aged 4–8 weeks were purchased from Envigo (San Pietro al Natisone, Italy). Care and husbandry of animals were in conformity with the institutional guidelines in compliance with national and international laws and policies. Mice were housed in cages in 22 °C monitored rooms with 12 h light/dark cycles with *ad libitum* access to food and water and weaned at 23 days old by sex. The procedures were approved by the local animal-health and ethical Committee (Università del Piemonte Orientale) and were authorized by the national authority (Istituto Superiore di Sanità; authorization number N. 22/2013). All mice were euthanized under deep CO_2-induced anaesthesia.

2.2. Drugs

Oxaliplatin (OHP), trazodone, haloperidol, ketanserin, cetirizine, loratadine, histamine (HIST), capsazepine, capsaicin and icilin were purchased from Sigma-Aldrich Inc., Italy. Nigericin, BCECF (2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) and Fura 2-AM were purchased from Life Technologies, Italy. These drugs, with the exception of capsaicin (reconstituted in 100 % EtOH), capsazepine (reconstituted in 100 % methanol) and histamine (reconstituted in milliQ (MilliPore) water), were dissolved in 100 % dimethyl sulfoxide (DMSO) and stored at −20 °C, according to manufacturers' specifications. Working concentrations of these drugs were freshly prepared for each experiment by diluting DMSO, methanol or EtOH to 0.1 % in milliQ (MilliPore) water.

2.3. Isolation and primary cell culture of mouse DRG neurons

DRG obtained from adult BALB/C mice (4/8-wk-old) were excised and collected in a dish containing cold F12 (Nutrient Mixture F12 Ham) medium (Sigma Aldrich Inc.). Working under a dissecting microscope and using fine forceps, the surrounding membranes were gently teased away from each DRG; nerves and sheath were cut. All de-sheathed DRG were then transferred into a sterile 35 mm dish containing collagenase from *Clostridium* hystoliticum 0.125 % (Sigma Aldrich Inc., Italy) and DNase (Sigma Aldrich Inc., Italy) in F12 (Nutrient Mixture F12 Ham) medium and incubated at 37 °C for 1 h. After incubation, DRG were triturated using a 1000 µl tip. Myelin and nerve debris were eliminated by centrifugation through a bovine serum albumin (BSA) cushion. Cell pellets were re-suspended in Bottenstein and Sato medium (BS) composed of 30 % F12 (Nutrient Mixture F12 Ham medium), 40 % DMEM (Dulbecco's Modified Eagle's medium (Sigma Aldrich Inc., Italy), 30 % Neurobasal A medium (Life Technologies, Italy), 100 X N2 supplement (Life Technologies, Italy), penicillin 10 U/mL and streptomycin 100 mg/mL (Sigma Aldrich Inc., Italy), supplemented with Recombinant Human β-NGF, Recombinant Murine GDNF and Recombinant Human NT3 (Peprotech, USA) and plated onto 24 mm glass coverslips pre-coated with laminin (Sigma Aldrich Inc., Italy).

2.4. Cell cultures

Human embryonic kidney (HEK293t) cells were obtained from ATCC (Rockville, MD, USA) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Inc., Italy), supplemented with 10 % heat-inactivated FBS (Gibco, Italy), L-glutamine 50 mg/mL (Sigma-Aldrich, Italy), 10 U/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich Inc., Italy) at 37 °C, under a 5 % CO_2 humidified atmosphere.

Human primary lung airway smooth muscle cells (ASM) were obtained from ATCC (Rockville, MD, USA) and were cultured in Vascular Cell Basal Medium (VCBM; ATCC, Rockville, MD, USA) supplemented with 5 % heat-inactivated fetal bovine serum (FBS), L-glutamine 50 mg/mL (Sigma-Aldrich, Italy), 10 U/mL penicillin, and 100 mg/mL streptomycin, 0,5 % antibiotic-antimycotic (ThermoFisher, Italy), 5 ng/ml of basic-fibroblasts growth factor and 5 ng/ml epidermal growth factor (Immunotools), 50 µg/ml of ascorbic acid, 10 ng/ml of insulin (Sigma-Aldrich Inc., Italy).

For calcium experiments, cell lines were seeded on poly-L-lysine (Sigma-Aldrich Inc., Italy) coated glass coverslips at concentrations 10×10^3 per mL (24 mm diameter coverslips in 6-well plates).

2.5. Generation of Hek Cells overexpressing H1 and TRPV1 receptors

HEK293 t cells overexpressing H1 receptors were maintained in a 37 °C, 5 % CO_2 humidified incubator as described for HEK293 t cells. The human H1 (pH1R-P2A-mCherry-N1)receptor was purchased from Addgene. The Rat pcDNA3-TRPV1 was a kind gift from Prof. Asia Fernandez Carvajal and was confirmed by sequencing. For cell transfection, 7 µg of plasmid DNA was transfected using lipid reagent lipofectamine (Lipofectamine 2000 Transfection Reagent, Life Technologies). 24 h after transfection, PCR was performed to evaluate the gene expression.

2.6. Gene expression evaluation by PCR

Total RNA was isolated from HEK, HEK-H1, HEK-H1-TRPV1 cells using TRI-Reagent® and reverse transcribed according to the manufacturer's instructions (SENSIFAST, Aurogene, Italy). cDNA was then stored at −20 °C until further used. The PCR reaction was conducted using 2x DreamTaq Hot Start Green PCR Master Mix (ThermoFisher, Italy), forward and reverse specific primers (H1: FW: 5′- CTTGGTCAC AGTAGGGCTCA-3′, REV: 5′-TGGGGAACCTGTACATCGTC-3′; TRPV1: TRPV1, FW:5′-CCTGCATTGACACCTGTGAG-3′; REV: 5′-AGAAGATGC GCTTGACAAATC-3′), and cDNA obtained as previously described. The electrophoresis of the PCR products were performed in a 2 % agarose gel (Euroclone spa, Italy).

2.7. Fura-2 Ca^{2+} measurements and image analysis

DRG cultures, HEK or HEK-H1 cells were loaded with 5 µM Fura-2 AM in presence of 0.02 % of Pluronic-127 (both from Life Technologies, Italy) and 10 µM sulfinpyrazone (Sigma Adrich Inc., Italy) in Krebs–Ringer buffer (KRB, 135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) containing 2 mM $CaCl_2$ (30 min, room temperature). Cells were washed and incubated with KRB for 15 min to allow de-esterification of Fura-2. Coverslips were mounted into acquisition chamber and places on the stage of a Leica DMI6000 epifluorescent microscope equipped with S Fluor ×40/1.3 objective. The probe was excited by alternate 340 and 380 nm using a Polychrome IV monochromator (Till Photonics, Munich, Germany) and the Fura-2 emission light was filtered through 520/20 bandpass filter and collected by a cooled CCD camera (Hamamatsu, Japan). The fluorescence signals were acquired and processed using MetaFluor software (Molecular Device, Sunnyvale, CA, USA). To quantify the differences in the amplitudes of Ca^{2+} transients the ratio values were

normalized using the formula ΔF/F0. Only responsive cells were used for statistical analysis. Responsive cells were defined as having increase that varied by at least an order of magnitude compared to the the basal calcium.

2.8. Measurement of intracellular pH in DRG neurons by epifuorescent microscopy with BCECF

DRG cells were plated onto 24 mm round cover-slips, incubated with 1 μM BCECF (Life Technologies, Italy) in KRB containing 2 mM $CaCl_2$ (15 min, room temperature) and re-suspended in KRB (pH 7.4). Coverslips were mounted into acquisition chamber and places on the stage of a Leica DMI6000 epifluorescent microscope equipped with S Fluor ×40/1.3 objective. Cells were alternatively excited at 490/450 nm (monochromator Polychrome IV, Till Photonics, Germany) and the fluorescent signals were collected and analysed every 10 s using MetaFluor software (Molecular Devices, Sunny-vale, CA, USA). Intracellular pH was calculated by comparing 525/610 nm emission fluorescence ratios with calibration curves obtained by pH equilibration using the proton ionophore nigericin (10 μM) and the Intracellular pH Calibration Buffer Kit (pH 7.5-5.5, Life Technologies, Italy).

2.9. Statistical analysis

Data are presented as mean ± SEM or Median and IQR. The normality of data distributions was assessed using Shapiro–Wilk test. Parametric (unpaired *t*-test and One-way analysis of variance (ANOVA) followed by Tukey's post-hoc) or non parametric (Mann-Whitney *U* test and One-way Kruskal-Wallis H test followed by Dunn's post-hoc) statistical analysis was used for comparisons of data. All statistical assessments were two-sided and a value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., USA). In *in vitro* experiments, the n number was calculated on the number of cells, and the number of independent experiments (defined as cultures performed on different days) is given in the respective figure legends.

3. Results

3.1. Oxaliplatin increases intracellular calcium through activation of Gq coupled receptors

Contrasting evidence exists in the literature on whether acute treatment with OHP induces an acute Ca^{2+}-response in cells [4]. To analyse this aspect, we exposed DRG cultures to concentrations between 0.003 μg/mL and 0.3 μg μg/mL (75 − 750 nM), and found that OHP dose-dependently increased intracellular Ca^{2+} (Fig. 1A and B) with small differences between the different concentration used (responding cells were: OHP 0.03 μg/mL: 38 ± 11.1 %; OHP 0.1 μg/mL 53 ± 10 %; OHP 0.3 μg/mL: 60 ± 17 %). Therapeutically relevant concentrations of OHP (0.1 μg/mL) induced a rapid rise in intracellular calcium, which was evident also when cells were placed in a Ca^{2+}-free solution (Fig. 1C and E for quantification), suggesting that the source of the ion was an intracellular store. When cells were analysed further, no oscillations or other signals were observed over the course of 10 min apart from the first spike (data not shown). To further investigate the mechanism of the Ca^{2+}-rise, cells were pre-incubated for 10 min with a PLC inhibitor, U73122 (5 μM), or with a non-specific IP_3 channel inhibitor, 2-APB (50 μM). Both agents abolished the response to OHP (Fig. 1D and E), proving that OHP activates a receptor coupled to Gq and its downstream PLC-IP_3 receptor pathway.

3.2. Oxaliplatin is an agonist of the H1 histamine receptor

To pinpoint the receptor that might have been responsible for OHP-induced Ca^{2+}-release, we used a number of multi-receptor antagonists and compared the number of responding cells in the presence or absence of these antagonists (OHP 0.1 μg/mL responding cells were 53 ± 10 %; n = 122). In the presence of haloperidol (500 nM), that at these concentrations blocks D2, D3, D4, H1, M1, α1, and 5HT2A-C [14], or trazodone (1 μM), a H1, 5HT2A-C, 5HT1, α1 antagonist [14], OHP elicited responses in 11 % (2/18) and 0 % (0/33) of cells, respectively. On the other hand, ketanserin, a specific 5HT2A-C antagonist was largely unable to affect responses, with 67 % of cells (30/45) responding to OHP. This led us to hypothesize that H1 receptors might have mediated the OHP response.

To verify the hypothesis generated with multi-receptor antagonists, we then challenged DRG neurons pre-treated with H1 selective antagonists (cetirizine, 50 nM; loratidine, 100 nM). As it can be observed in Fig. 2A and B, both antagonists fully blocked the response to OHP. To further validate this hypothesis, we took advantage of the desensitization of H1 receptor after exposure with high histamine concentration [15] and evaluated whether Ca^{2+}-responses to OHP and histamine cross-desensitized. As shown in Fig. 2C and D, when cells were challenged with high concentrations of histamine (300 μM) they were unable to respond to OHP (0.1 μg/mL) while they still responded to ATP (100 μM), indicating that the effect was not due to a non specific desensitazion of its downstream cascade. The opposite experiment was also true (Fig. 2E and F), as when cells were challenged with high concentrations of OHP (3 μg/ml) they were unable to respond to a challenge to histamine, but still responded to ATP. Homologous or heterologous desensitization did not occur, instead, when lower concentrations of either OHP (0.1 μg/mL) or histamine (10 μM) were used (supplementary Fig. S1). These data indicate that, surprisingly, OHP behaves as an agonist of the H1 receptor. In our cultures, the % of histamine-responsive neurons was 52.4 ± 6.3 % (n = 246) of the total population, which was comparable to the OHP-sensitive population (53 ± 0.1 %; see above). It should be noticed that previous reports suggested that the number of histamine-sensitive neurons was lower [16], and this discrepancy might be explained by culture methods, strains of mice used or other factors.

The hypothesis that H1 is the receptor mediating OHP-induced Ca^{2+}-release is further substantiated by the fact that we observed a response both to histamine or OHP in ASM (Fig. S2), a bronchial smooth muscle cell line which is known to respond to histamine [17], while we did not observe a response with either agent in HEK293 t cells (Fig. 3), in which we could not detect a transcript for H1 (Fig. 3A, inset). To conclusively confirm that OHP releases Ca^{2+} *via* H1 receptors, we therefore decided to transiently express H1 in HEK293 t cells (Fig. 3A, inset). As shown in Fig. 3A and B, the presence of H1 receptors conferred sensitivity of cells to both histamine (10 μM) and OHP (0.1 μg/ml).

3.3. H1 activation and cytosolic pH acidification are distinct phenomena

We have previously shown that OHP induces intracellular acidification in DRG neurons and that this sensitizes TRPA1 and blunts responses to TRPV1 channels [8]. In the original report [8], we showed that this acidification occurs after a 6-h treatment although further characterization has shown that acidification occurs as early as 30 min [18]. We therefore decided to evaluate whether these two phenomena were linked, *i.e.* whether Ca^{2+}-responses are linked to cytosolic acidification, or, *viceversa*, whether it is the acidification that is responsible for the Ca^{2+}-release. We first determined whether we could detect a significant pH change in the first five minutes of addition of OHP, but this was not the case (pH CTRL 7.04 ± 0.02 n = 41; OHP 6.97 ± 0.03 n = 56). Furthermore, we evaluated whether OHP, in the presence of H1 antagonists was still able to acidify the cytosol after 30 min. Indeed, this was the case (pH CTRL 7.04 ± 0.02; n = 38; OHP 6.73 ± 0.049 n = 49; CTZ + OHP: 6,77 ± 0.026 n = 25). Last, we could detect a pH change in HEK293 t cells after 30 min [18], despite the fact that these cells do not possess H1 receptors transcripts and neither OHP nor

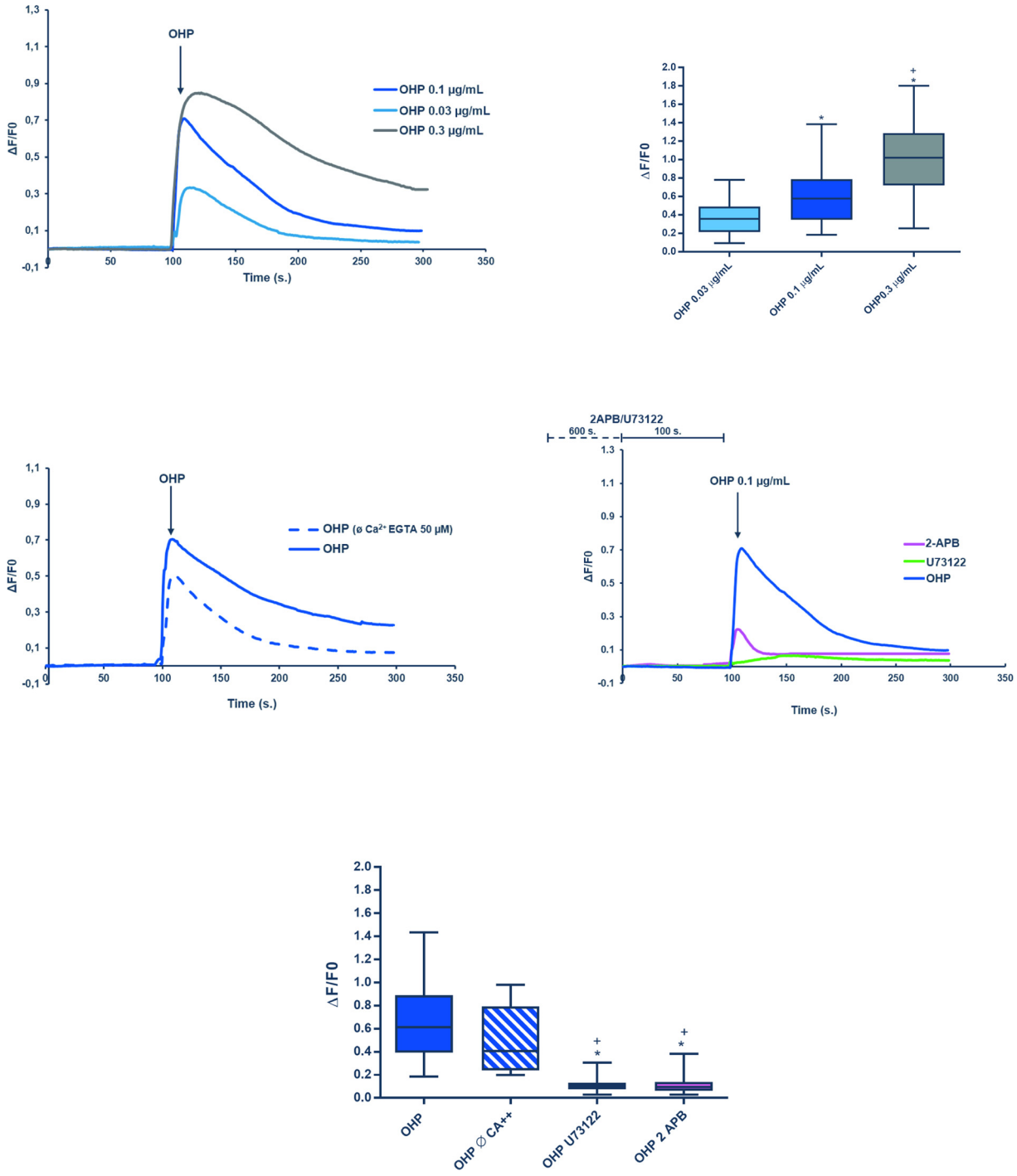

Fig. 1. Oxaliplatin increases intracellular calcium through activation of G_q coupled receptors.
A Representatives Ca^{2+}-traces from DRG neurons treated with OHP. Traces are representative of three independent cultures. The number of cells evaluated is indicated in the figure. B Box and whisker plots show median and IQR of peak of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* OHP 0.03 µg/mL, $^{+}$ $P < 10^{-6}$ *vs* OHP 0.1 µg/mL. C Representative Ca^{2+}-traces from DRG neurons treated with 0.1 µg/mL of OHP in the presence or absence of EGTA 50 µM. Traces are representative of three independent cultures. The number of cells evaluated is indicated in the figure. D Representative Ca^{2+}-traces from DRG neurons treated with 0.1 µg/mL of OHP with or without pre-incubation with the PLC inhibitor U73122 (5 µM) or the non-specific IP_3R blocker 2-APB (50 µM). Traces are representative of three independent cultures. The number of cells evaluated is indicated in the figure. E Box and whisker plots show median and IQR of peak of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* OHP, $^{+}$ $P < 10^{-6}$ *vs* OHP Ø Ca^{2+}.

histamine induced Ca^{2+}-release. These data suggest that the effect of OHP on H1 receptors and the effect on intracellular pH are mediated by separate mechanisms.

3.4. OHP, like histamine, activates TRPV1 receptors

It has been previously reported that histamine is able to modulate TRPV1 responses, partially contributing to histamine-induced itching [12]. As shown in Fig. 4A, pre-treatment of DRG cells with histamine led to a significant reduction in the subsequent Ca^{2+}-response induced

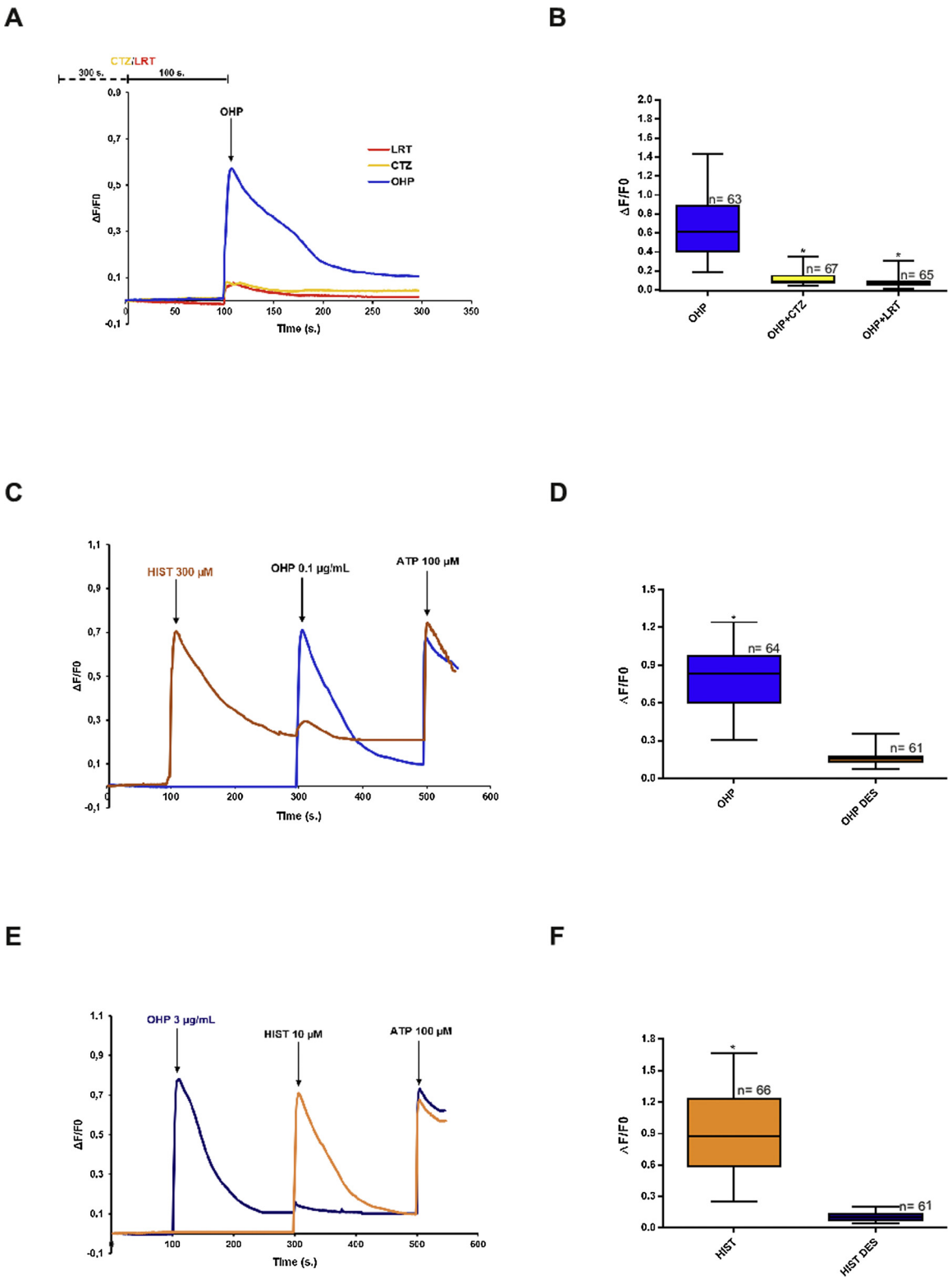

Fig. 2. H1 antagonists prevent OHP-induced Ca^{2+}-increases.
A Representatives Ca^{2+}-traces from DRG neurons treated with 0.1 µg/mL of OHP in the presence or absence of cetirizine (50 nM) or loratadine (100 nM). Traces are representative of four independent cultures. The number of cells evaluated is indicated in the figure. B Box and whisker plots show median and IQR of peak of Ca^{2+} changes. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* OHP. C and E Representative Ca^{2+}-traces from DRG neurons treated with 300 µM histamine (C) or 3 µg/mL OHP (E) to desensitize the H1 receptor and then treated with OHP 0.1 µg/mL (C) or histamine 10 µM (E), respectively. ATP was used as a control at the end of each experiment. Traces are representative of four independent cultures. The number of cells evaluated is indicated in the figure. D Box and whisker plots show median and IQR of OHP peak of Ca^{2+} induced by OHP. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* OHP DES. F Box and whisker plots show median and IQR of the Ca^{2+}-peak induced by histamine. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* HIST DES.

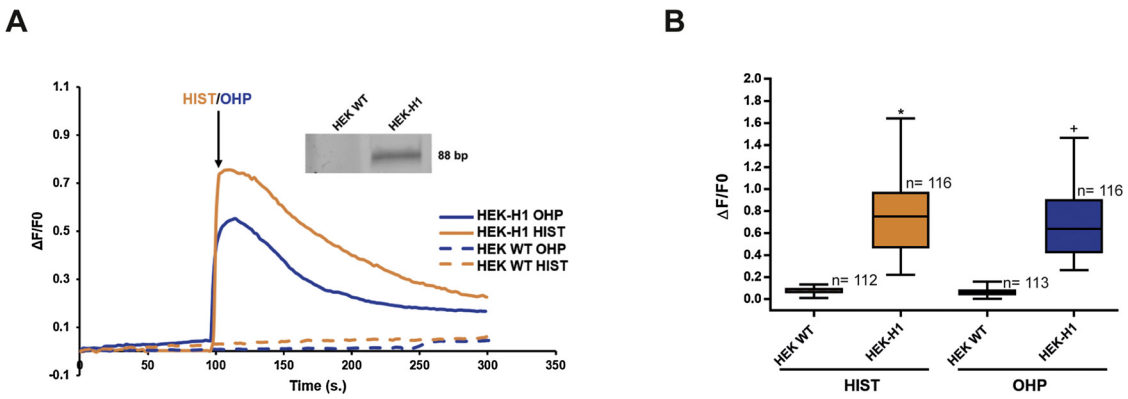

Fig. 3. H1 receptors expression is required for OHP-induced intracellular calcium rises.
A Representative Ca^{2+}-traces from HEK WT and HEK-H1 treated with 10 μM histamine or 0.1 μg/mL OHP. Traces are representative of three independent experiments. The number of cells evaluated is indicated in the figure. B Box and whisker plots show median and IQR of peak of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* HEK WT, $^{+}P < 10^{-6}$ *vs* HEK WT.

by capsaicin both when compared to cells that did not respond to histamine (presumably due to the absence of the H1 receptor). An identical result was obtained when cells were pre-treated with OHP. More importantly, the effect was totally reversed when experiments were performed in the presence of cetirizine (Fig. 4B, C). Furthermore, the number of capsaicin responsive cells decrease after treatment with histamine or OHP, an effect that suggest a desensitization of TRPV1 receptors and that was abolished by pre-treatment with cetirizine (Fig. 4D). It should be noticed that in our hands the number of capsaicin-sensitive cells is higher (90.5 ± 3.1 %) than what previously reported by others [19,20], as is the case for histamine-sensitive neurons. The characterization of the diameters of our cell culture population has been recently published [8]. Last, we performed experiments in the presence of the selective antagonist of TRPV1, capsazepine (10 μM). As shown in Fig. 4E–G, this inhibitor reduced both the amplitude of the Ca^{2+}-peak and the area under the curve, providing further evidence for the involvement of TRPV1 in the H1-induced signals.

To verify whether OHP had a direct effect on TRPV1 receptors, we tested OHP on HEK cells expressing TRPV1. In these cells, no activity by OHP or histamine was observed (Fig. S3). To further investigate whether the abolition of the capsaicin response observed in DRG neurons pre-treated with OHP or histamine was the consequence of the desensitization of TRPV1 channels, we co-expressed H1 and TRPV1 receptors in HEK293 t cells (HEK H1-V1) and we compared the response elicited by OHP or histamine with cells transfected exclusively with H1. HEK-H1-V1 cells, as expected, responded to histamine, OHP and capsaicin. Yet, if cells were pre-treated with OHP or histamine, no response to capsaicin was observed (Fig. 5A and B). Yet, when comparing the area under the curve (Fig. 5D) of the responses to histamine or OHP, this was significantly greater in HEK-H1-V1 cells compared to HEK-H1 cells. There was no difference, instead, in the peak Ca^{2+} elicited in the two cell types (Fig. 5C), suggesting that Ca^{2+}-entry through the TRPV1 channel contributed to the overall response to histamine/OHP and that the lack of response to capsaicin observed in DRG neurons is due to TRPV1 desensitization after activation *via* H1 receptors. Indeed, capsazepine was able to reduce the Ca^{2+}-responses to histamine or OHP in HEK-H1-V1 cells, although statistical significance was evident only for the area under the curve (Fig. 5E–G).

4. Discussion

In the present manuscript we show, that OHP, at concentrations that are therapeutically relevant in chemotherapy, triggers a Ca^{2+}-response that is mediated by H1 receptors. Furthermore, we show that such activation, similarly to histamine [12], is able to trigger the activation of TRPV1 receptors in DRG neurons, a receptor implicated both in pruritus and in neuropathic pain [21,22].

Our data are in contrast to previous reports that OHP, at similar concentrations, was unable to elicit acute Ca^{2+}-signals. A number of factors may account for this discrepancy, including difference in species and age of the donors for the culture (early postnatal rats *vs* adult mice).

Hypersensitivity reactions to OHP are rather frequent [3]. A large trial with OHP (MOSAIC) has reported an incidence of 10.3 % of patients but observational studies suggest that in real practice the incidence could well be higher [23]. To minimize the possibility of hypersensitivity reactions, a pre-medication protocol with immunosuppressants, mainly dexamethasone is usually employed [3]. Yet, antihistamine agents have also been empirically used [23]. When a retrospective cohort analysis was undertaken in which patients treated with dexamethasone alone were compared with patients treated with dexamethasone and anti-histamine agents, hypersensitivity reactions were reduced from 53 % to 11 % [13]. Unfortunately, to our knowledge this retrospective finding was never used to design a prospective trial.

The serendipitous finding reported in the present manuscript was obtained in DRG neurons, and it is possible that the pruritus and itch observed in some OHP-treated patients is secondary to this system. While we did not investigate the effect of OHP on extra-neuronal cellular systems (except for bronchial smooth muscle), which most likely are the major contributors to the hypersensitivity reactions, the agonistic effect of OHP is likely to be similar, as shown also in heterologous HEK293t cells.

In conclusion, therefore, therapeutically relevant concentration of OHP, *in vitro*, both in DRG neurons, ASM cells and in transfected HEK293t cells acts as a H1 agonist. We believe that these data provide a strong rationale to envisage antihistamine agents as supportive therapy for OHP-treated patients and for a trial to be conducted.

Author contribution

AP designed, performed and analysed all experiments and wrote the manuscript. BR performed some experiments and data-analyses. AAG supervised the project and wrote the manuscript.

Declaration of Competing Interest

The authors declares no conflict of interest.

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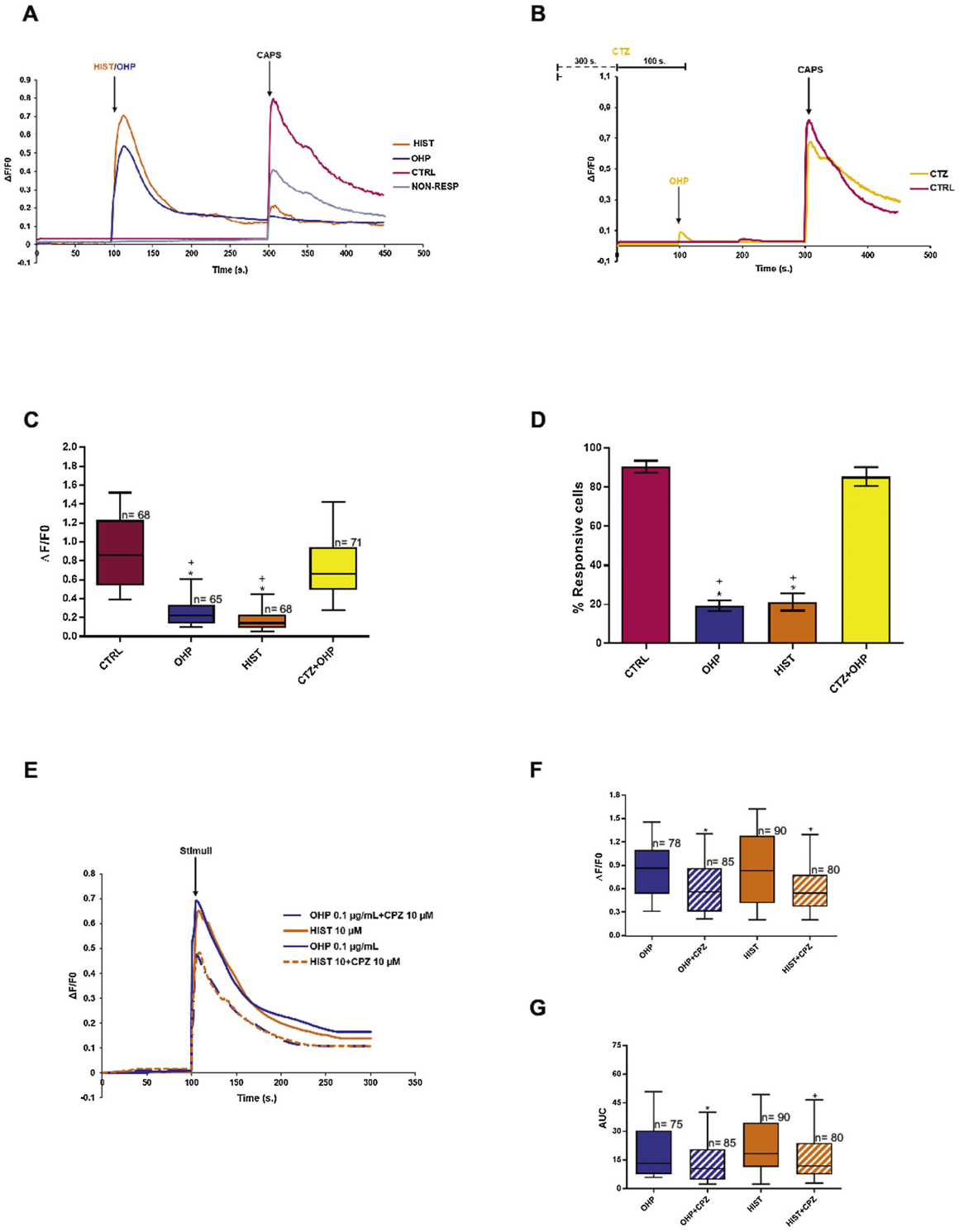

Fig. 4. H1 receptors agonist activates TRPV1 channels in DRG neurons.
A Representative Ca^{2+}-traces from DRG neurons treated with or without 0.1 μg/mL of OHP or 10 μM histamine and then exposed to capsaicin (200 nM). B Representative Ca^{2+}-traces from DRG neurons treated with 0.1 μg/mL of OHP in the presence or absence of CTZ and then exposed to capsaicin. Traces shown in A, B are representative of four independent cultures. The number of cells evaluated is indicated in the figure. C Box and whisker plots show median and IQR of peak of capsaicin induced calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* CTRL, $^{+}$ $P < 10^{-6}$ *vs* CTZ + OHP. D Bar graph showing the percentage of capsaicin responsive neurons. Histograms show the mean ± S.E.M of four separate experiments. One-way analysis of variance followed by Tukey's post-hoc.*$P < 10^{-6}$ *vs* CTRL, $^{+}$ $P < 10^{-6}$ *vs* CTZ + OHP. E Representative Ca^{2+}-traces from DRG neurons treated with OHP or histamine in the presence or absence of capsazepine (10 μM). Traces shown in A, B are representative of three independent cultures. The number of cells evaluated is indicated in the figure. F Box and whisker plots show median and IQR of peak of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. * $P = 0.017$, $^{+}$ $P = 0.012$ G Box and whisker plots show median and IQR of AUC of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc.* $P = 0.005$, $^{+}$ $P = 0.04$.

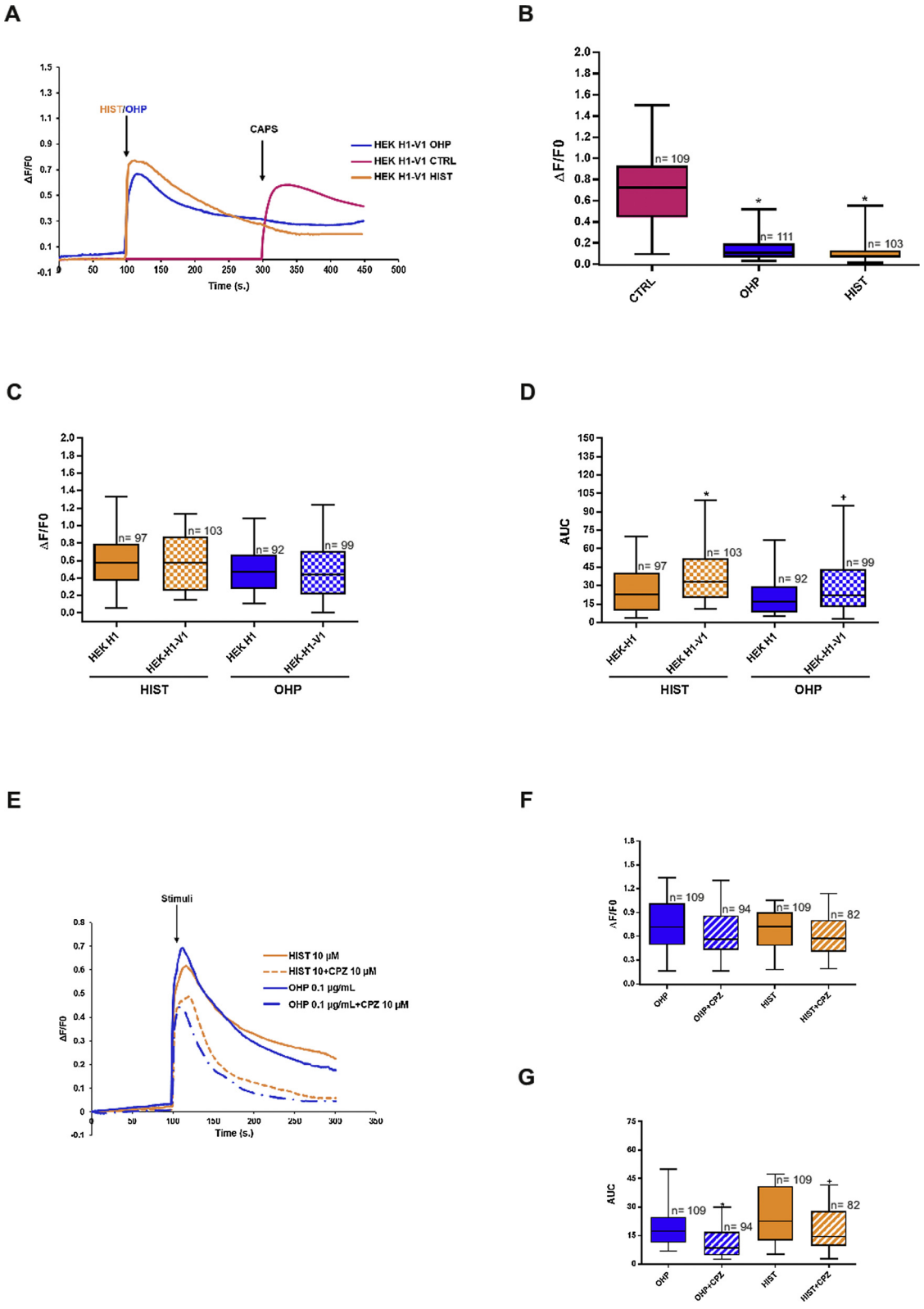

Fig. 5. H1 receptors agonists activate and desensitize TRPV1 in HEK cell co-expressing H1-TRPV1 receptors.
A Representative Ca^{2+}-traces from HEK H1-V1 cells treated with or without 10 μM histamine or 0.1 μg/mL OHP. Traces are representative of three independent experiments. For each treatment about 100 cells were evaluated. B Box and whisker plots show median and IQR of peak of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* CTRL. C, D Comparison of max peak (C) and AUC (D) of HEK cells transfected with H1 and TRPV1 receptors or H1 alone. *$P = 0.0028$ *vs* HEK-H1, $^{+}P = 0.0495$ *vs* HEK-H1. E Representative Ca^{2+}-traces from HEK H1-V1 cells treated with OHP or histamine in the presence or absence of capsazepine (10 μM). Traces are representative of three independent experiments. The number of cells evaluated is indicated in the figure. F Box and whisker plots show median and IQR of peak of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. G Box and whisker plots show median and IQR of AUC of calcium changes. Kruskal-Wallis H test followed by Dunn's post-hoc. *$P < 10^{-6}$ *vs* OHP, $^{+}P = 0.0008$ *vs* HIST.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ceca.2019.102128.

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