

# G protein-coupled receptor signalling potentiates the osmo-mechanical activation of TRPC5 channels

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**Abstract** TRPC5 is an ion channel permeable to monovalent and divalent cations that is widely expressed in different tissues. Although implicated in the control of neurite extension and in the growth cone morphology of hippocampal neurons, as well as in fear-related behaviour, the mechanisms by which TRPC5 is activated remain poorly understood. TRPC5 is known to be activated downstream of Gq-coupled receptors and by membrane stretch, and since there is evidence that mechanical stress may directly activate Gq-coupled receptors, we examined the relationship between the activation of TRPC5 by the type 1 histamine receptor and osmotic stress. Using calcium imaging and patch clamp recordings, we found that a higher proportion of cells expressing TRPC5 respond to hypoosmotic solution when they co-express H<sub>1</sub>R. This response is associated with a phospholipase C-dependent increase in the cells internal calcium concentration, which is abolished on depletion of calcium stores. We also found that the hypoosmotic stimulus that provokes mechanical stress drives the translocation of TRPC5 to the plasma membrane by a mechanism dependent on PI<sub>3</sub>K. This increase in TRPC5 at the plasma membrane augments the proportion of cells that respond to hypoosmotic stimulation. Together, these

results suggest that hypoosmotic cell-swelling activates Gq-coupled receptors, which in turn enhance the activation of TRPC5 by regulating this channel membrane trafficking. Gq-coupled receptors and TRPC5 are co-expressed in several tissues such as those of the vascular system and in somatosensory neurons, suggesting that this mechanism of TRPC5 activation may have interesting and important implications in arterial pressure sensing and mechanotransduction.

**Keywords** TRP channels · G protein-coupled receptors · Osmo-mechanical activation · H<sub>1</sub> receptor

## Introduction

Several molecules have been implicated in cellular mechanotransduction, including transient receptor potential (TRP) channels, acid-sensing ion channels (ASICs), 2P-domain K<sup>+</sup> channels and, more recently, the piezo protein family [9, 11, 12, 28, 30, 34]. Members of the canonical TRP family of channels (TRPC) have been shown to be activated by mechanical stimuli. TRPC1 contributes to mechanosensitization in light touch by primary afferent subtypes [15] and to mechanical hyperalgesia [2]. TRPC6 is thought to be a direct sensor of both pressure and osmotic stimulation [46], although later studies claimed it was indirectly activated through G $\alpha_{q/11}$ -coupled receptors (GPCRs) [43, 44]. Further evidence that TRPC6 and TRPC3 participate in low-threshold mechanical responses was recently obtained in DRGs [37]. TRPC5 is activated by swelling and pressure-induced membrane stretch [16], although it is not clear what role it plays in the physiological transduction of mechanical forces. TRPC5 is also activated by extracellular reduced thioredoxin (rTRX) [54], La<sup>+3</sup> and Gd<sup>+3</sup> [24], and by intracellular Ca<sup>2+</sup> [5, 18, 23, 36], although the role of calcium in TRPC5 activation remains controversial and high micromolar

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concentrations of  $\text{Ca}^{2+}$  are inhibitory to this channel [33, 58]. It has been proposed that TRPC5 acts as a direct sensor of lysophospholipids [13], suggesting that this channel is sensitive to the structure of the lipid bilayer. TRPC5 shares some features with TRPC6 as it is activated by receptors that couple to phospholipase C (PLC) [42] and by stretch, and it is inhibited by the tarantula peptide GsMTx-4 [16] that is known to specifically inhibit mechanosensitive channels by modifying the external lipid-channel boundary [46]. However, in contrast to other members of the TRPC family, TRPC5 is not activated by diacylglycerol DAG [19]. Another characteristic of the TRPC5 channel involves its rapid translocation and insertion into the plasma membrane after stimulation of growth factor receptors via Rac, phosphatidylinositol 3 kinase (PI<sub>3</sub>K) and phosphatidylinositol 4-phosphate 5-kinase (PIP<sub>5</sub>K) [4] as well as by muscarinic stimulation [47] and activation of G $\alpha_s$  [20].

TRPC5 is expressed in different tissues, including the brain, gonads, lung, heart, adrenal glands, endothelium, kidney and vascular and gastric smooth muscle [3, 32, 35, 40]. In terms of its functions *in vivo*, TRPC5 activity regulates neurite extension and growth cone morphology in hippocampal neurons [17] and it is activated via neuronal G protein-coupled receptors. Studies in TRPC5 knockout mice have shown that this channel fulfils an essential role in innate fear [39]. Moreover, TRPC5 mediates the  $\text{Ca}^{2+}$  influx that inhibits endothelial cell, fibroblast and podocyte migration [10, 49] and that evokes vascular smooth muscle cell motility [53].

In addition to the aforementioned channels, there is evidence that GPCRs are directly activated by mechanical stress without the involvement of their ligands, as evident in many G $\alpha_{q/11}$ -coupled receptors, e.g. histamine 1 (H<sub>1</sub>R), angiotensin II (AT<sub>1</sub>R), acetylcholine (M<sub>5</sub>R) and vasopressin (V<sub>1A</sub>R) in decreasing order of mechanosensitivity [43]. These studies further indicate that activation of TRPC channels by mechanical stimuli occurs through activation of GPCRs. TRPC6, TRPC3 and TRPC7 are similarly activated by hypotonicity, and these receptors are stimulated when co-expressed with GPCRs, indicating that all DAG-sensitive TRPC channels are indirectly sensitive to membrane stretch.

Given that TRPC5 is directly activated by osmotic stress and pressure, as well as by GPCRs that are also osmo-mechanically activated, we investigated the contribution of G $\alpha_{q/11}$  signalling to the mechanism of TRPC5 activation in response to hypoosmotic stimulation by co-expressing TRPC5 with the histamine 1 Gq-coupled receptor (H<sub>1</sub>R). We combined ratiometric  $\text{Ca}^{2+}$  imaging with whole cell patch clamp recordings to show that the response of TRPC5 to hypoosmotic stimulation was potentiated by this GPCR. This potentiation is dependent on PLC activity and the intracellular calcium concentration. We propose that hypoosmotic stimulation increases the expression of TRPC5 at the plasma membrane through a process that is dependent on PI<sub>3</sub>K, which potentiates this channel's response when it is co-expressed

with H<sub>1</sub>R. On the basis of these data, we hypothesise that TRPC5 and GPCRs act in synergy, reflecting a novel mechanism that drives TRPC5 channel activation in different tissues.

## Materials and methods

### Cell culture and transfection

For 24–48 h before transfection, human embryonic kidney (HEK) 293 cells (ECACC, Salisbury, UK) were grown in poly-L-lysine-coated dishes in medium supplemented with 10 % FBS and 1 % of penicillin/streptomycin. HEK-293 cells were transiently transfected with the murine TRPC5 cDNA fused to the green fluorescent protein (GFP) alone (1  $\mu\text{g}/\text{ml}$ ; kindly donated by D.E. Clapham, Harvard University, Cambridge, MA, USA) or along with the cDNA encoding the rat histamine 1 (H<sub>1</sub>R) receptor (0.2  $\mu\text{g}/\text{ml}$ ; a generous gift from T.D. Plant, Philipps-Universität Marburg, Germany). The cells were transfected on glass coverslips for 4–6 h using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA; 5  $\mu\text{l}/\mu\text{g}$  DNA). In some experiments, HEK-293 cells were transfected with TRPC5 and the bradykinin type II receptor (BK<sub>2</sub>) or the thyrotropin-releasing hormone type 1 receptor (TRHR1; both kind gifts from D.A. Bayliss, University of Virginia, USA), and in another set of experiments, the cells were transfected only with these latter receptors and the GFP protein (Life Technologies, Carlsbad, CA, USA). The mTRPC5 cDNA was cloned into the pCINeo vector and thus, cells were transfected with the pCINeo/IRES-GFP vector as controls (kindly donated by J. Eggermont, KU Leuven, Belgium). All constructs were verified by sequence analysis, and the transfected cells were identified by GFP fluorescence.

### Bathing solutions and reagents

We employed external solutions of different osmolarities that was measured with a cryoscopic osmometer (Gonotec, Berlin, Germany). The isotonic bathing solution used contained (in millimolar): 90 NaCl, 5 KCl, 1.3 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, 10 HEPES and 10 D-glucose, with a pH adjusted to 7.4 with NaOH. Mannitol (100 mM) was added to maintain the osmolarity constant at 300 mOsm  $\text{kg}^{-1}$ , while the hypoosmotic solution (210 mOsm  $\text{kg}^{-1}$ ) was prepared by omitting mannitol. For  $\text{Ca}^{2+}$ -free solutions, calcium was omitted and replaced with 5 mM EGTA. All the experiments were performed at room temperature (22–26 °C). Carbachol, histamine, diphenhydramine, guanosine 5'-[ $\beta$ -thio]diphosphate (GDP- $\beta$ -S), thapsigargin and wortmannin were all purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), while U-73122 and U-73343 was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany).

### Fluorometric calcium measurement

Cells were incubated with 5  $\mu\text{M}$  Fura-2AM (Life Technologies, Carlsbad, CA, USA) for 45 min at 37 °C in a 5 %  $\text{CO}_2$  incubator. Recordings were performed in a low-volume chamber continuously perfused at  $\sim 1 \text{ ml min}^{-1}$ . Fluorescent signals were measured using a Nikon TE2000-U microscope, exciting Fura-2 at 340 and 380 nm with a monochromator (TILL Photonics GmbH, Gräfelfing, Germany) and filtering the fluorescence emitted with a 510-nm long-pass filter. Images were acquired using an Orca ER CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and analysed with Metafluor software (Molecular Devices, Sunnyvale, CA, USA). Increases in cytosolic  $\text{Ca}^{2+}$  are presented as the ratio of the emission intensities at 340 and 380 nm ( $F_{340}/F_{380}$ / fluorescence arbitrary units (FAU)). Recordings were made alternately from transfected and non-transfected cells, monitoring GFP fluorescence with an excitation wavelength of 480 nm. A positive calcium response was considered when the fluorescence signal ( $F_{340}/F_{380}$ ) deviated by at least four times the standard deviation of the baseline.

### Electrophysiology

Whole cell recordings were obtained using 3–5  $\text{M}\Omega$  borosilicate glass capillary patch pipettes. Current signals were recorded with a Multiclamp 700B amplifier, and ramp voltage clamp commands were applied using pCLAMP software and a Digidata 1322A digitizer (Molecular Devices, Sunnyvale, CA, USA). Cells were held at a potential of  $-60 \text{ mV}$ , and current–voltage ( $I$ – $V$ ) relationships were obtained from voltage ramps from  $-100$  to  $+100 \text{ mV}$ , with a duration of 400 ms applied every 5 s. The current was sampled at a frequency of 20 kHz. A series resistance compensation of  $>50 \%$  was used, and current amplitudes were measured at  $-80$  and  $+80 \text{ mV}$ . The internal solution contained (in millimolar): 135 CsCl, 2  $\text{MgCl}_2$ , 10 HEPES, 1 EGTA and 5  $\text{Na}_2\text{-ATP}$ , adjusted to pH 7.2 with CsOH and 300  $\text{mOsm kg}^{-1}$ . In some experiments, the pipette solution was modified by adding 2 mM GDP- $\beta$ -S. The bath solutions for whole-cell recordings were the same as those used to measure fluorimetric calcium.

### Cell surface biotinylation

Biotinylation assays were performed using the Pierce cell surface protein isolation kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). TRPC5 and  $\text{H}_1\text{R}$  were expressed in HEK-293 cells plated in  $60 \times 15 \text{ mm}$  wells and 24 h post-transfection;  $\sim 2 \times 10^6$  cells were washed twice with an isotonic solution before incubation with the hypoosmotic solution for different times. Cells were then immediately transferred to ice; they were washed with ice-cold phosphate-buffered saline

(PBS) and then incubated with 1 mg/ml of sulfo-NHS-SS-biotin in phosphate-buffered saline on ice for 30 min. The biotinylation reaction was stopped by adding quenching solution for 15 min followed by two washes with ice-cold PBS. The biotinylated cells were lysed for 10 min on ice in 900  $\mu\text{l}$  of radioimmune precipitation assay (RIPA buffer: PBS, pH 7.4; 1 % (v/v) Igepalca630; 0.5 % (w/v) sodium deoxycholate; 0.1 % (v/v) sodium dodecyl sulfate (SDS) supplemented with a protease inhibitor mixture (Roche Applied Science, Indianapolis, IN, USA). The cells were recovered in an eppendorf on ice and harvested at 12,000 rpm for 15 min at 4 °C. For each experimental condition, the protein content of the samples was normalised, and they were then incubated with 70  $\mu\text{l}$  of streptavidin-conjugated agarose for 20 min at room temperature and then overnight at 4 °C after collecting a small aliquot as an input control. After three washes with RIPA buffer, agarose-bounded biotinylated proteins were eluted in sample buffer (50 mM Tris–Cl [pH 6.8], 4 % (w/v) SDS, 10 % (v/v) glycerol, 4 % (v/v) 2-beta-mercaptoethanol, 0.001 % (w/v) bromophenol blue) to be analysed by western blotting.

### Western blotting

Eluted proteins and whole lysates (20–50  $\mu\text{g}$ ) were denatured at 95 °C for 5 min, loaded onto a 10 % SDS–polyacrylamide gel and resolved by electrophoresis. Proteins were transferred to a nitrocellulose membrane, blocked with 10 % skimmed milk in TBS and incubated with antibodies against rabbit anti-GFP (diluted 1:1,000; Sigma-Aldrich, Inc., St. Louis, MO, USA). A horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibody (Sigma-Aldrich) was used at a final concentration of 1:2,000 for detection and the signal was developed by enhanced chemiluminescence (Amersham Biosciences UK Limited, Buckinghamshire, England). The signals were recorded using an image analyzer LAS-1000Plus (Fuji Photo Film Co., Ltd., Tokyo, Japan) and quantified with ImageGauge Version 4.0 software (Fuji Photo Film Co., Ltd.).

### Data analysis

The percentage of responding cells was analysed by pooling the total number of responding cells relative to the total number of cells analysed. Electrophysiological analysis was performed with WinASCD software (G. Droogmans, KULeuven, Belgium) and Origin 7.5 (OriginLab Corporation, Northampton, MA, USA). All values were expressed as means  $\pm$  SEM. Statistical tests included the  $Z$  test and student's  $t$  test, as indicated, with  $P < 0.05$  considered statistically significant.

## Results

### Co-expression of TRPC5 with a G protein-coupled receptor potentiates swelling (osmotic stress)-induced TRPC5 activation

To investigate the role of GPCRs in the osmo-mechanical activation of TRPC5, we studied the calcium responses to hypoosmotic solution in HEK-293 cells co-transfected with the TRPC5 channel and the type 1 histamine receptor (H<sub>1</sub>R). When exposed to the 210 mOsm kg<sup>-1</sup> solution, 61 % of these GFP(+) cells responded with a transient rise of [Ca<sup>2+</sup>]<sub>i</sub> (1,258 of 2,054 cells; Fig. 1a, b), a significantly higher proportion of cells than those expressing TRPC5 alone (i.e. without H<sub>1</sub>R, *P* < 0.001; Fig. 1d [16]). By contrast, the hypoosmotic stimulus did not produce such a response in a significant proportion of the cells transfected with the empty vector alone (pCINeo/IRES-GFP, 2.75 %; Fig. 1c, d). Most of the GFP (+) cells also responded to 100 μM histamine (Fig. 1a, b) which indicates that both plasmids were incorporated into the cells. Responses to hypoosmotic solution in cells transfected with TRPC5/H<sub>1</sub>R decreased to 28.3 % (Fig. 1d) when calcium was removed from the extracellular medium and to 23.3 % when cells were incubated with 100 μM diphenhydramine (DPH), an antagonist of the histamine receptor (Supplementary Fig. S1a, b). Thus, the osmotically evoked [Ca<sup>2+</sup>]<sub>i</sub> response of TRPC5 co-expressed with H<sub>1</sub>R results from a dual mechanism: calcium entry from the extracellular medium and calcium release from intracellular stores. Co-expression of the H<sub>1</sub>R with TRPC5 also produced a significant increase in the maximal calcium concentration during hypoosmotic stimulation when compared to the responses in cells expressing TRPC5 alone (Fig. 1e). This increase in calcium was significantly reduced when the histamine receptor was blocked by DPH (Supplementary Fig. S1c).

Hypoosmotic stimulus evoked responses in 28 % of GFP (+) cells when the cells were transfected with the H<sub>1</sub>R receptor alone (Fig. 1d), when increases in [Ca<sup>2+</sup>]<sub>i</sub> would only be due to calcium release from intracellular stores. However, when calcium was removed from the external solution, the proportion of these cells that responded to hypoosmotic solution (25 %) and the increase in intracellular calcium were only slightly, albeit significantly, lower than that obtained in the presence of external calcium (Fig. 1d, e). This decrease in calcium concentration may be due to a lower calcium level at the beginning of the experiment since the cells were maintained in calcium-free conditions. These results indicated that in addition to direct activation of the TRPC5 channels, hypoosmotic stimulation of H<sub>1</sub>R receptor induces Ca<sup>2+</sup> release from internal stores, exerting a positive effect on TRPC5. More cells co-expressing TRPC5 and H<sub>1</sub>R responded to hypoosmotic solution and with a stronger increase in [Ca<sup>2+</sup>]<sub>i</sub> than cells expressing TRPC5 or H<sub>1</sub>R alone (Fig. 1d,

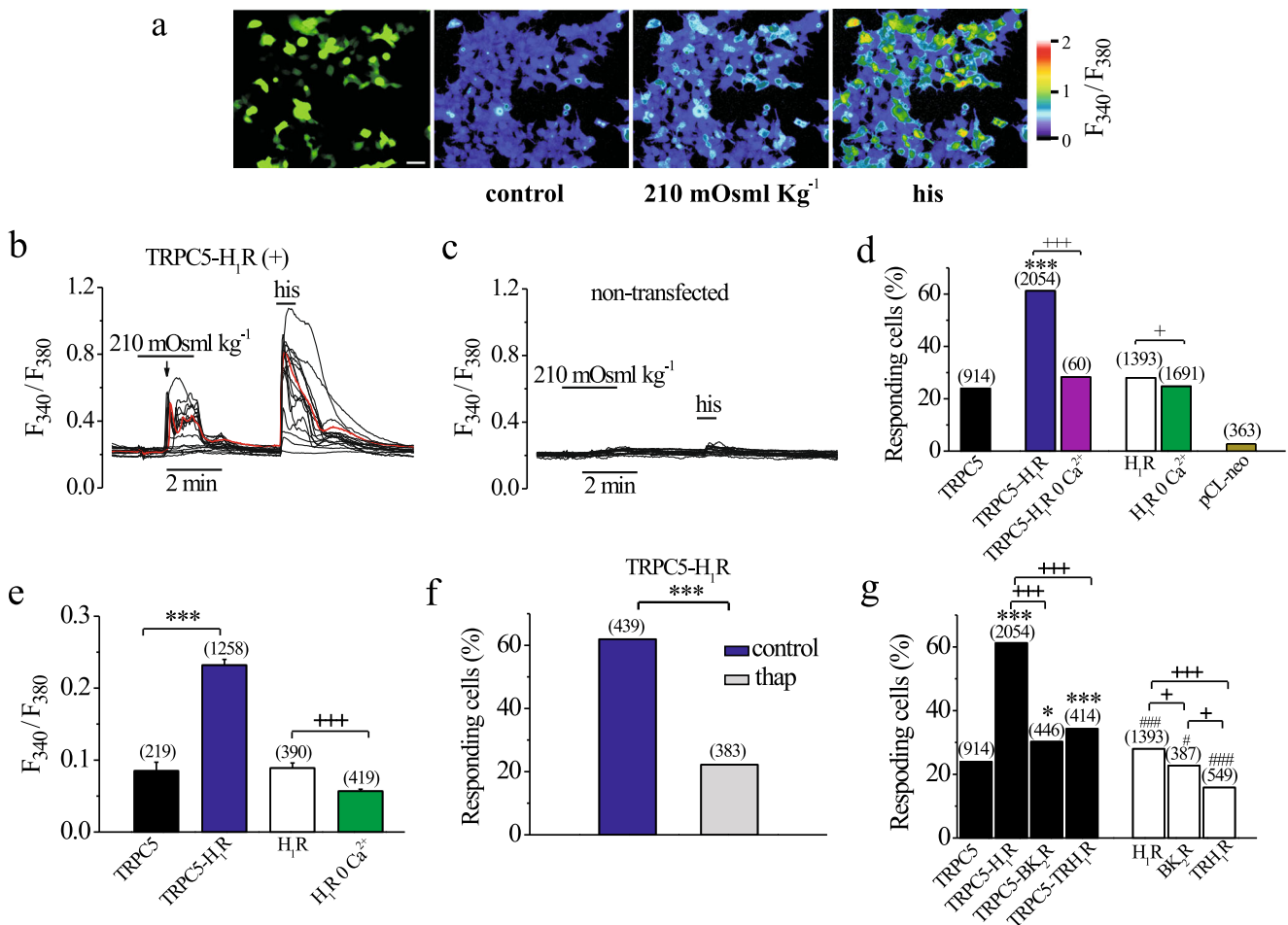
e). The total increase in calcium is likely to reflect the calcium flux across the plasma membrane plus the histamine-induced release from calcium stores. We investigated whether the calcium released from intracellular stores through H<sub>1</sub>R activation contributed to the potentiation of hypoosmotic TRPC5 activation by depleting the intracellular stores. When co-transfected cells were exposed for 1 h with thapsigargin (Thap; 2 μM), an inhibitor of the endoplasmic reticulum Ca<sup>2+</sup>-ATP<sub>ase</sub> [48], there was a significant decrease in the proportion of TRPC5/H<sub>1</sub>R responding cells compared to that in cells expressing the TRPC5 channel alone (Fig. 1f). If the potentiation of TRPC5 activation was due to the summation of the individual TRPC5 and H<sub>1</sub>R responses to hypoosmotic stimulation, we would only have found a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> and not the increase in the number of responding cells that was observed.

Hypoosmotic potentiation is also observed in cells co-transfected with BK<sub>2</sub>R and TRH<sub>1</sub>R

To establish whether H<sub>1</sub>R specifically potentiates the hypoosmotic activation of TRPC5, we co-transfected TRPC5 with other Gαq/11-coupled receptors, BK<sub>2</sub>R and TRH<sub>1</sub>R. The proportion of TRPC5/BK<sub>2</sub>R or TRPC5/TRH<sub>1</sub>R expressing cells that respond to hypoosmotic solution reached 30.3 and 34.3 %, respectively (Fig. 1g). These values were significantly higher than the hypoosmotic responses of cells that expressed the BK<sub>2</sub> (23 %) and TRH<sub>1</sub> (16 %) receptors, or TRPC5, alone. However, these values were significantly lower than those obtained in cells that expressed both the TRPC5 and H<sub>1</sub>R. Like H<sub>1</sub>R, BK<sub>2</sub>R and TRH<sub>1</sub>R are activated by hypoosmotic solution, although the proportion of cells that responded to this stimulus was significantly lower than those that expressed H<sub>1</sub>R (Fig. 1g, white bars). Hence, there appears to be differences in stress sensitivity among these receptors, as what occurs with other GPCRs [43], or differences in the expression of these receptors at the plasma membrane.

Co-expression of TRPC5 with the H<sub>1</sub>R potentiates the TRPC5 currents

To demonstrate that activation of H<sub>1</sub>R directly potentiates the TRPC5 channel activity provoked by osmotic stress, we measured whole cell ion currents in response to the application of a hypoosmotic solution to cells co-transfected with both these constructs. Accordingly, the development of currents was monitored with periodic applications of voltage ramps from -100 to +100 mV. Application of hypoosmotic solution and 100 μM histamine activated a current with similar characteristics, including double rectification current-voltage relationship and a reversal potential close to zero (-7.7±0.8 mV; for the hypoosmotic stimulation, *n*=48 and -8.9±0.8 mV; for responses to histamine, *n*=25; Fig. 2a). The proportion of



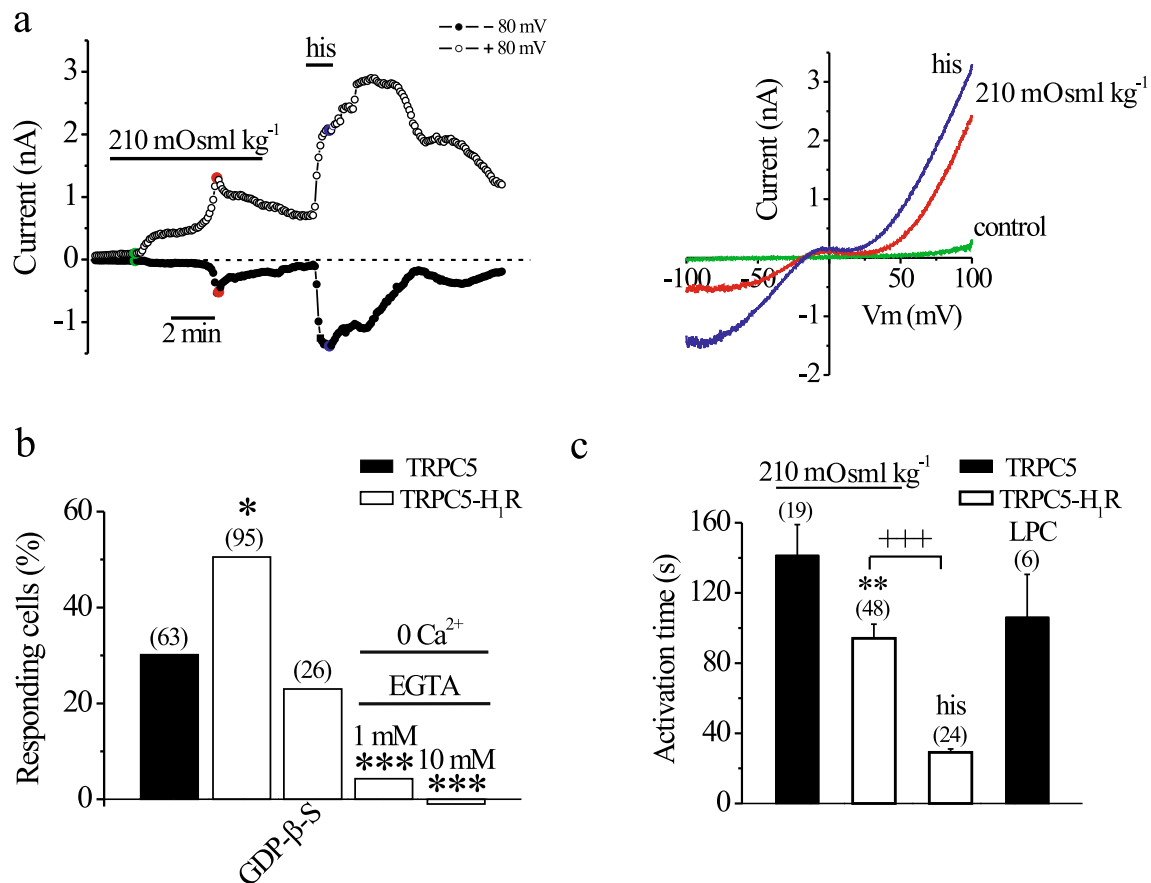
**Fig. 1** Hypoosmotic G protein-coupled receptor activation potentiates the activation of TRPC5 by membrane stress. **a** Pseudocolour images of TRPC5-GFP/H<sub>1</sub>R-transfected cells showing GFP fluorescence (*left image*) and ratiometric [Ca<sup>2+</sup>]<sub>i</sub> responses to control, 210 mOsm kg<sup>-1</sup> and 100 μM histamine solution. Changes in [Ca<sup>2+</sup>]<sub>i</sub> are reflected by the ratio of fura-2 emission at 340 to 380 nm excitation (see *colour bar*). Scale bar, 50 μm. **b**, **c** Time course of the calcium changes on exposure of cells transfected with TRPC5 and the histamine type 1 receptor (**b**) or non-transfected cells in the same field (**c**) to hypoosmotic and 100 μM histamine solution. Image in 210 mOsm kg<sup>-1</sup> corresponds to the time course of the calcium increase indicated by the *arrow* in **b**. *Red trace* shows an example of an individual response to hypoosmotic stimulation. **d** Proportion of cells responding to exposure to a 210-mOsm kg<sup>-1</sup> solution in cells expressing the cDNAs indicated (\*\*\*, +++*P*<0.001, Z test; each condition in > three independent experiments); an *asterisk*

denotes statistical significance between cells expressing TRPC5/H<sub>1</sub>R and each of the other conditions, while *plus sign* denotes statistical significance between cells in the conditions indicated by the gap. **e** Bar graph summarising the mean evoked increase in [Ca<sup>2+</sup>]<sub>i</sub> under the conditions indicated (\*\*\*, +++*P*<0.001, student's *t* test). **f** Bar histogram showing the percentage of TRPC5/H<sub>1</sub>R cells responding to hypoosmotic stimulus after incubation with 2 μM thapsigargin for 60 min compared to the control cells (\*\*\*, +++*P*<0.001, Z test). **g** Proportion of cells expressing TRPC5 and the indicated GPCRs (*black bars*) responding to hypoosmotic solution or cells expressing only the GPCR (*white bars*); *asterisk* denotes statistical significance between cells co-expressing TRPC5 and GPCRs and the cells transfected with TRPC5 alone; *number sign* denotes statistical significance between cells that express the GPCRs alone and cells co-expressing TRPC5 and GPCRs (\*\*\*, +++*P*<0.001, Z test). The number of cells is given above each *bar*

cells co-transfected with TRPC5 and H<sub>1</sub>R that showed TRPC5-like current was 50.5 % (*n*=95). Removal of calcium from the extracellular solution strongly diminished the hypoosmotic-activated TRPC5 current and increasing the EGTA concentration in the pipette from 1 mM (*n*=17) to 10 mM completely abolished these residual responses (*n*=8; Fig. 2b). Thus, calcium entry appeared to be necessary to support the activation of TRPC5 channels by hypoosmotic stimulation.

The proportion of cells co-transfected with TRPC5 and H<sub>1</sub>R that produced these currents was significantly higher

than that of cells that only expressed TRPC5 (30.16 %; Fig. 2b). To indeed confirm that the potentiation is mediated by the activation of GPCR, we modulated G protein activity by including the non-hydrolysable guanine nucleotide analogue GDP-β-S in the pipette solution. In the presence of GDP-β-S, the proportion of TRPC5/H<sub>1</sub>R cells that responded to hypoosmotic stimulation was significantly reduced to 23 % (*n*=26; Fig. 2b), a similar value obtained in cells that only expressed TRPC5. The ionic currents activated by histamine were completely abolished (Supplementary Fig. S2).



**Fig. 2** Co-expression of TRPC5 with the H<sub>1</sub>R potentiates the hypoosmotic-activated TRPC5 current. **a** Time course of current development from -80 and +80 mV in a TRPC5/H<sub>1</sub>R HEK-293 cell in a 210-mOsm kg<sup>-1</sup> solution and in the presence of histamine (100 μM). Currents were evoked by a 400-ms-long -100/+100 mV voltage ramp delivered every 5 s, and the right panel shows the I-V relationship corresponding to the colour code ramps of the current time course. **b** Bar graph summarising the proportion of cells expressing TRPC5 or TRPC5/H<sub>1</sub>R that responds to hypoosmotic solution with 2 mM

GDP-β-S in the pipette solution, in the presence or absence of calcium, and with different EGTA concentrations in the pipette. **c** Comparison of the time of current activation between cells co-expressing TRPC5/H<sub>1</sub>R and cells transfected with TRPC5 alone when exposed to a hypoosmotic solution, histamine (100 μM) or LPC (10 μM). The latency of current activation in cells transfected with TRPC5 alone was also compared in response to hypoosmotic solution (141±18 s) and LPC (10 μM, 106±25 s); asterisk denotes statistical significance between cells co-expressing TRPC5/H<sub>1</sub>R and cells transfected with TRPC5 alone

These results indicate that this potentiation is due to stronger channel activity or more channels at the plasma membrane. These hypothetical mechanisms are dependent on the increase in intracellular calcium; they are not mutually exclusive and potentiation may not be attributed to the summation of the individual responses mediated by TRPC5 and H<sub>1</sub>R. This may suggest that the unresponsive cells that only express TRPC5 could respond were H<sub>1</sub>R activated. The mean amplitude of the inward currents, measured at -80 mV, was very similar in cell transfected with TRPC5/H<sub>1</sub>R (0.33±0.06 nA, n=48) to that in cells that only expressed TRPC5 (0.375±0.09 nA, n=19). However, the delay between the application of the hypoosmotic solution and the onset of the current was significantly shorter in cells co-transfected with TRPC5 and H<sub>1</sub>R than in cells transfected with TRPC5 alone (94.2±8 versus 141±18 s, *P*<0.01; Fig. 2c). Moreover, in cells expressing TRPC5, there were no significant differences in

current activation when the cells were stimulated with the direct activator of TRPC5, lysophosphatidylcholine (LPC; 10 μM) or the hypoosmotic solution. However, the latency of the current activated by the histamine receptor agonist was significantly faster compared to activation by the hypoosmotic solution (Fig. 2c). Together, these results further indicate that the co-expression of TRPC5 and H<sub>1</sub>R potentiated the osmo-mechanical activation of TRPC5 in a calcium dependent manner.

H<sub>1</sub>R-mediated TRPC5 potentiation is dependent on PLC activity

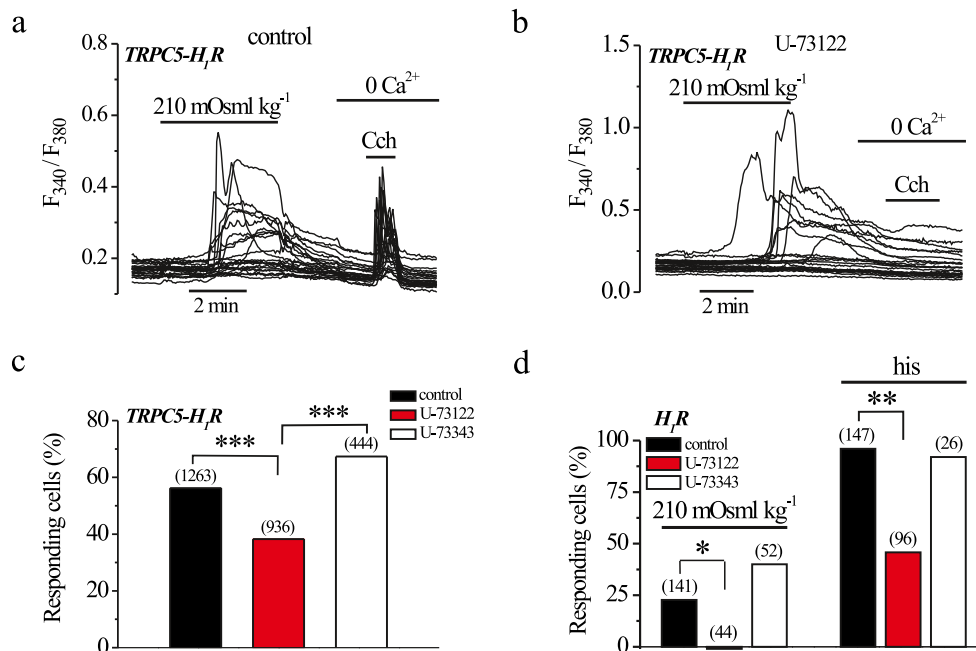
The opening of TRPC5 channels following the activation of H<sub>1</sub>R receptors is mediated by phospholipase C (PLC) [42]. To assess the role of PLC in the potentiation of the responses to hypoosmotic stimulation in cells co-expressing TRPC5 and

H<sub>1</sub>R, we used the PLC inhibitor U-73122. As seen previously and as it is shown in the representative experiment in Fig. 3a, exposure to a 210-mOsm kg<sup>-1</sup> solution evoked an increase [Ca<sup>2+</sup>]<sub>i</sub> in 56.2 % (*n*=1263; Fig. 3c) of the cells expressing TRPC5 and H<sub>1</sub>R while addition of carbachol (Cch; 10 μM) elevated the [Ca<sup>2+</sup>]<sub>i</sub> in the majority of these cells by activating endogenous muscarinic receptors. After incubation with U-73122 (5 μM; Fig. 3b), the proportion of cells that responded to the 210 mOsm kg<sup>-1</sup> solution fell to about 35.25 % (*n*=936; Fig. 3c), a value nearly identical to that obtained in cells expressing TRPC5 alone (see Fig. 1d). Responses to Cch were abrogated or greatly diminished after incubation with U-73122 (Fig. 3b), indicating that U-73122 effectively inhibited PLC signalling. In the presence of U-73343, an analogue with little or no effect on PLC activity, the potentiation of the response to hypoosmotic solution was not affected (67.34 %, *n*=444) and it was significantly higher than the cells exposed to U-73122 as summarised in Fig. 3c, showing the significant dampening of the response to hypoosmotic stimulation when PLC is inhibited. Cells transfected with the H<sub>1</sub>R alone responded to hypoosmotic stimulation by elevating [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 1d) and thus, we assessed whether this elevation in calcium was mediated by PLC. The increase in [Ca<sup>2+</sup>]<sub>i</sub> induced by hypoosmotic stimulation in cells expressing H<sub>1</sub>R was fully abrogated in the presence of U-73122 (Fig. 3d), and the response to histamine also diminished significantly. Incubation with U-73343 did not affect the activation of H<sub>1</sub>R either by hypoosmotic solution or by histamine. Thus,

in H<sub>1</sub>R-transfected cells, the hypoosmotic-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> can be attributed to calcium release from intracellular stores, secondary to PLC activation. Accordingly, we propose that PLC participates in the potentiation of the TRPC5 responses provoked by hypoosmotic stimulation through H<sub>1</sub>R activation.

Hypoosmotic stimulation increases the expression of TRPC5 at the plasma membrane in cells co-transfected with H<sub>1</sub>R

Not all TRPC5-transfected cells respond to hypoosmotic stimulation, which is perhaps due to individual differences in channel expression. TRPC5 is a membrane protein that is translocated to the plasma membrane in intracellular vesicles [17] through epidermal growth factor activity, muscarinic stimulation and activation of Gα<sub>s</sub>, a process dependent on PI<sub>3</sub>K. To establish whether the potentiation of the responses observed here may be due to stronger expression of the channel at the plasma membrane, we used cell surface biotinylation to evaluate the membrane expression of TRPC5 following hypoosmotic stimulation in cells expressing TRPC5 alone or TRPC5 and H<sub>1</sub>R. Cell surface proteins were selectively biotinylated with the membrane-impermeant amino reagent sulfo-NHS-SS-biotin, and the proteins were then solubilised and recovered with streptavidin-agarose before probing these lysates with an anti-GFP antibody. To quantify the surface biotinylation, we calculated the ratio between the



**Fig. 3** Potentiation of TRPC5-hypoosmotic activation is dependent on phospholipase C activation. **a**, **b** Hypotonicity- and carbachol-activated increase in [Ca<sup>2+</sup>]<sub>i</sub> in TRPC5/H<sub>1</sub>R-transfected cells, pretreated or not with U-73122 (5 μM) for 30 min–1 h. Extracellular Ca<sup>2+</sup> was eliminated through the Cch (10 μM) stimulation. **c** Summary of the proportion of

responding cells in experiments such as those in **a** and **b** in both control cells and following pretreatment with U-73122 and U-73343 (5 μM). **d** Summary of the proportion of cells expressing the histamine receptor alone that respond to 210 mOsm kg<sup>-1</sup> and histamine (100 μM) in control conditions and following treatment with U-73122 and U-73343 (5 μM)

immunoreactivity of the surface protein and that of the total protein extract.

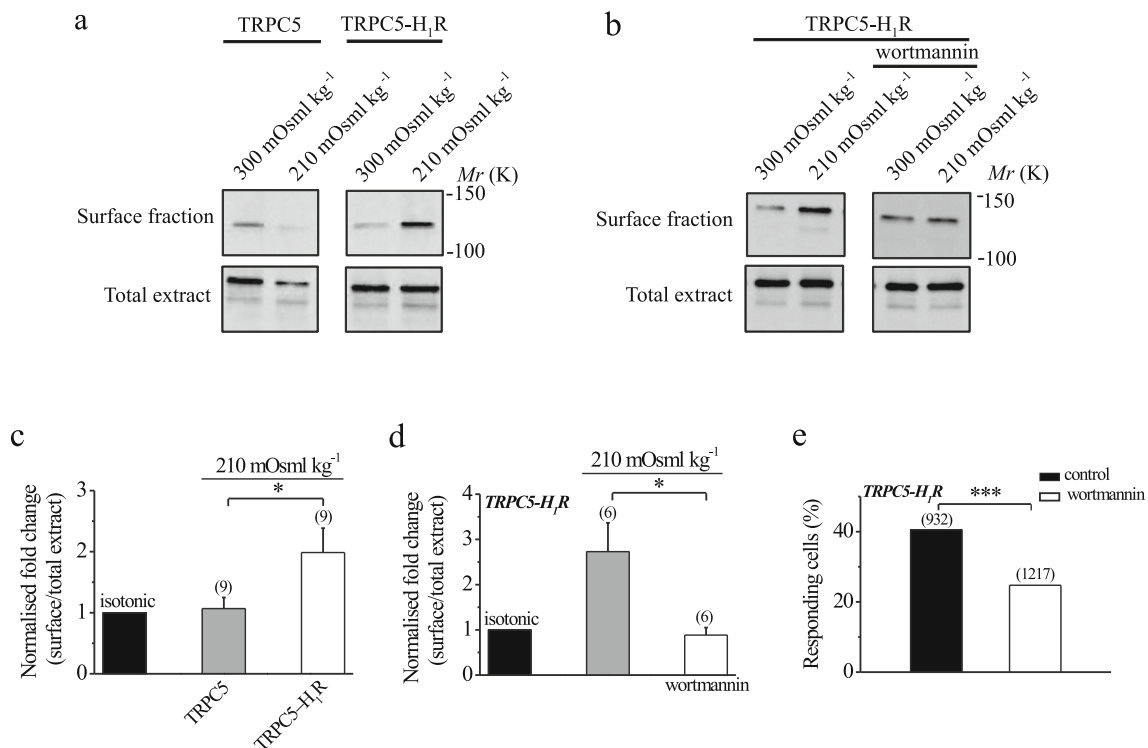
In cells transfected with TRPC5 alone, the surface expression of this channel (Fig. 4a, left panel) was no different following exposure to isotonic (300 mOsm kg<sup>-1</sup>) or hypoosmotic solution for 1.5–3 min. However, there was an increase in TRPC5 surface expression in cells expressing TRPC5 and H<sub>1</sub>R (right panel) when they were maintained in the hypoosmotic solution, augmenting the relative amount of TRPC5 at the cell surface by 1.9-fold over the basal level in isotonic solution ( $n=9$  or in 9 of 15 assays; Fig. 4c).

The rapid translocation of TRPC5 to the plasma membrane is thought to be mediated by PI<sub>3</sub>K activity [4]. To determine whether the translocation of the channel to the plasma membrane is involved in the potentiation of the response to osmotic stress, we studied surface membrane biotinylation in calcium imaging experiments after preincubating the cells with wortmannin (20 nM), a selective inhibitor of PI<sub>3</sub>K. Preincubation of the cells co-expressing TRPC5 and H<sub>1</sub>R with wortmannin for 30 min (Fig. 4b right panel) significantly

reduced the hypoosmotic-induced increase in TRPC5 surface expression observed after hypoosmotic exposure (Fig. 4b, left panel, d). Moreover, pretreatment with wortmannin for 30 min reduced the proportion of cells transfected with TRPC5 and H<sub>1</sub>R that responded to the hypoosmotic stimulus (24.7 %,  $n=1,217$ ) compared to the control cells pretreated with the vehicle alone (DMSO, 40.56 %,  $n=932$ ;  $P<0.001$ ; Z test; Fig. 4e). Therefore, these data demonstrate that osmotic stress stimulation promotes the translocation of TRPC5 to the plasma membrane by a PI<sub>3</sub>K-dependent mechanism that potentiates the response.

## Discussion

In the present study, we show that the osmotic stress activation of TRPC5 is further potentiated by osmotic modulation of the G protein-coupled H<sub>1</sub>R through PLC-coupled and Ca<sup>2+</sup>-dependent signalling pathways. Hypoosmotic stimulation directly activates both the TRPC5 channel and the histamine receptor and this dual activation elevates intercellular calcium by



**Fig. 4** Hypoosmotic stimulation increases the surface-accessible TRPC5 channels in cells that also express GPCRs. **a, b** Surface expression was analysed by probing western blots of biotinylated surface proteins with an anti-GFP antibody. TRPC5 expression in whole cell extracts (total) prior to incubation with agarose–streptavidin is also shown. The membrane protein expression of TRPC5 was normalised relative to the total protein. **c** Quantification of the biotinylation experiments. The ratio increased from  $1.07\pm 0.18$  in cells expressing TRPC5 to  $1.99\pm 0.4$  in cells expressing TRPC5 and H<sub>1</sub>R after hypoosmotic stimulation (9 of 15 assays;  $*P<$

$0.05$ ; student's  $t$  test). **d** The increase in TRPC5 expression is dependent on PI<sub>3</sub>K. TRPC5/H<sub>1</sub>R cells were incubated with wortmannin (20 nM) for 30 min prior to exposure to hypoosmotic solution. The ratio decreased from  $2.7\pm 0.6$  in control cells pre-incubated with DMSO alone to  $0.9\pm 0.2$  in cells expressing exposed to wortmannin (6 assays;  $*P<0.05$ ; student's  $t$  test). **e** Bar histogram showing the proportion of TRPC5/H<sub>1</sub>R expressing cells responding to the hypoosmotic stimulus after a 30-min incubation with wortmannin compared to cells incubated with 2  $\mu$ M DMSO (control;  $***P<0.001$ ; Z test)



promoting calcium influx through TRPC5 channels, and through PLC and IP<sub>3</sub> mediated  $[Ca^{2+}]_i$  release from intracellular stores. Consequently, significantly more cells respond to hypoosmotic stimulation, and there is a significant increase in  $[Ca^{2+}]_i$ . The potentiation of the TRPC5 response is abolished by the removal of extracellular calcium, depletion of endoplasmic reticulum  $Ca^{2+}$  or blocking H<sub>1</sub>R or PLC.

Potentiation of the response to hypoosmotic stimulation is also observed in cells that co-express TRPC5 and two other G<sub>q/11</sub>-coupled receptors, namely the bradykinin type 2 and the thyrotropin-releasing hormone type 1 receptor, although a stronger effect is observed in cells that express TRPC5 and H<sub>1</sub>R. Our results are consistent with the suggestion that G<sub>q/11</sub>-coupled receptors function as sensors of membrane stretch [43], where the activation of TRPC channels by mechanical stimuli is proposed to occur through activation of GPCRs. Our findings support a synergistic mechanism that transduces the stretch stimulus into the direct activation of TRPC5, independently of GPCR activation as well as in the activation of a GPCR that activates an intracellular pathway that potentiates the response of TRPC5.

This potentiation was also observed when we recorded the TRPC5 currents with patch clamp techniques and it was abolished by inactivating GPCR. The latencies of channel activation in response to osmotic stress or LPC are significantly longer than the responses to receptor ligand. Such differences may indicate the involvement of conformational changes of the proteins due to membrane stretch, as what occurs with the angiotensin type 1 receptor [55], and of intracellular signalling process in the activation of TRPC5 and H<sub>1</sub>R by hypoosmotic stimulation. Whatever the mechanism employed, it involves events that persist for longer than agonist activation.

The activation latency in response to hypoosmotic solution was significantly faster in cells expressing TRPC5 and H<sub>1</sub>R than in cells expressing TRPC5 alone, suggesting that osmotic stress activation of H<sub>1</sub>R may also affect the gating of the channel. Removing extracellular  $Ca^{2+}$  and chelating intracellular  $Ca^{2+}$  prevent current activation, suggesting that  $Ca^{2+}$  entry and calcium release from intracellular stores are necessary for channel activation. In addition, we suggest that the increase in the intracellular calcium concentration due to the activation of H<sub>1</sub>R potentiates the TRPC5 response by increasing the channel's activity or its expression at the plasma membrane. If the increase in calcium were to augment channel activity, we would have expected differences in current amplitude between cells expressing TRPC5 alone or TRPC5 and H<sub>1</sub>R. By contrast, increased surface expression of TRPC5 may allow the cells to reach the minimum threshold number of channels required to produce a response. If this were the case, the current amplitude would remain similar but more cells would respond, as our

results indeed reveal. We cannot rule out the possibility that a strong increase in intracellular calcium may have a dual effect, enhancing the translocation of the channel to the plasma membrane and partially blocking channel activity [33, 58], thereby preventing an increase in current. However, intracellular calcium-mediated potentiation of agonist-activated TRPC5 was previously seen to occur through a combination of increased channel number or channel open probability, but not by changes in single-channel conductance [5].

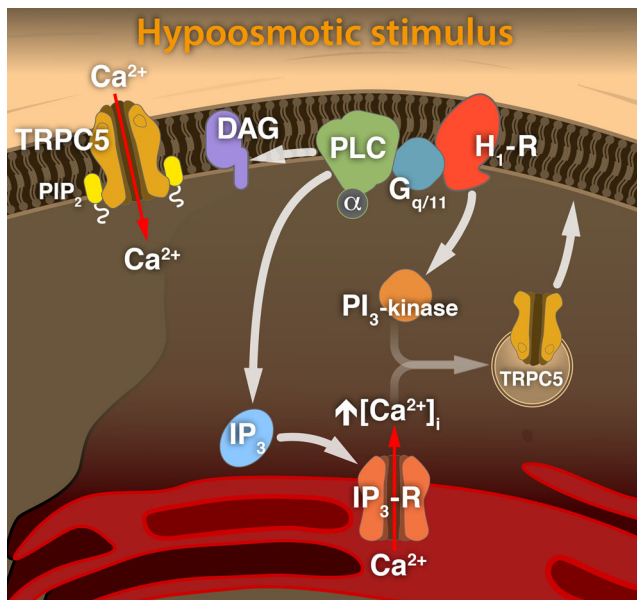
Mechanical forces can initiate the rapid translocation of other TRP channels to the plasma membrane. For example, TRPV2, TRPV4 and TRPM7 have been shown to translocate to the plasma membrane following mechanical forces applied to endothelial cells or myocytes [22, 27, 31]. TRPC6 channels are inserted into the membrane in response to receptor stimulation by CCh, presumably via an exocytotic mechanism [8]. Moreover, PI<sub>3</sub>K has been shown to play a key role in the translocation of TRPC6 to the plasma membrane when GPCRs are stimulated [29]. There are eight PI<sub>3</sub>K isoforms divided into three classes [50]. Class I includes four isoforms that are activated by tyrosine kinase receptors and GPCRs, and it has been suggested that the group 1 isoform p110 $\gamma$  is involved in the translocation of TRPC6 [29].

Activation of several receptor classes enhances the insertion of TRPC5 channels into the plasma membrane [4, 20]. A fraction of TRPC5 channels are located in intracellular vesicles, and they can be inserted into the plasma membrane in response to activation of growth factor receptors. The time course of TRPC5 trafficking in our biochemical experiments was consistent with those observed in functional assays, and those observed elsewhere in HEK cells and hippocampal neurons activated by EGF or NGF and by muscarinic receptor agonists, respectively [4, 47]. Our results reveal that TRPC5 is expressed at the plasma membrane in control conditions and that exposure to a hypoosmotic solution does not alter its expression in cells expressing TRPC5. Thus, the influx of calcium due to direct activation of TRPC5 may be insufficient to induce channel translocation. However, surface expression of TRPC5 does increase after hypoosmotic stimulation of cells expressing TRPC5 and H<sub>1</sub>R. Indeed, pre-incubation with wortmannin (20 nM) reduces the surface expression of TRPC5 and the number of cells that respond to hypoosmosis. PI<sub>3</sub>K inhibitors can also inhibit PI<sub>4</sub>K and thus, the effect of PI<sub>3</sub>K inhibitors may be due to the depletion of membrane PIP<sub>2</sub> rather than the inhibition of PI<sub>3</sub>K activity [1, 41]. However, the low concentration of wortmannin (20 nM) used should ensure that the levels of IP<sub>3</sub> induced by H<sub>1</sub>R activation and PIP<sub>2</sub> are not affected [29].

Based on our findings, we propose the following mechanism of activation in cells expressing TRPC5/H<sub>1</sub>R. Membrane stretch directly activates TRPC5 channels at the plasma

membrane (see also [16]) as well as  $G_{q/11}$ -coupled receptors, without the involvement of their ligands. This GPCR activation stimulates the PLC pathway, increasing  $IP_3$  levels and rapid calcium release from intracellular stores. Thus, in cells expressing TRPC5 and  $H_1R$ , the osmotically-evoked increase in  $[Ca^{2+}]_i$  is a consequence of calcium entry from the extracellular medium and calcium release from intracellular stores. In addition, osmotic stress activation of  $H_1R$  may activate  $PI_3K$  driving the translocation of TRPC5 channels to the plasma membrane (Fig. 5). Thus, the potentiation of TRPC5 activity observed in response to hypoosmotic stimulation in cells expressing TRPC5 and  $H_1R$  is dependent on both cascades operating simultaneously: the increase in intracellular calcium concentration and in the number of channels at the plasma membrane. It is possible that  $PI_3K$ -dependent translocation does not provoke channel activation but that the increase in intracellular calcium causes the activation or stabilisation of the channel at the plasma membrane. Alternatively,  $PI_3K$  activation and  $Ca^{2+}$  may be linked pathways.

In addition to these mechanisms, we cannot rule out the involvement of other pathways in the translocation of the channel to the plasma membrane. The SNARE complex interacts with different TRP channels, and the SNARE complex is involved in vesicle fusion with the plasma membrane. SNAP-23 and SNAP-25, as well as syntaxin-3, have been seen to associate with TRPC1 or TRPC3, participating in their



**Fig. 5** Scheme illustrating the pathways of TRPC5 channel activation by mechanical stress. TRPC5 is activated by mechanical stress (hypoosmotic solution) leading to calcium entry into the cell. The same stimulus activates the histamine-sensitive GPCR, which in turn activates PLC, as a result of which  $IP_3$  releases calcium from the internal stores, and  $PI_3K$ .  $PI_3K$  and calcium contribute directly or indirectly to the translocation of the TRPC5 channel to the plasma membrane and to increase the osmo-mechanical activation of TRPC5

plasma membrane insertion [7, 38, 45]. In addition, the potentiation of TRPC5 activity following hypoosmotic-induced calcium release from the endoplasmic reticulum may be coupled to activation of a store-operated pathway. Two proteins could play a role in this mechanism, Orai and STIM1 [52, 57]: STIM1 senses the depletion of  $Ca^{2+}$  in the endoplasmic reticulum, while Orai is a regulatory subunit of TRPC channels that could transduce the STIM1 signal into TRPC5 channel activation [26]. Indeed, STIM1 is required for receptor-mediated TRPC5 activation but not for its activation by  $La^{3+}$  [57]. All these hypotheses are not mutually exclusive and further studies will be necessary to identify which of these mechanisms are involved in potentiating the response to hypoosmotic stimulation.

To date, the physiological role of TRPC5 remains unclear. TRPC5 is expressed not only in somatosensory neurons but also in other tissues that are subjected to mechanical forces like cardiomyocytes, fibroblasts, endothelial cells and vascular and gastric smooth muscle [3, 14, 32, 35, 40, 56]. Recent results emphasised a role for TRP channels in myogenic constriction of blood vessels (the Bayliss effect) [6, 21, 51]. Additionally, we show that  $G_{q/11}$ -coupled receptors function as sensors of membrane stress, as reported elsewhere [43].  $G_q$ -coupled receptors are endogenously expressed in all cell types and thus, one may speculate that membrane stress stimulation could produce enhanced responses in cells co-expressing TRPC5 and GPCRs and that this mechanism may have considerable physiological relevance in the vascular system, contributing to cell motility or essential hypertension. On the other hand, there is no potentiation of TRPC5 responses to osmotic stress in the absence of  $H_1R$  overexpression and thus, as indicated for TRPC6 [44], it will be important to establish whether mechanical activation of GPCR is physiologically relevant in tissues with their endogenous GPCR levels. Elsewhere, activation of GPCRs by their agonist was shown to sensitise a subpopulation of cutaneous C fibre nociceptors via the G protein-dependent potentiation of mechanically activated RA-type currents [25]. Our results suggest a possible G protein-dependent mechanism for the sensitization/modulation of mechanically activated currents.

In light of this data, we propose a synergistic mechanism by which osmo-mechanical stimulation activates a GPCR that induces the surface expression of TRPC5, potentiating the responses of this channel to mechanical stimulus in a calcium, PLC and  $PI_3K$  dependent manner.

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**Conflict of interests** The authors have no conflicts of interest to declare.

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