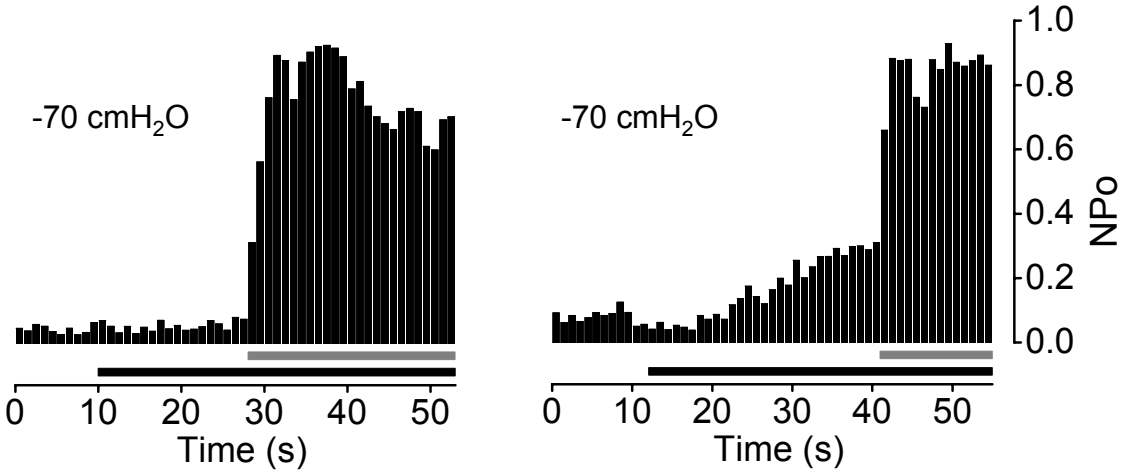
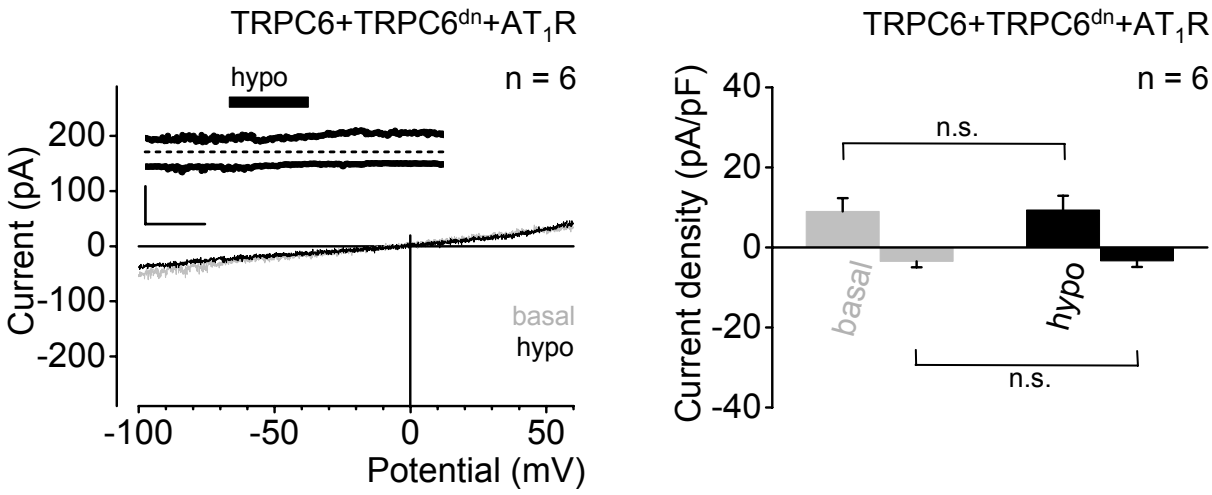
Supplementary Figure S1



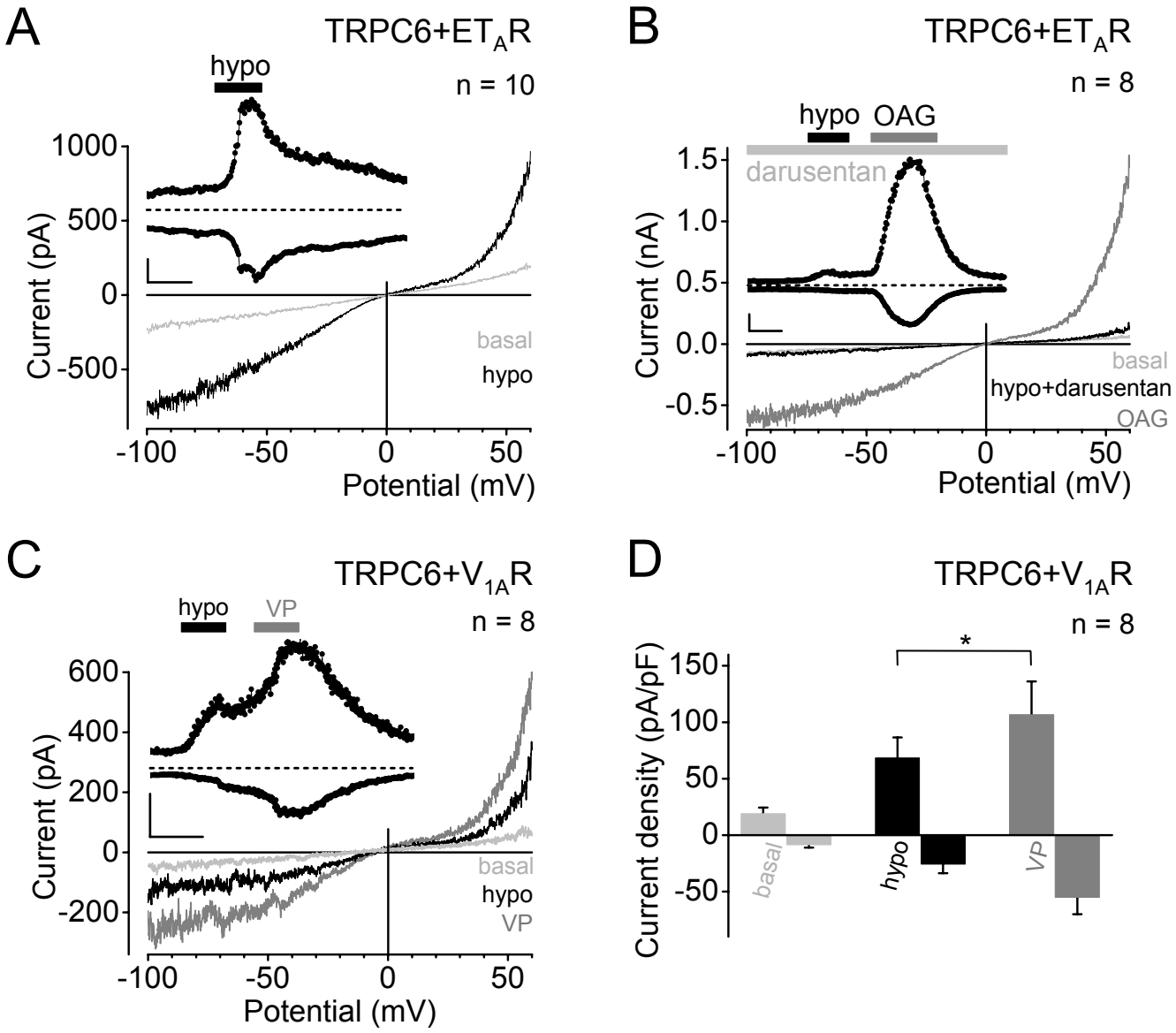
Supplementary Figure S1. Effect of exuberant membrane stretch on TRPC6 activation. Two of four graphs of consecutive open probabilities (NPo) in 1-s steps from inside-out patches from TRPC6 expressing HEK293 cells at -60 mV are shown. The graphs display the smallest (*left*) and the largest effect (*right*) of -70 cmH_2O pressure on TRPC6 channel activity. The durations of pressure application (black bars) and bath application of 10 µM SAG (grey bars) are shown.

Supplementary Figure S2



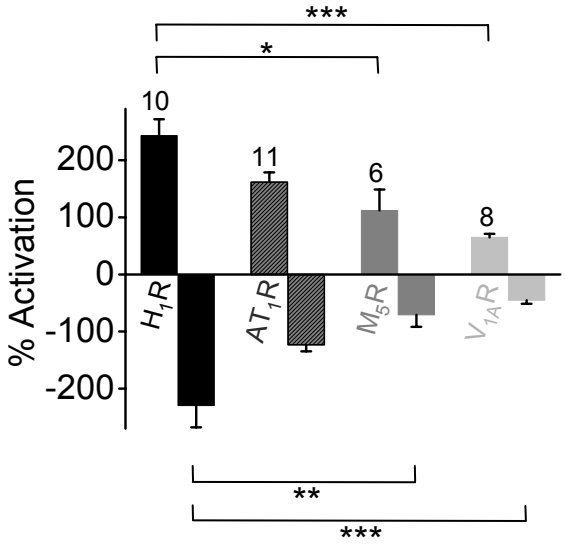
Supplementary Figure S2. Activation by hypotonicity is fully suppressed by a dominant-negative TRPC6 channel mutant (TRPC6^{dn}). Whole-cell recordings from HEK293 cells co-expressing TRPC6 and TRPC6^{dn} (1:1) and AT_1R. IV relationships before 'basal' and during hypotonic solution 'hypo' (250 mOsm kg^{-1}) are displayed (*left*). Inset, Current time courses at ±60 mV, hypotonic stimulation, zero current level (stippled line) and scale bars (100 pA, 50 s) are shown. The corresponding current density analysis is shown in the *right panel*.

Supplementary Figure S3



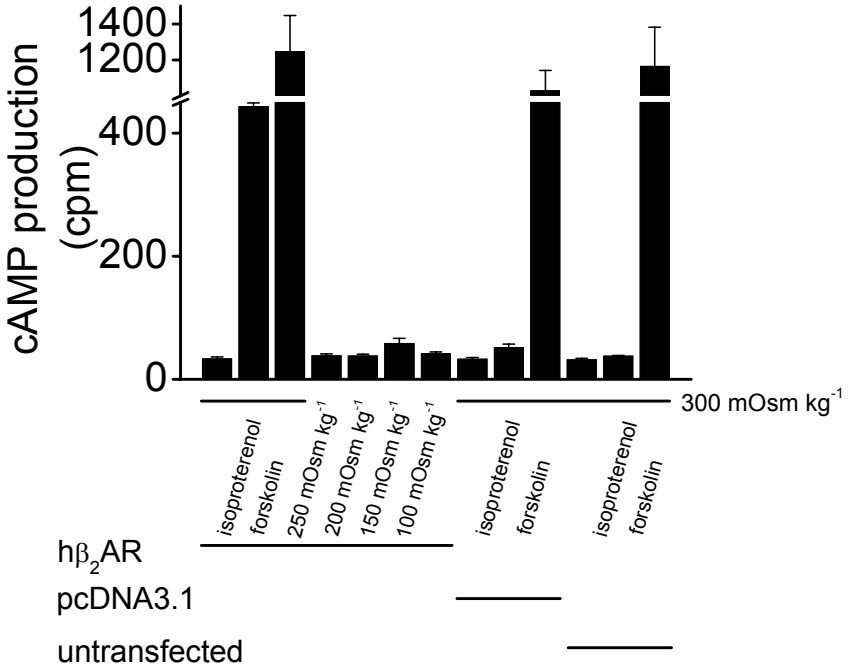
Supplementary Figure S3. Activation of ET_A and V_1 receptors by hypotonicity. Whole-cell recordings from HEK293 cells co-expressing TRPC6 and the ET_AR (A, B) or the $V_{1A}R$ (C, D). IV relationships before 'basal', during hypotonic solution 'hypo' (250 mOsm kg^{-1}) without (A and C) and with 10 µM darusentan, during agonist stimulation with 1 µM vasopressin 'VP' (C) and during direct TRPC6 stimulation with 100 µM OAG (B) are displayed. A-C, insets, Current time courses at ±60 mV, hypotonic stimulation, vasopressin application, OAG application, darusentan application, zero current level (stippled lines) and scale bars (200 pA, 50 s) are shown. D, Current densities before hypotonic stimulation and during vasopressin stimulation.

Supplementary Figure S4



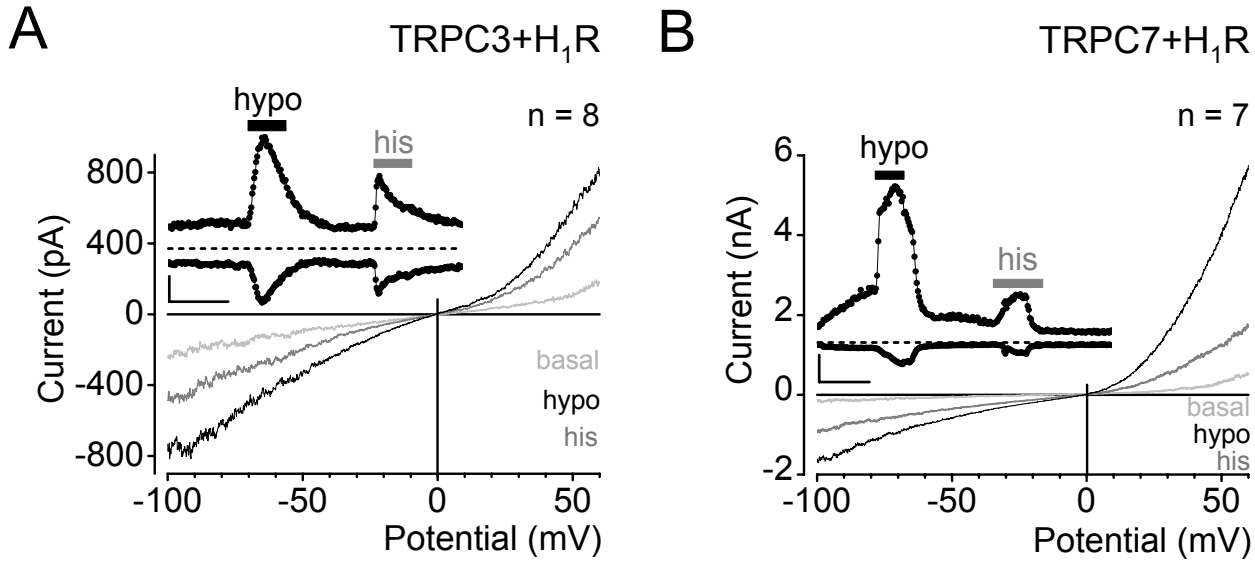
Supplementary Figure S4. Comparison of the mechanosensitivity of $G_{q/11}$-coupled receptors. The summary of whole-cell recordings from HEK293 cells transiently expressing TRPC6 and the indicated $G_{q/11}$-coupled receptors at ±60 mV is shown. The percentage of current activation by hypotonicity is normalized to a subsequent agonist stimulus at maximally effective concentrations. For TRPC6 and AT_1R co-expressing cells the hypotonicity-induced current was normalized to an initial agonist response of the same cell-transfection. The number of summarized cells for the indicated combinations (numbers over bars) is displayed.

Supplementary Figure S5



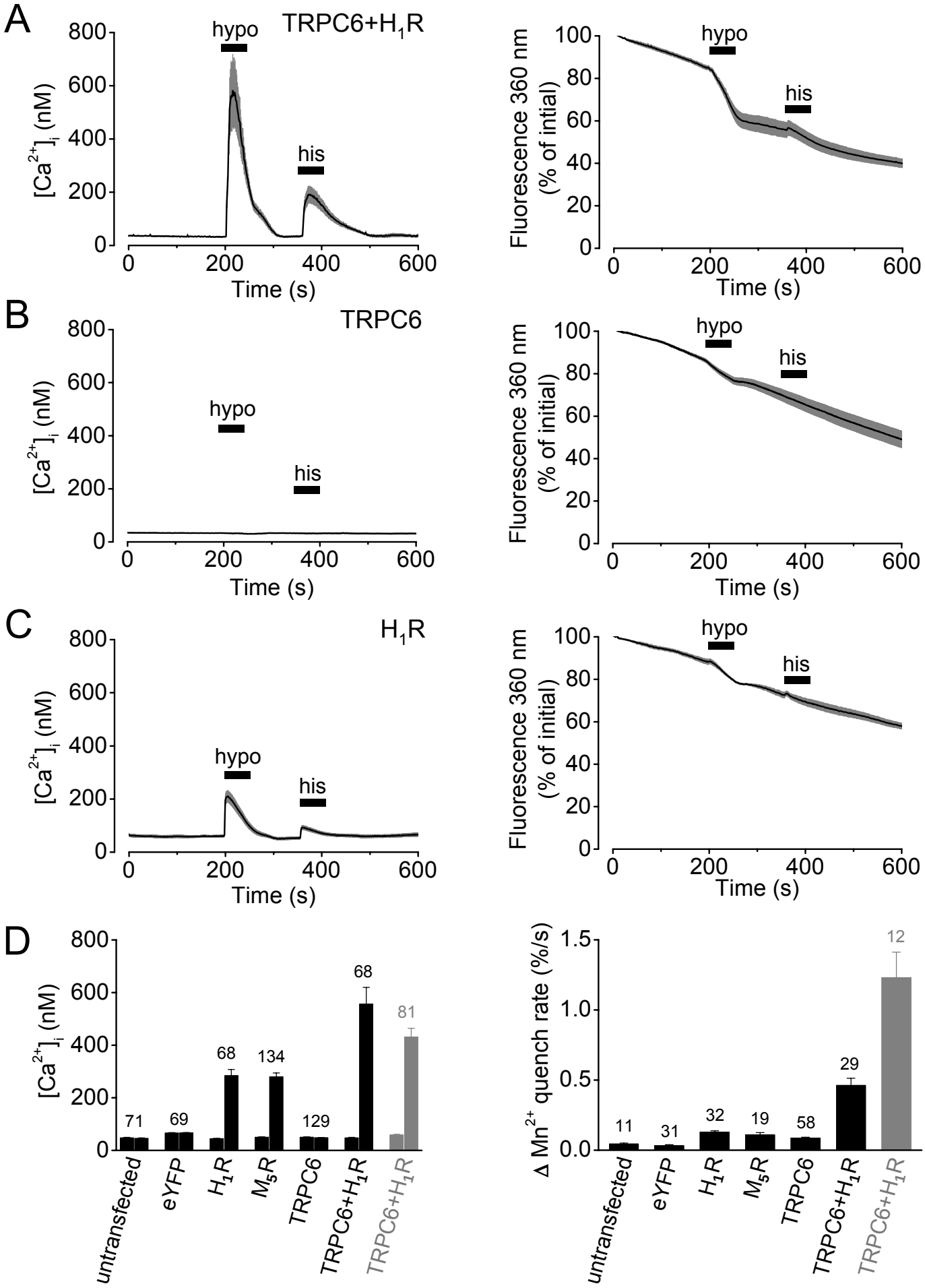
Supplementary Figure S5. Adrenoceptors are insensitive to membrane stretch. One exemplary assay of 3 independent cAMP assays with untransfected and transfected HEK293 cells transiently expressing β_2AR or empty vector as control. cAMP productions measured in counts per minutes (cpm) are displayed as mean of quadruplets. Different hypotonic solutions, receptor activation with 100 µM isoproterenol and adenylyl cyclase stimulation with 100 µM forskolin for 30 minutes at 37°C are indicated.

Supplementary Figure S6



Supplemetary Figure S6. Hypotonicity-induced TRPC3 and -7 currents. A and B, Whole-cell patch-clamp recordings from transfected HEK293 cells co-expressing TRPC3 and the H_1R (A) and co-expressing TRPC7 and the H_1R (B). IV relationships before 'basal', during hypotonic 'hypo' and receptor stimulation with 100 µM histamine 'his' (A and B) are displayed. A and B, insets, Time-dependent changes of inward and outward currents at ±60 mV are shown with hypotonic stimulation and agonist stimulation. Stippled lines represent the zero current level. The time scale bar is 50 s and the current scale bar is 200 pA (A) and 1 nA (B) (insets).

Supplementary Figure S7



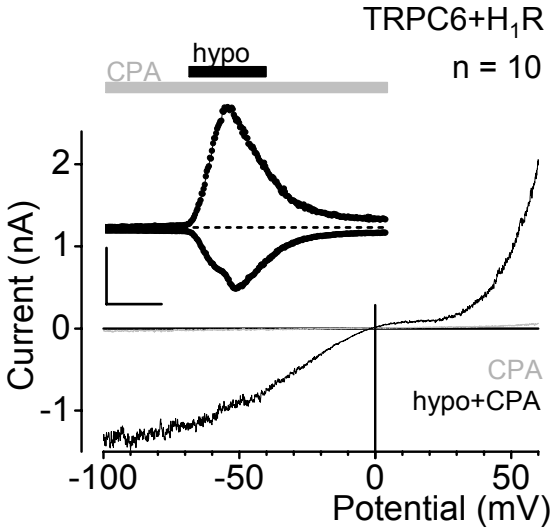
Supplementary Figure S7 continued

Supplementary Figure S7. Cell swelling-induced Ca^{2+} increases and Mn^{2+} influxes. A-C, *left*, Representative $[Ca^{2+}]_i$ imaging with five fura-2 loaded HEK293 cells transfected with TRPC6 and the H_1R (A), TRPC6 alone (B), the H_1R (C) and corresponding Mn^{2+} quench experiments (A-C, *right*) with 200 µM Mn^{2+} are displayed. The hypotonic stimulus was 250 mOsm kg^{-1} 'hypo' and the agonist stimulation was performed with 100 µM histamine 'his'. D, *left*, Summary of Ca^{2+} imaging. Each indicated transfection is shown by two bars: left bars: basal $[Ca^{2+}]_i$, right bars: maximal $[Ca^{2+}]_i$ during hypotonic stimulation. D, *right*, Summary of Mn^{2+} quench experiments. Each indicated transfection shows the normalized Mn^{2+} quench rate expressed as percentages over time. D, *left* and *right*, Agonist stimulation instead of hypotonic stimulation (grey bars) and the number of summarized cells for the indicated transfection (numbers over bars) are displayed.

Cell Swelling-induced Ca^{2+} Increases and Mn^{2+} Influxes We reconstituted proximal components of the smooth muscle signaling cascade by transiently expressing TRPC6 with or without the H_1R in HEK293 cells. Cells expressing TRPC6 and the H_1R responded to osmotically-induced membrane stretch with a transient rise of $[Ca^{2+}]_i$ (Supplementary Figure S7 A, *left*). Subsequent receptor activation by histamine elicited a desensitized Ca^{2+} transient serving as a transfection control. In contrast, cells only transfected with TRPC6 cDNA did not respond to the hypoosmotic stimulus (Supplementary Figure S7 B, *left*). Next, we tested the responsiveness of cells only expressing the H_1R to a hypotonic stimulus, and observed a transient rise of $[Ca^{2+}]_i$ (Supplementary Figure S7 C) whose amplitude was significantly lower ($P<0.001$) (Supplementary Figure S7 D, *left*). Similar results were obtained when the M_5R was expressed in HEK293 cells (Supplementary Figure S7 D, *left*).

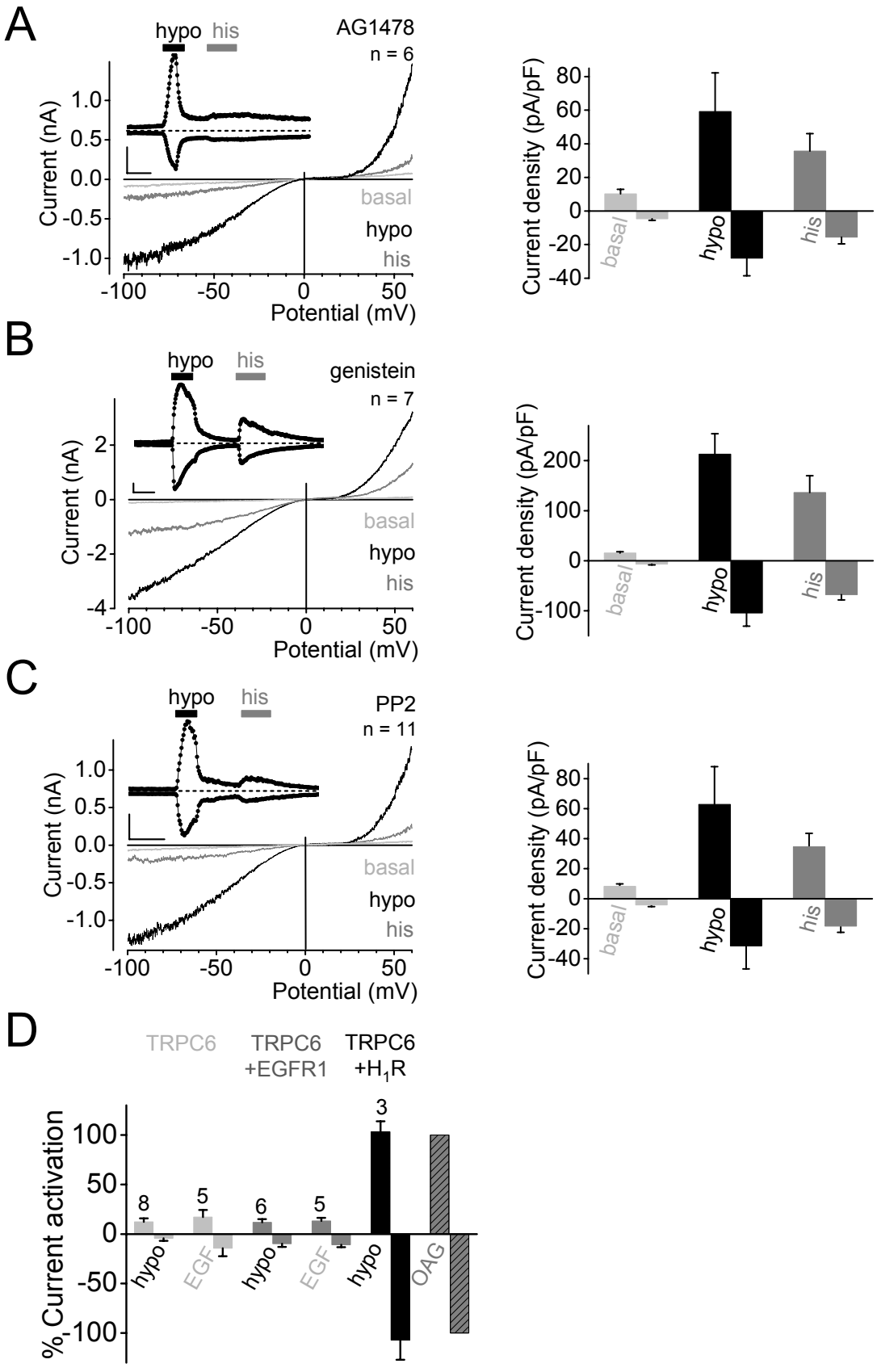
To discriminate between Ca^{2+} release from intracellular stores and Ca^{2+} entry from the external medium, quenching of fura-2 fluorescence by influx of extracellular Mn^{2+} was measured. A pronounced quench of fura-2 fluorescence was observed only in TRPC6- and H_1R-expressing cells (Supplementary Figure S7 A, *right* and D, *right*). Expression of TRPC6 (Supplementary Figure S7 B, *right*) or H_1R alone (Supplementary Figure S7 C, *right*) did not give rise to increased fura-2 quenching demonstrating that the osmotically-induced increase in $[Ca^{2+}]_i$ in H_1R expressing cells (Supplementary Figure S7 C, *left*) is caused by Ca^{2+} release from internal stores and is not due to different receptor expression levels. The Mn^{2+} quench rate triggered by a hypotonic stimulus was not different in untransfected, eYFP-, H_1R- or M_5R-expressing cells ($P>0.05$) (Supplementary Figure S7 D, *right*) suggesting that hypotonic cell swelling-induced cation entry only occurs after co-expression of TRPC6 and the H_1R.

Supplementary Figure S8



Supplementary Figure S8. Store depletion does not influence hypotonicity-induced receptor activation. Whole-cell recordings from HEK293 cells co-expressing TRPC6 and the H_1R. IV relationships during cyclopiazonic acid 'CPA' (10 µM) application and during simultaneous application of hypotonic solution 'hypo' (250 mOsm kg^{-1}) and CPA are displayed. Inset, Current time course at ±60 mV, CPA application and hypotonic stimulation, zero current level (stippled line) and scale bars (1 nA, 50 s) are shown.

Supplementary Figure S9



Supplementary Figure S9 continued

Supplementary Figure S9. Receptor tyrosine kinases do not play a dominant role for mechanosensation. A-C, One example of a whole-cell recording from HEK293 cells transiently expressing TRPC6 and the H_1R. Pre-treatment with 100 nM AG1478 for 10 to 15 minutes (A), with 10 µM genistein for 10 minutes (B), with 1 µM PP2 for 30 minutes (C). A-C, *left*, IV relationships before 'basal', during hypotonic stimulation (250 mOsm kg^{-1}) 'hypo' and during receptor stimulation with 100 µM histamine 'his' are displayed. A-C, *left*, insets, Current time courses at ±60 mV, hypotonic stimulation and histamine stimulation, zero current level (stippled lines) and scale bars (500 pA, 50 s) are shown. A-C, *right*, Corresponding current density analysis of whole-cell measurements are represented. The current densities were determined at ±60 mV before, in the presence of hypotonic solution and histamine. D, Summary of whole-cell recordings from HEK293 cells transiently expressing TRPC6 (light grey), TRPC6 and the EGFR1 (grey) or TRPC6 and the H_1R (black). The percentage current activation is calculated as the ratio of maximal current in the presence of hypotonic solution (250 mOsm kg^{-1}) 'hypo' or during stimulation with 4 mg/ml EGF 'EGF' minus basal current to maximal current elicited by 100 µM OAG application (grey hatched bar). The number of summarized cells for the indicated transfection combinations (numbers over bars) is displayed.

Influence of Tyrosine Kinase To assess the contribution of tyrosine phosphorylation pathways to TRPC6 activation by hypotonic cell swelling, an EGFR tyrosine kinase inhibitor AG1478 (100 nM) was applied 10 to 15 minutes before the onset of whole-cell recordings in TRPC6 and H_1R co-expressing HEK293 cells. AG1478 at a final concentration of 100 nM to selectively target EGFR tyrosine kinase activity and at 100 µM to unspecifically inhibit a broader spectrum of tyrosine kinases had no effect on TRPC6 activation by osmotically induced membrane stretch or by histamine challenge (Supplementary Figure S9 A). Additionally, neither pre-treatment of cells with 10 µM genistein, an unspecific tyrosine kinase inhibitor, nor with PP2 (1 µM), a selective inhibitor of Src tyrosine kinases, impeded cell swelling-induced TRPC6 activation (Supplementary Figure S9 B and C). Furthermore, we analyzed the mechanosensitivity of the epidermal growth factor receptor 1 (EGFR1) in an over-expression system. We did not observe significant differences in TRPC6 current activation in response to either hypotonic or agonist stimulation. In addition, there were no differences between cells expressing endogenous EGF receptors or those over-expressing recombinant EGF receptors while co-expression of TRPC6 and histamine H_1 receptor (H_1R) entailed full channel activation in response to hypotonicity (Supplementary Figure S9 D). These results strongly support the view that EGF receptors are not mechanosensitive under the experimental conditions chosen. Furthermore, the EGF receptor tyrosine kinase pathway does not appear to play a dominat role for TRPC6 activation in HEK293 cells.

Online Data Supplement

Material and Methods

Fluorescence Imaging

Intracellular free Ca^{2+} measurements and Mn^{2+} quench experiments were conducted 48 to 60 h after transfection of HEK293 cells with the FuGENE6 reagent (Roche Applied Science, Mannheim, Germany). Transfected HEK293 cells were loaded for 30 min with fura-2-acetoxymethyl ester (5 µM; Molecular Probes Inc., Eugene, OR) in HEPES-buffered saline containing: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES (pH 7.4 with NaOH) and 0.1% (w/v) bovine serum albumin at 37°C. Coverslips were mounted on the stage of a monochromator-equipped (Polychrome II, TILL-Photonics, Martinsried, Germany) inverted microscope (Olympus IX 70 with an Uapo/340 40x/1.35 oil immersion objective). Fluorescence was recorded with a 12-bit CCD camera (IMAGO, TILL-Photonics). The fluorescence of fura-2 and enhanced yellow fluorescence protein (eYFP) was excited at 340, 360, 380 and 500 nm. Fura-2 was not excited at 500 nm and eYFP contributed negligibly to fluorescence excited at 340-380 nm. Intracellular free Ca^{2+} concentrations were calculated as described previously[1] For an imaging experiment, transfected HEK293 cells were reseeded on 5 to 6 coverslips. Measurements with 3-24 individual cells were made within 3 to 20 h. The means of three to eight independent experiments were calculated. Cells were continuously superfused at room temperature with a bath solution containing 110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4 with NaOH) which was supplemented with mannitol to 300 mOsm kg^{-1}. The hypoosmotic solution had the same salt concentration without added mannitol resulting in an osmolality of 249 to 253 mOsm kg^{-1}. Receptor stimulation was carried out by adding 100 µM histamine to the isotonic bath solution. In Mn^{2+} quench experiments, 200 µM $MnCl_2$ was applied. Mn^{2+} quench was calculated and normalized as described previously.[2]

Electrophysiological Techniques

HEK293 cells were transfected with cDNAs coding for human TRPC3, TRPC6 and mouse TRPC7 in pcDNA3, with the original cDNA coding for one of the following receptors: guinea pig histamine receptor 1 (H_1R), rat type-5 muscarinic acetylcholine receptor (M_5R), mouse angiotensin II AT_{1A} (AT_1R) and the enhanced green fluorescent protein (eGFP) reporter plasmid using FuGENE6 reagent. A7r5 cells were transfected with the AT_1R in pIRES2-EGFP (Clontech, Palo Alto, USA). Conventional whole-cell patch-clamp recordings were carried out at 23°C 48 h after transfection. The bath solution contained 110 mM NaCl, 5 mM CsCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4 with

NaOH) supplemented with mannitol to 300 mOsm kg^{-1}. The hypoosmotic solution without mannitol had an osmolality of 249-253 mOsm kg^{-1}. A7r5 cells were superfused with the isotonic bath solution plus 10 µM nicardipine and 50 µM 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB). Data were collected with an EPC10 patch clamp amplifier (HEKA, Lambrecht, Germany) using the Pulse software. IV relations were obtained from triangular voltage ramps from –100 to +60 mV with a slope of 0.4 V s^{-1} applied at a frequency of 1 Hz. Data were acquired at a frequency of 5 kHz after filtering at 1.67 kHz. Cell inflation was performed by applying positive pressure of 8-10 cmH_2O to the patch pipette through a water manometer. Membrane stretch was applied by raising the patch pipette vertically by 8-10 µm.

Single Channel Recordings

HEK293 cells were transiently transfected with 2 µg of cDNAs coding for human TRPC6 in pIRES2-EGFP (Clontech, Palo Alto, USA) using 6 µl of the FuGENE6 reagent. For this experiment, the cells were seeded on culture dishes treated with polylysine. Excised inside-out patch-clamp recordings were carried out at room temperature (23°C) 72 h after transfection. The bath and pipette solution contained 130 mM cesium methane-sulfonate, 102 µM CsCl, 1 mM $MgCl_2$, 3.949 mM $CaCl_2$, 10 mM BAPTA (100 nM free Ca^{2+}) and 10 mM HEPES (pH 7.2 with CsOH) resulting in an osmolality of 294 mOsm kg^{-1}. The bath solution also contained 50 µM 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB). Patch pipettes made of borosilicate glass (Science Products, Hofheim, Germany) were coated with R-6101 (Dow Chemical, Midland, USA) and had resistances of 8-10 MΩ. Data were collected with an EPC9 patch clamp amplifier (HEKA, Lambrecht, Germany) using the Pulse software and were acquired at a holding potential of -60 mV with a frequency of 20 kHz after filtering at 6.67 kHz. Membrane stretch during inside-out recordings was performed by applying negative pressure of 10 or 70 cmH_2O to the patch pipette through a water manometer. Finally, 10 µM 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG) was applied to the bath solution to obtain maximal channel activity in the patch. For the evaluation of consecutive channel activity ('NPo', the product of the number of channels and open probability) in 1-s steps, PC DAC 1.1.5 of Marburg University Software Team was used.

PI Assay

To monitor receptor-mediated activation of the PLC pathway, accumulation of inositol phosphates was assessed as described previously.[3]

Bioluminescence Resonance Energy Transfer (BRET) Assay

To detect β-arrestin-2 recruitment by the human AT_1R, the BRET[1] technique was performed using a Fluostar Optima (BMG LabTechnologies, Offenburg, Germany) as described previously.[4] Briefly, COS-7 cells were co-transfected with the cDNA encoding a AT_1R-venus fusion protein (10 µg) and 15 ng of a cDNA encoding a fusion protein between the humanized version of *Renilla* luciferase and rat β-arrestin-2 (βArr2-*R*luc) using the FuGENE6 reagent. Both cDNAs were kindly provided by the laboratory of Dr. Bouvier, Montrèal. 48 h post transfection, approximately 75000 cells were distributed in a 96-well microplate in 100 µl of a bath solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4) with an osmolality of 298-302 mOsm kg^{-1}. Agonist-promoted arrestin-recruitment was detected after adding 1 µM (final) ATII. Hypoosmotic stress was induced by adding 10 µl pure H_2O (resulting in an osmolality of 273 mOsm kg^{-1}). After 60 s, the osmotic stress was relieved from the cells by adding 10 µl of a 2-fold concentrated bath solution. Effects of the AT_1R antagonist losartan were determined by adding 1 µM (final) of the antagonist 4 minutes prior to agonist or hypoosmotic stimulation. The *R*luc substrate coelenterazine H (5 µM) was added 25 minutes after stimulation and the BRET signal was continually sampled. The BRET signal (ratio of the light intensity measured at 535±30 over 475±30 nm) was determined in quadruplicates. BRET signals of cells expressing energy donor and acceptor were not significantly different (P=0.27) from cells expressing only the energy donor. The means of three independent experiments (monitored within 50 minutes) were calculated.

Radioimmunoassay

To determine the AII concentration in medium superfusing brain arteries, the radioimmunoassay was performed with a double antibody for quantitative *in vitro* diagnosis (Bühlmann, Schönenbuch Switzerland) according to the manufactures instructions. To prevent degradation of AII, bestatin was added. The detection limit for AII in the latter assay was 300 fM.

cAMP Assay

For determination of cAMP production, 4x106 HEK293 cells were transfected with 10 µg of the human β2 adrenoceptor (β2AR) or the empty vector using Fugene6 or cells were not transfected. Cells were transferred into 12-well plates ($1.5x10^5$ cells per well). After 48 h, 2 µCi of [2,8-^{3}H]-adenine (31.7 Ci/mmol, PerkinElmer Life Science, USA) was added to 1 ml growth medium per well. After an 18-h labelling period, cells were washed once with 1 ml of serum-free DMEM supplemented with 1 mM 3-isobutyl-1-methylxanthine (IBMX). Control stimulation was carried out by replacing 0.5 ml isotonic bath solution with 145 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4 with NaOH) and 1 mM IBMX resulting in an osmolality of 298 mOsm kg^{-1} at 37°C. Stimulation with hypotonic solutions was similar to that with isotonic solution except that NaCl was reduced to 120, 95, 70 and 45 mM yielding total osmolalities of 250, 200, 150 and 100 mOsm kg^{-1} with a tolerance of ±6 mOsm kg^{-1}. Receptor stimulation was performed with 100 µM DL-isoproterenol with 0.05% sodium metabisulfite at 37°C. Forskolin was added at a final concentration of 100 µM at 37°C. After 30 minutes, stimulation was stopped by adding 1 ml 5% trichloroacetic acid, followed by a 30-min incubation at 4°C. cAMP separation was carried out according to Salomon et al. (1974)[5] and counted with a scintillation counter (LS 6000IC, Beckman, USA). The cAMP fraction was determined as quadruplets.

References

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