

G_q-coupled receptors as mechanosensors mediating myogenic vasoconstriction

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Despite the central physiological function of the myogenic response, the underlying signalling pathways and the identity of mechanosensors in vascular smooth muscle (VSM) are still elusive. In contrast to present thinking, we show that membrane stretch does not primarily gate mechanosensitive transient receptor potential (TRP) ion channels, but leads to agonist-independent activation of G_{q/11}-coupled receptors, which subsequently signal to TRPC channels in a G protein- and phospholipase C-dependent manner. Mechanically activated receptors adopt an active conformation, allowing for productive G protein coupling and recruitment of β -arrestin. Agonist-independent receptor activation by mechanical stimuli is blocked by specific antagonists and inverse agonists. Increasing the AT₁ angiotensin II receptor density in mechanically unresponsive rat aortic A7r5 cells resulted in mechanosensitivity. Myogenic tone of cerebral and renal arteries is profoundly diminished by the inverse angiotensin II AT₁ receptor agonist losartan independently of angiotensin II (AII) secretion. This inhibitory effect is enhanced in blood vessels of mice deficient in the regulator of G-protein signalling-2. These findings suggest that G_{q/11}-coupled receptors function as sensors of membrane stretch in VSM cells.

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Introduction

The mechanisms underlying the translation of mechanical stimuli into biochemical information have a fundamental

function in physiology and pathophysiology, but are only poorly understood (Kung, 2005; Orr *et al*, 2006). Local blood flow is dynamically regulated to match the metabolic demand of peripheral tissues and organs. More than 100 years ago, Bayliss made the seminal observation that small-resistance arterial blood vessels have the intrinsic property to constrict in response to rises in intraluminal pressure (Bayliss effect). As disruption of the endothelium does not impair pressure-induced myogenic vasoconstriction, it is now assumed that myogenic responsiveness is an inherent property of vascular smooth muscle (VSM) and that it can be fine-tuned by endothelial and neurohumoral factors (Davis and Hill, 1999; Murphy *et al*, 2002) acting at G protein-coupled receptors (Pierce *et al*, 2002). The vasomotor response is of prime physiological relevance because it determines basal vascular tone and peripheral vascular resistance and regulates capillary hydrostatic pressure and organ perfusion. Consequently, impaired myogenic responsiveness and blood flow autoregulation are encountered in various pathological states, such as systemic hypertension, diabetes mellitus and stroke.

However, the underlying signalling pathways and the molecular identity of mechanosensors in VSM are largely unknown. Increased intravascular pressure causes depolarization of the arterial myocyte cell membrane (Davis and Hill, 1999), thereby activating voltage-dependent L-type Ca²⁺ channels (Ca_vs) (Moosmang *et al*, 2003). However, pressure-induced depolarization is not affected by L-type Ca²⁺ channel blockers (Knot and Nelson, 1995), implying that another stretch-activated ion channel is responsible for smooth muscle cell (SMC) depolarization.

The mechanisms linking mechanical stimuli to ion channel activation appear to rely critically on biochemical signalling cascades. There is a compelling body of evidence that phospholipase C (PLC) activation is a prerequisite for pressure-induced myogenic vasoconstriction (Thorneloe and Nelson, 2005; Inoue *et al*, 2006). However, the mechanism of PLC activation in response to mechanical stimuli is still elusive. As classical vasoconstrictors such as angiotensin II (AII) or endothelin exert their action by activating the PLC pathway, it is tempting to speculate that intravascular pressure and receptor agonists may engage similar signalling cascades leading to smooth muscle contraction.

A number of proteins, including stretch-sensitive ion channels, cell adhesion proteins, the cytoskeleton, receptors, G proteins, enzymes and the phospholipid bilayer of the plasma membrane itself, have been discussed as potential mechanosensors (Martinac, 2004; Kung, 2005; Ingber, 2006; Orr *et al*, 2006). During the past decade, a large portfolio of ion channels has been shown to make crucial contributions to the regulation of smooth muscle contractility (Beech *et al*, 2004; Thorneloe and Nelson, 2005). Of late, several members of the transient receptor potential (TRP) family of cation channels (Montell *et al*, 2002; Clapham, 2003) have been proposed as mechanosensitive ion channels, for instance, TRPC6 (Spasova *et al*, 2006).

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However, in TRPC6-deficient mice, the Bayliss effect in cerebral arteries is not affected (Dietrich *et al*, 2005), proving *in vivo* that TRPC6 *per se* is not required for pressure-induced vasoconstriction. In view of these contradictory findings, we investigated the mechanism of mechanosensation and TRPC channel activation in VSM.

Results

TRPC6 is not a classical mechanosensitive ion channel

To investigate whether TRPC6 channels are mechanosensitive, the effect of osmotically induced membrane stretch (250 mOsm kg⁻¹) on TRPC6-expressing HEK293 cells was monitored. By applying the whole-cell patch-clamp recording technique, rapidly developing transient outward and inward currents at holding potentials of ± 60 mV were observed only in response to application of the membrane-permeable DAG analogue 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; 100 μ M) demonstrating the functional expression of TRPC6, but they were

not elicited by hypotonicity (Figure 1A). The current–voltage (IV) relations revealed functional hallmarks of the TRPC3/6/7 subfamily (Hofmann *et al*, 1999), that is, dual inward and outward rectification, a rise in current fluctuations with increasing driving force and a reversal potential close to 0 mV.

To further investigate the potential mechanosensitivity of TRPC6 in response to direct membrane stretch, we used isolated inside-out membrane patches from TRPC6-expressing HEK293 cells. Pressure application of -10 cm H₂O through the patch pipette failed to activate ion currents at a holding potential of -60 mV, whereas subsequent addition of 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG; 10 μ M) to the bath evoked a marked increase in TRPC6 channel activity (Figure 1B). NPo values, the product of channel number and open probability, between 0.044 and 0.089 with negative pressure applied (Figure 1C) but before SAG addition, reflect a low basal channel activity of around 10%. In these experiments, the concentration of Cl⁻ ions was reduced to 10 mM, and 50 μ M 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB)

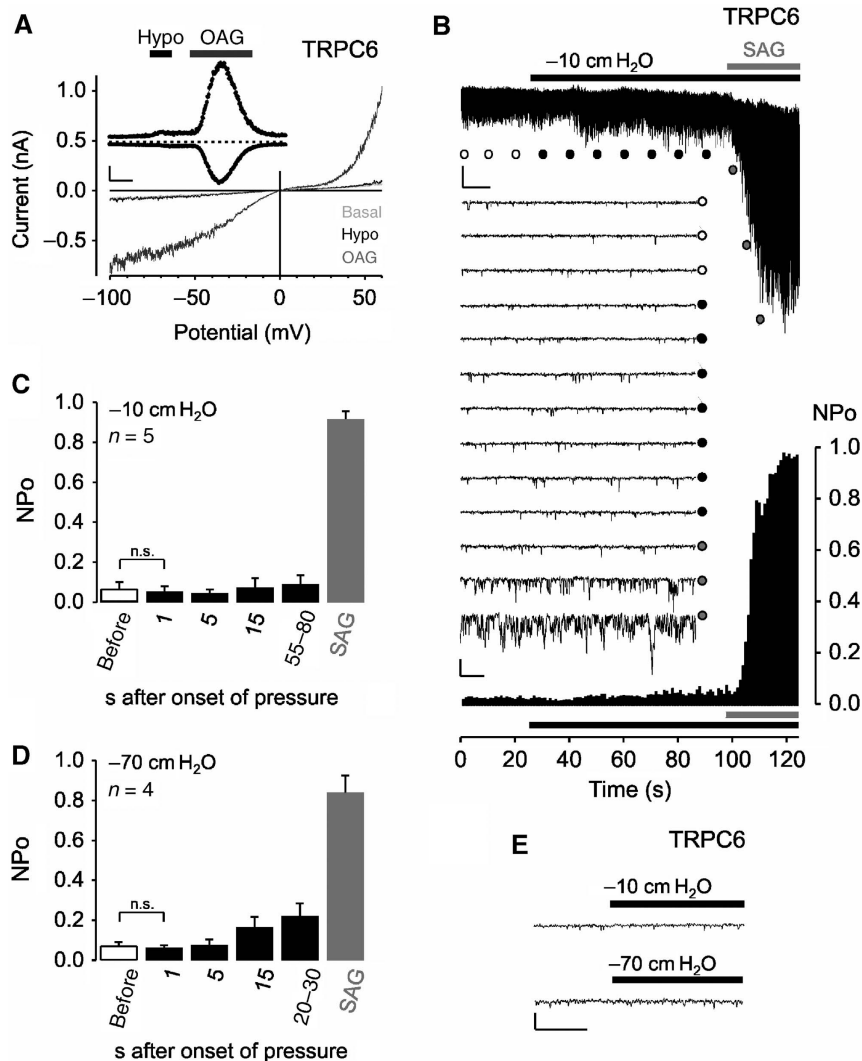


Figure 1 TRPC6 *per se* is not mechanosensitive. (A) Whole-cell recordings from HEK293 cells expressing TRPC6; application of hypotonic stimulus (250 mOsm kg⁻¹), 'Hypo', and of 100 μ M OAG are indicated. Current time courses at ± 60 mV, zero current levels (stippled lines), time scale bar (50 s) and current scale bar (200 pA). (B–E) Single-channel recordings in inside-out patches with negative pipette pressure and subsequent SAG bath application. (B) Original current trace, scale bar (2 pA, 10 s) (top); 13 consecutive traces on an expanded time-scale at the indicated time points, scale bar (5 pA, 20 ms) (middle); the respective graph of consecutive open probabilities (NPo) in 1-s steps (bottom) is displayed. (C, D) Analysis of mean NPo values. (E) Representative expanded current traces around the time point of pressure application, scale bar (5 pA, 10 ms).

was present in the bath solution. Without these measures, the pressure stimulus promptly gated stretch-activated Cl⁻ channels characterized by a large conductance of approximately 70 pS and amplitudes of 4–5 pA at -60 mV. Increasing the negative pipette pressure up to -70 cm H₂O did not trigger a significant increase in TRPC6 activity after 1, 5 or 15 s of mechanical stimulation (Figure 1D). In some membrane patches, a slow saturable increase in NPo values up to 0.35 was observed at later time points (Figure 1D and Supplementary Figure S1), but instantaneous channel activation during the first few milliseconds was never observed (Figure 1E), suggesting that TRPC6 channels *per se* are not mechanosensitive.

Ligand-independent activation of AT₁R elicits TRPC6-dependent cation currents

It has been reported that mechanical stress can activate the AT₁ receptor (AT₁R) independently of AII (Zou *et al*, 2004), leading to an active receptor conformation (Yasuda *et al*, 2008). Therefore, we co-expressed TRPC6 together with the AT₁R in HEK293 cells. Osmotically induced membrane stretch invariably activated TRPC6-dependent cation currents (Figure 2A). The hypotonicity-induced TRPC6 activation could be completely suppressed by a dominant-negative TRPC6 mutant (Hofmann *et al*, 2002) (Supplementary Figure S2). Interestingly, the currents were substantially reduced in the presence of the selective inverse agonists losartan (1 μM) (Figure 2B) or candesartan (100 nM) (data not illustrated). Subsequent addition of OAG in the presence of the inverse agonists directly activated TRPC6 currents. As AII is not synthesized in HEK293 cells (Zou *et al*, 2004), these

observations imply ligand-independent activation of the AT₁R by membrane stretch.

Hypotonic cell swelling may trigger additional unspecific effects apart from exerting membrane stretch by increasing the cell volume. When applying a more direct form of membrane stretch, that is, positive pipette pressure, TRPC6-dependent cation currents were readily detectable (Figure 2C). However, in the presence of losartan, TRPC6 activation by positive pipette pressure was completely abrogated (Figure 2D). These observations strongly argue for an indirect activation of TRPC6 by membrane stretch.

Mechanical activation is a common feature of many G_{q/11}-coupled receptors

We performed similar experiments with cells co-expressing the H₁ histamine receptor (H₁R) and TRPC6. These cells responded to direct membrane stretch exerted by positive pipette pressure (Figure 3A) and as a second direct mechanical stimulus to vertical membrane stretch (Figure 3B) with enhanced TRPC6 currents. Hypotonic stimulation also activated TRPC6 currents (Figure 3C), and subsequent receptor activation by histamine (100 μM) elicited a second TRPC6 activation serving as an expression control. Similar results were obtained when the muscarinic receptor (M₅R) was co-expressed (Figure 3D). Similar to AII, histamine or acetylcholine are not endogenously produced in HEK293 cells, indicating that the observed receptor activation is ligand independent. In both cases, membrane stretch-induced currents were strongly reduced by the application of diphenhydramine (100 μM) and atropine (1 μM), respectively (Figure 3E and F).

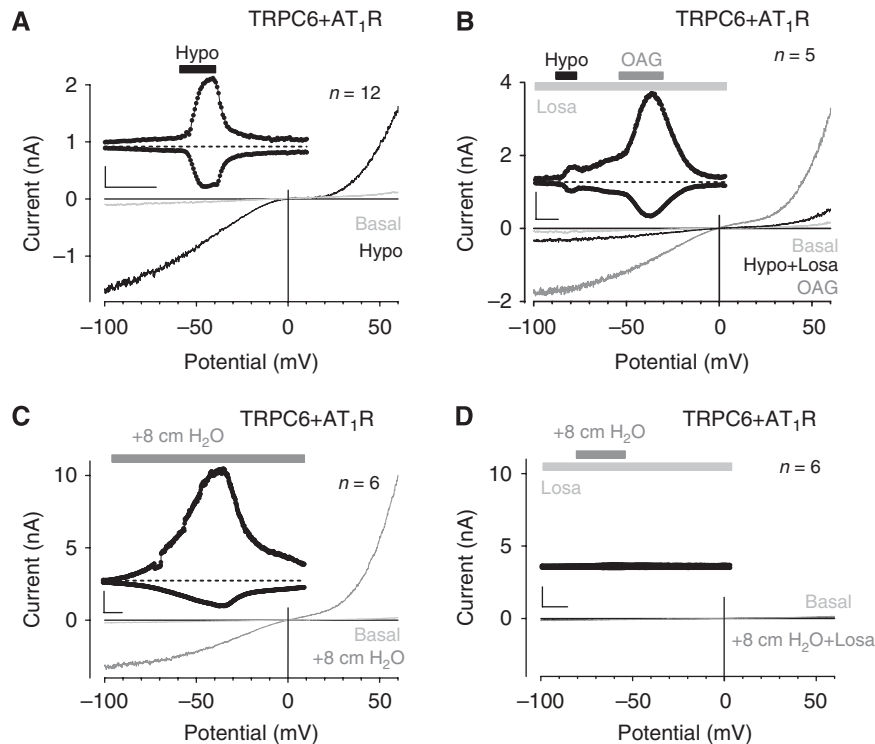


Figure 2 Indirect TRPC6 activation by membrane stretch through AT₁R. Whole-cell recordings from transiently AT₁R (A, B) or AT₁R-Venus (C, D) and TRPC6 co-expressing HEK293 cells. (A–D) IV relationships before ‘Basal’ and during hypotonicity (A, B) and direct membrane stretch (C, D) with and without 1 μM losartan, ‘Losa’, are displayed; current time courses at ±60 mV, zero current levels (stippled lines), time scale bars: 50 s (A, B), 100 s (C, D) and current scale bars: 500 pA (A, B), 2 nA (C, D).

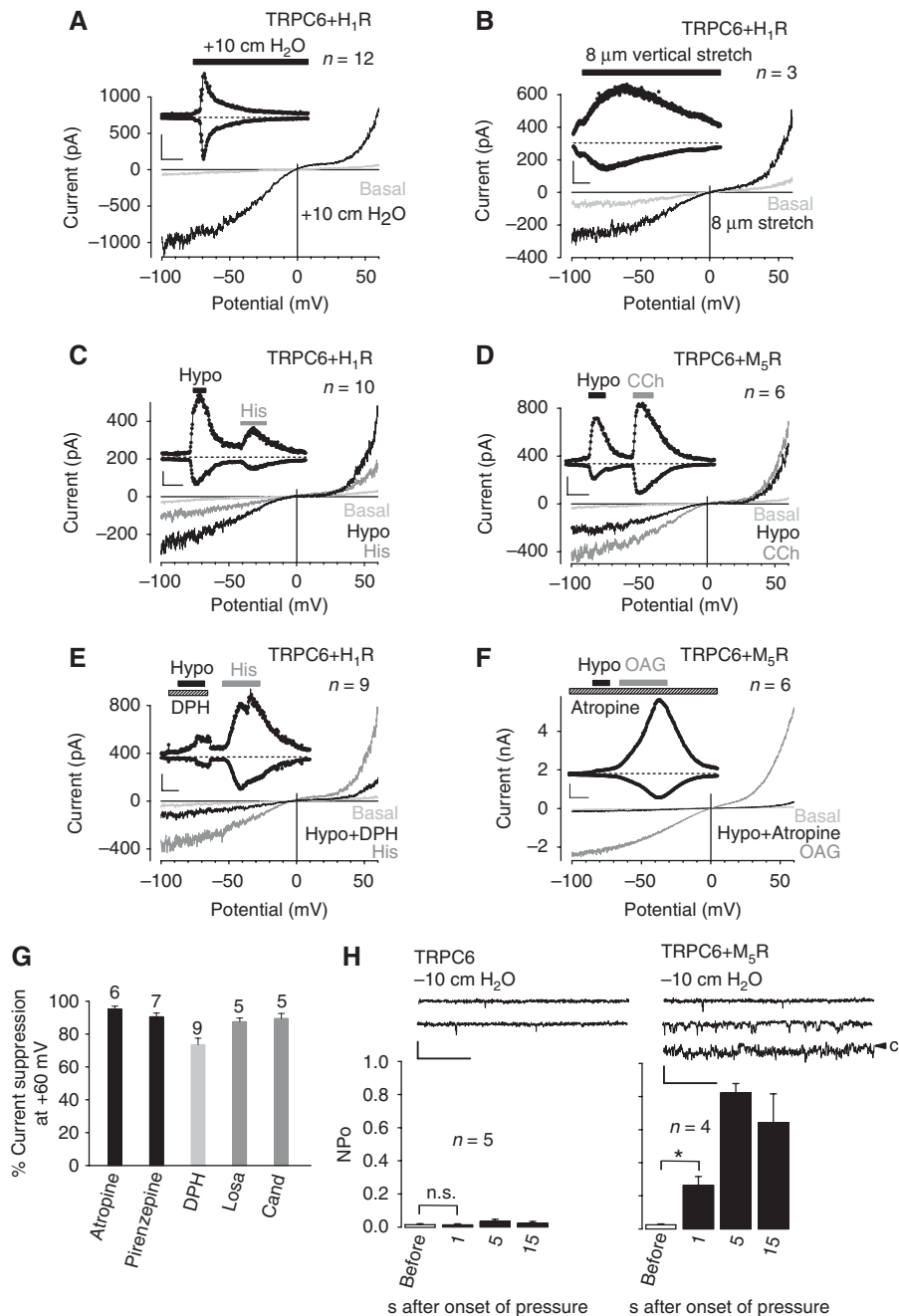


Figure 3 Inverse agonists and antagonists prevent mechanical activation of GPCRs. Whole-cell measurements of H₁R (A–C, E) or M₅R 100 s (D, F) with stimulation by positive pipette pressure (A), with vertical stretch (B) or with hypotonicity (C–F) in the presence or absence of 100 μM diphenhydramine, ‘DPH’ (E) or 1 μM atropine (F). Agonist stimulation was performed with 100 μM histamine, ‘His’ or with 100 μM carbachol ‘CCh’. Direct TRPC6 stimulation was performed with 100 μM OAG. Current time courses at ± 60 mV, zero current levels (stippled lines), scale bars: 500 pA (A), 200 pA (B–E) or 1 nA (F) and 50 s (A, C–F) or 100 s (B). (G) Comparison of TRPC6 current suppression by atropine (1 μM), pirenzepine (1 μM), diphenhydramine (100 μM), losartan (1 μM) and candesartan, ‘Cand’, (100 nM) with hypotonic stimulation of HEK293 cells co-expressing TRPC6 and the respective receptors. (H) Analysis of NPo values in cell-attached patches. (H) (top) Representative current traces before, during the first (left and right) and the fifth (right) second after application of –10 cm H₂O pipette pressure. The closed state is indicated with ‘c’ (right), scale bars (5 pA, 20 ms).

To compare the inhibitory effects of antagonists or inverse agonists used, we transiently expressed a select array of G_{q/11}-coupled receptors, that is, M₅R, H₁R and AT₁R and monitored suppression of TRPC6 currents at + 60 mV elicited by osmotically induced membrane stretch (Figure 3G). Current reductions were normalized to maximal currents in response to either OAG (M₅R and AT₁R) or histamine (H₁R). At maximally effective concentrations, the various compounds tested

reduced cation currents by 72–95%. As the chemical nature of the substances is very diverse, direct unspecific effects on TRPC proteins are unlikely.

In addition, cells transiently expressing other vasopressor receptors, ET_A endothelin (ET_AR) or V_{1A} vasopressin receptors (V_{1A}R), responded to hypotonic bath solutions with TRPC6 activation that was inhibitable by the selective antagonist darusentan in the case of ET_AR (Supplementary

Figure S3). A comparison between different G_{q/11}-coupled receptors revealed the following order of mechanosensitivity: H₁R > AT₁R > M₅R > V_{1A}R (Supplementary Figure S4). On the contrary, we did not obtain any evidence for mechanosensitivity of G_s-coupled receptors as exemplified by the β₂-adrenergic receptor (Supplementary Figure S5).

Using the cell-attached patch-clamp configuration, which preserves intracellular signalling pathways, only co-expression of GPCRs, that is, M₅Rs (Figure 3H) or AT₁Rs (data not illustrated) with TRPC6 resulted in an indirect channel activation by membrane stretch. Altogether, these observations strongly argue against a direct activation of TRPC6 by membrane stretch.

Notably, TRPC6 could be replaced by TRPC3 or TRPC7, which were similarly activated by hypotonicity and receptor stimulation (Supplementary Figure S6), indicating that all DAG-sensitive TRPC channels are indirectly sensitive to membrane stretch. In addition to electrophysiological measurements, we corroborated our findings using single-cell fluorescence imaging of fura-2-loaded intact cells (Supplementary Figure S7).

TRPC6 activation by membrane stretch depends on G proteins and PLC activation

Next, we examined the intracellular signalling cascade from mechanically stimulated receptor to channel activation. In H₁R and TRPC6 co-expressing HEK293 cells, the PLC inhibitor U73122 prevented TRPC6 activation in response to membrane stretch and agonist challenge of the H₁R (Figure 4A),

whereas cells promptly reacted to both kinds of stimuli after addition of the inactive analogue U73343 (Figure 4B).

PLC-β isoforms can be stimulated by GTP-loaded G_{α_{q/11}} subunits or by Gβγ dimers released from activated G_{i/o} proteins (Rhee, 2001). Pertussis toxin (PTX) pretreatment of HEK293 cells co-expressing the H₁R and TRPC6 to uncouple G_{i/o} proteins from receptors did not affect TRPC6 activation by hypotonic cell swelling or by agonist challenge (Figure 4C).

To demonstrate a participation of G proteins, the non-hydrolysable GTP analogue GDP-β-S was included in the pipette solution. In the presence of GDP-β-S, ionic currents triggered by hypotonic cell swelling or by the addition of histamine were completely abolished (Figure 4D), suggesting that G_{q/11}-type G proteins mediate membrane stretch-induced PLC and subsequent TRPC6 activation. Notably, membrane stretch-induced TRPC6 activation is independent of store depletion as induced by cyclopiazonic acid (Supplementary Figure S8).

Apart from GPCRs, TRPC channels can also be engaged by receptor tyrosine kinases. However, we observed that tyrosine kinase activation does not have an important function for TRPC6 activation by osmotically induced membrane stretch in HEK293 cells (Supplementary Figure S9).

Mechanically induced active receptor conformations are prevented by inverse agonists

COS-7 cells transiently expressing the M₅R reacted to stepwise reductions of medium osmolality with increased agonist-independent inositol phosphate formation (Figure 5A).

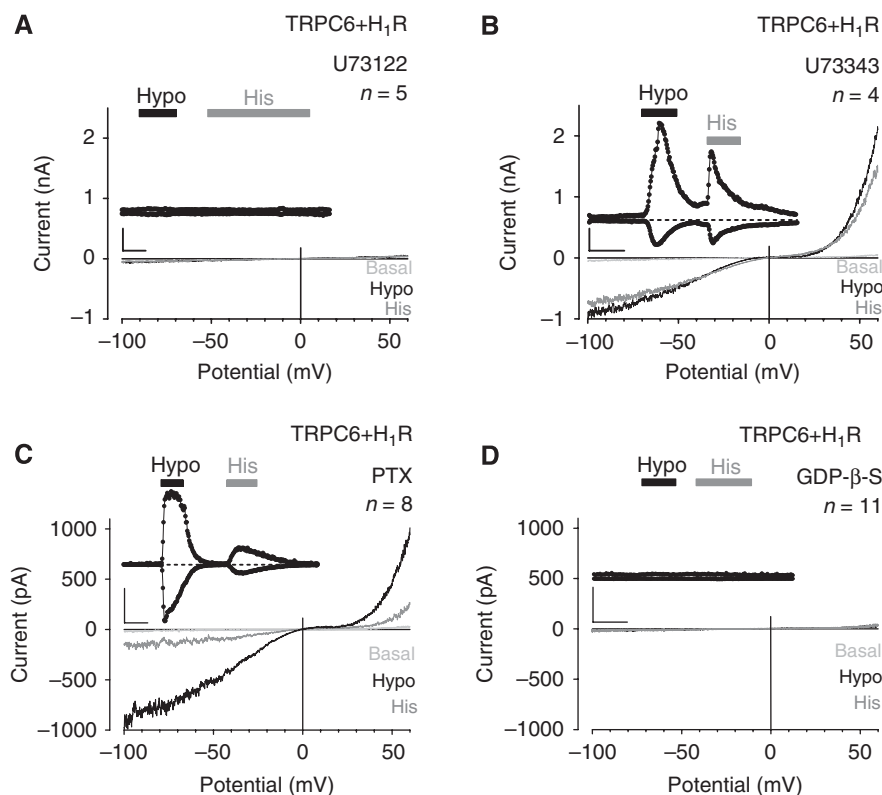


Figure 4 Osmotically induced membrane stretch activates phospholipase C and G proteins. (A–D) Whole-cell recordings from HEK293 cells co-expressing TRPC6 and H₁R. Recording with 10 μM U73122 (A) or with 10 μM U73343 (B) in the pipette solution. Recordings of cells pretreated with 100 ng ml⁻¹ PTX for 16 h (C) or with 2 mM GDP-β-S in the pipette solution (D). IV relationships before ‘Basal’, during hypotonic stimulation, ‘Hypo’ and receptor stimulation with 100 μM histamine, ‘His’ are shown. (A–D) Insets show current time courses at ± 60 mV, hypotonic and histamine stimulations, zero current levels (stippled lines); scale bars: 500 pA, 50 s.

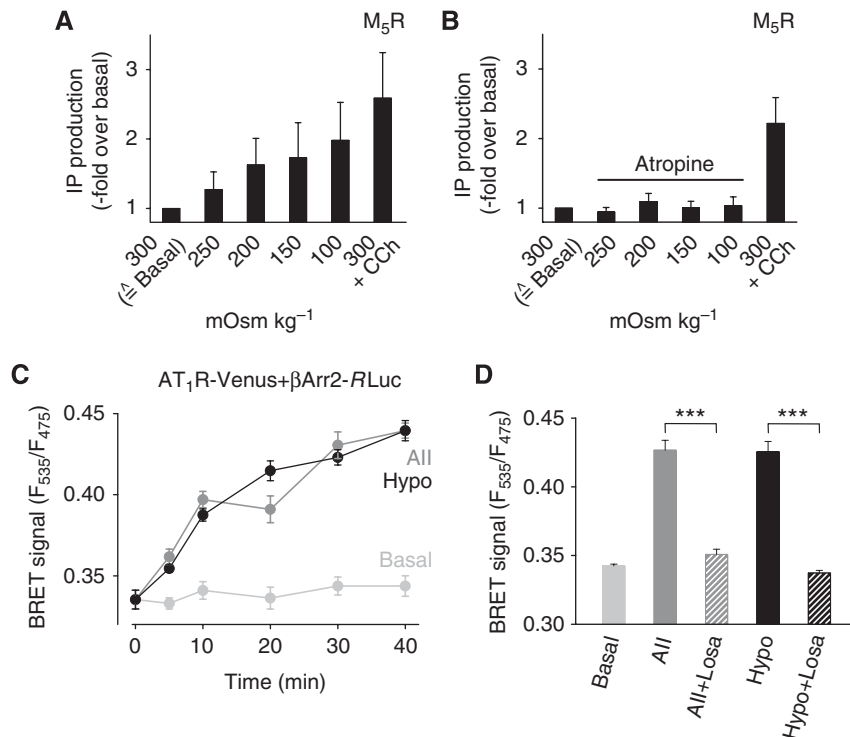


Figure 5 Active receptor conformations induced by membrane stretch. (A, B) Inositol phosphate (IP) accumulation as measured in one out of 3 representative assays with M₅R expressing COS-7 cells. Agonist stimulation with 100 μM carbachol, 'CCh', is also displayed. (C, D) BRET¹ assay with COS-7 cells co-expressing AT₁R-Venus and βArr2-RLuc. (C) Representative time courses of BRET signals determined as quadruplets. (D) Summary of three independent BRET assays. After 30 min, BRET signals elicited by stimulation with an isotonic solution, 'Basal', 100 nM AII, with a hypotonic solution, 'Hypo' (273 mOsm kg⁻¹), and under 1 μM losartan, 'Losa'.

Receptor occupation by atropine completely prevented inositol phosphate formation triggered by hypotonic stimuli, whereas muscarinic receptors were efficiently activated by carbachol in the absence of atropine (Figure 5B). Similarly, H₁R-expressing COS-7 cells reacted to a decrease in osmolality with increased inositol phosphate production (data not shown).

To gather evidence for membrane stretch-induced conformational changes in the receptor, we applied the bioluminescence resonance energy transfer (BRET) technique and co-expressed eYFP (Venus)-fused AT₁R and β-arrestin-2 covalently linked to *Renilla* luciferase (RLuc) in COS-7 cells. Hypotonic cell swelling, as well as receptor activation at 22°C, led to a marked increase in the BRET signal (Figure 5C). Conformational changes in the receptor as evidenced by β-arrestin-2 recruitment in response to both stimuli were abrogated by preincubation with losartan (Figure 5D). Membrane stretch recruits β-arrestins to the receptor as efficiently as maximal stimulation by agonist, demonstrating that membrane stretch results in an active receptor conformation, which can be precluded by previous inverse agonist binding.

Expression of AT₁R imparts mechanosensitivity to aortic SMCs

A7r5 cells are derived from embryonic rat thoracic aorta, an elastic vessel not developing myogenic tone. Rapidly developing TRPC6-like currents were observed only in response to receptor activation by vasopressin (Figure 6A) but not in response to osmotically induced membrane stretch (Jung *et al*, 2002). In the aorta (Nickenig *et al*, 1998) devoid

of myogenic responsiveness, AT₁R density is around 5 fmol mg⁻¹, whereas it amounts to around 200 fmol mg⁻¹ in the afferent arterioles (Ruan *et al*, 1997). As AT₁R are sparsely expressed in A7r5 cells, addition of AII did not affect TRPC6 activity (Figure 6A, right, inset). However, after increasing AT₁R density in A7r5 cells, hypotonic bath solutions caused highly significant TRPC6-like currents (Figure 6B) as did the addition of exogenous AII (Figure 6B, right, inset). Basal or vasopressin-induced current densities of untransfected and transfected A7r5 cells were not significantly different (Figure 6A and B). Ligand-binding studies revealed an AT₁R density of 88 fmol mg⁻¹ in native A7r5 cells, whereas an A7r5 cell line stably expressing AT₁R had a 15-fold higher receptor density of 1305 fmol mg⁻¹. In both cell lines, the vasopressin receptor (V_{1A}R) density was around 118 fmol mg⁻¹. Furthermore, the number of functional TRPC6 channels per cell in untransfected as well as in AT₁R over-expressing A7r5 cells (approximately 360) was comparable with the number of channels in transfected HEK293 cells (approximately 470).

To test whether AII potentially stored in A7r5 cells was released upon hypotonic cell swelling to activate AT₁R in an autocrine manner, we blocked AII with a neutralizing antibody. Although a concentration of 100 μg ml⁻¹ in the bath solution was sufficient to preclude the response to 100 nM exogenous AII (Figure 6C), the antibody had no effect on the cation current increase in response to osmotically induced membrane stretch (Figure 6D), clearly demonstrating that secreted AII is not involved in stretch-induced AT₁R activation.

To investigate whether increasing AT₁R densities correlate with cellular responses to membrane stretch, we monitored

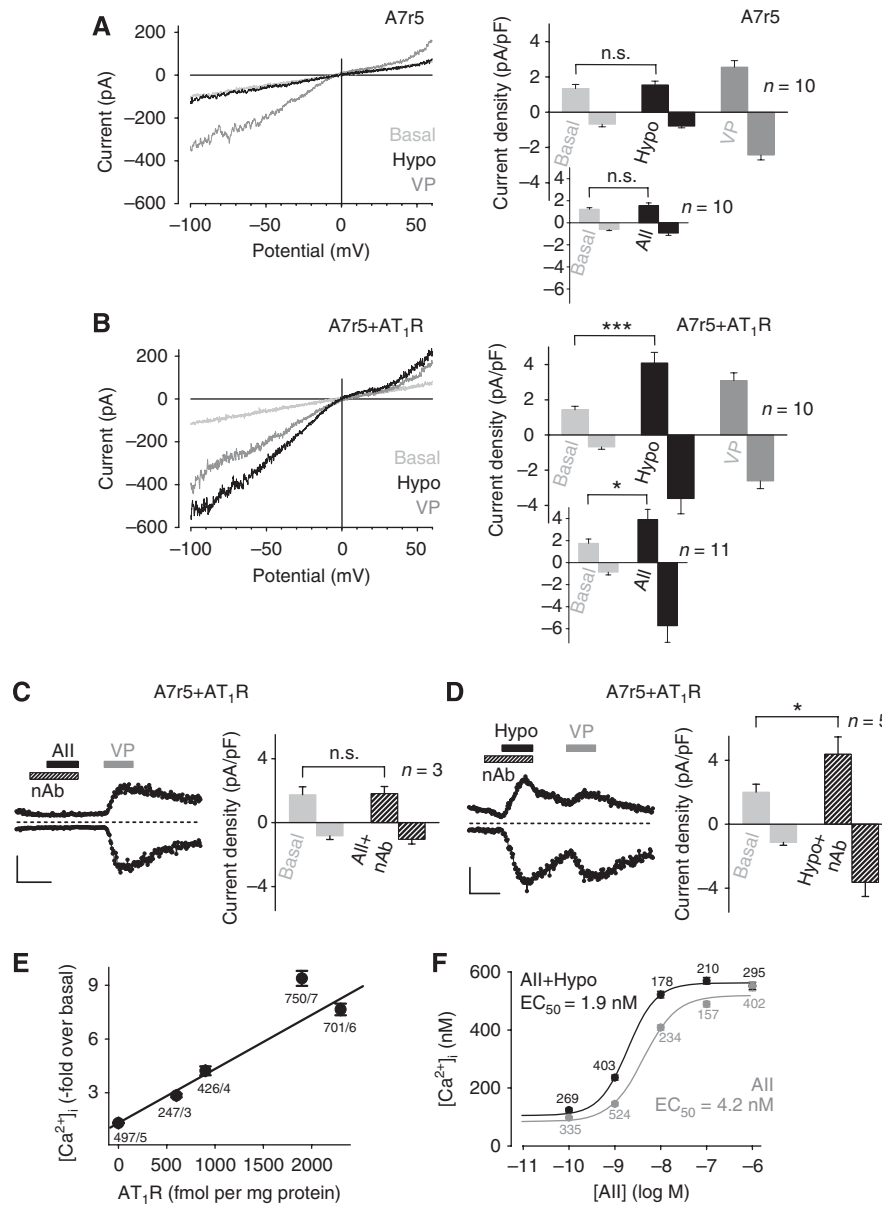


Figure 6 Expression of AT₁R leads to mechanosensitivity of aortic SMCs. Whole-cell recordings from non-transfected (A) and A7r5 cells over-expressing AT₁R (B–D). (A, B) IV relationships before ‘Basal’, during hypotonic stimulation, ‘Hypo’, and receptor stimulation with 1 μM vasopressin ‘VP’ (left) and current density analyses at ±60 mV (right) are displayed. (C, D) Current time courses at ±60 mV with zero current levels (stippled lines); time scale bars 60 s and 100 pA (left) and current densities in the presence of neutralizing antibody, ‘nAb’ (right) are displayed. (E) [Ca²⁺]_i increases by hypotonic stimulation in HEK293 cells transfected with different amounts of AT₁R-Venus cDNAs. Venus fluorescence was correlated with receptor densities. Numbers indicate the number of summarized cells and of independent transfections. (F) Concentration–response curves to AII of fura-2-loaded HEK293 cells stably expressing AT₁R-Venus in isotonic and in hypotonic (273 mOsm kg⁻¹) solutions.

the increase in [Ca²⁺]_i in response to hypoosmotic bath solutions in HEK293 cells transiently expressing AT₁R at different receptor densities. Up to 2300 fmol AT₁R per mg protein, there is a linear correlation between receptor density and the amplitude of the evoked Ca²⁺ transients (Figure 6E). When increasing AT₁R density further, saturation was reached. Thus, AT₁R density appears to be a critical variable determining whether ligand-independent receptor activation by membrane stretch is translated into detectable biological responses.

Under physiological conditions, vascular vasopressor receptors are simultaneously exposed to membrane stretch and

agonists. As shown in Figure 6F, in HEK293 cells expressing recombinant AT₁R, osmotically induced membrane stretch results in a leftward shift of the concentration–response curve to AII, thus sensitizing the response to agonist.

AT₁R blockade reduces myogenic tone

Next, we studied single isolated primary renal VSMCs with a high endogenous density of AT₁R and measured the responses to osmotically induced membrane stretch. Fura-2-loaded VSMCs repeatedly responded to hypotonic bath solutions (250 mOsm kg⁻¹) with transient rises of [Ca²⁺]_i. The Ca²⁺ response was greatly diminished upon losartan

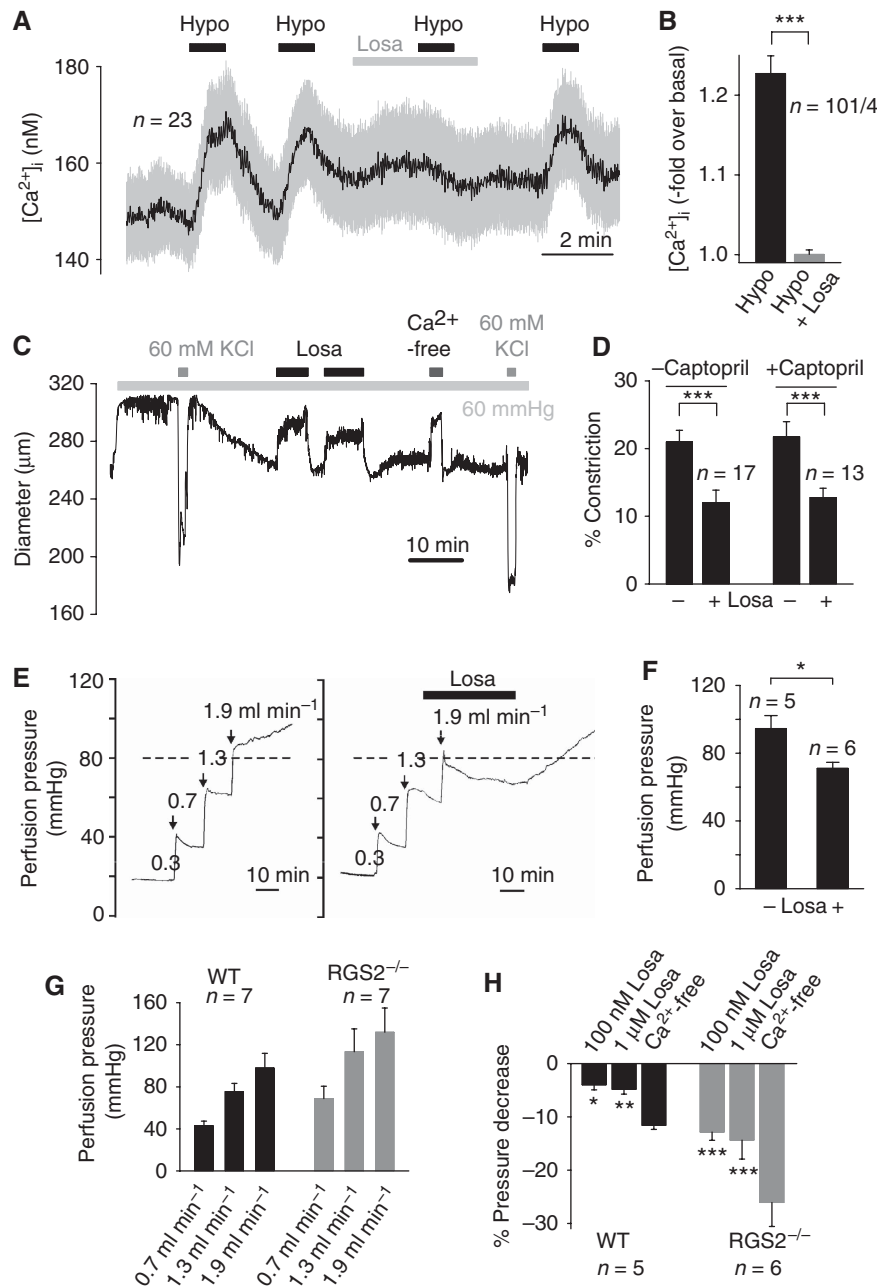


Figure 7 Physiological relevance of agonist-independent activation of AT₁R by membrane stretch. (A) Representative [Ca²⁺]_i recordings in fura-2-loaded renal VSMCs. Application of hypotonic stimuli, ‘Hypo’, and losartan, ‘Losa’, are indicated. (B) Summary of maximal [Ca²⁺]_i increases in renal VSMCs. (C) Representative trace of a diameter recording from an isolated rat cerebral artery continuously superfused with oxygenated PSS. Application of 60 mmHg intravascular pressure, applications of 60 mM high K⁺ solution, application of nominally Ca²⁺-free solution and applications of 1 μM losartan are represented. (D) Summary of measured cerebral arteries showing the reduction of vessel constriction with losartan in the presence or absence of 1 μM captopril. (E) Representative traces of perfusion pressure recordings from isolated kidneys of mice in the absence and presence of losartan. (F) Summary of perfusion pressures. (G) Summary of perfusion pressures at indicated flow rates measured in perfused isolated kidneys from wild-type, ‘WT’, and RGS2^{-/-} mice. (H) Summary of flow pressure decrease after 5 min measured at a flow rate of 1.9 ml min⁻¹ illustrating the pressure decrease with losartan compared with a Ca²⁺-free bath solution. Captopril had no effect on perfusion pressure.

application (Figure 7A and B), indicating that membrane stretch leads to AT₁R activation in VSMCs.

To investigate whether the mechanosensitivity of AT₁Rs has an important function for myogenic vasoconstriction, we monitored the effect of intravascular pressure on the diameter of isolated rat cerebral arteries in the presence and absence of the inverse AT₁R agonist losartan. An intravascular pressure of 60 mmHg was applied to initiate myogenic

vasoconstriction (Figure 7C). Maximal constriction of cerebral arteries was achieved by elevation of external K⁺ to 60 mM leading to activation of Ca_vs and a subsequent increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i). Addition of losartan diminished pressure-induced vasoconstriction, an effect that was reversed by washout of the drug. Maximal vasodilation was observed in a nominally Ca²⁺-free bath solution.

To investigate the possible contribution of AII locally released in the vessel wall, vessels were incubated with captopril to block the angiotensin-converting enzyme (ACE). In contrast to humans, only ACE is responsible for local AII formation in vascular tissues of rats (Jin *et al*, 2000). Figure 7D shows that myogenic constriction was markedly suppressed by losartan irrespective of previous captopril treatment. Accordingly, we did not obtain any evidence for a release of AII into the bath solution during the experiments (Supplementary data).

To monitor the myogenic response of a whole vascular bed, we performed perfusion experiments on isolated mouse kidneys (Figure 7E). The perfusion pressure monitored 10 min after applying an increase in flow to 1.9 ml min^{-1} was reduced by losartan infusion by 23.5 mmHg ($P = 0.013$) (Figure 7F). Similar results were obtained in the presence of captopril (100 nM and $1 \mu\text{M}$; $n = 5$ each). The regulator of G protein signalling (RGS)-2 is a selective and negative regulator of G_{q/11} proteins in the cardiovascular system (Xie and Palmer, 2007). Accordingly, RGS2^{-/-} mice are characterized by an increased myogenic tone of isolated renal interlobar arteries (Hercule *et al*, 2007). In line with this observation, we observed an increased perfusion pressure at different flow rates in RGS2^{-/-} kidneys compared with wild-type kidneys (Figure 7G). The increased perfusion pressure in RGS2^{-/-} and wild-type kidneys was not affected by captopril ($1 \mu\text{M}$, at 0.7 and 1.9 ml min^{-1}). Figure 7H shows that losartan had a more pronounced inhibitory effect on the myogenic response in RGS2^{-/-} as compared with wild-type kidneys most likely reflecting the inhibition of a G_{q/11}-mediated signalling pathway by the inverse agonist. The pressure decrease by losartan was concentration dependent. Collectively, our findings are compatible with the notion that ligand-independent signalling of the AT₁R is an essential component of dynamic mechanochemical signalling in VSM contributing to myogenic tone.

Discussion

Although TRPC channels have been implicated as essential elements mediating myogenic vasoconstriction, the mechanism by which pressure activates vascular TRPC channels is incompletely understood. TRPC channels have been shown to be activated by PLC (Clapham, 2003), possibly through direct actions of DAG (Hofmann *et al*, 1999; Inoue *et al*, 2001) generated through PLC activity. Pressure increases PLC activity (Osol *et al*, 1993) as well as the concentration of DAG (Narayanan *et al*, 1994) in isolated cerebral arteries. In this study, we provide evidence that G_{q/11}-coupled receptors are essential mechanosensing components in vascular smooth muscle cells (VSMCs) and function as sensors of membrane stretch leading to TRPC channel activation in a G protein- and PLC-dependent manner. Mechanically activated receptors adopt an active conformation allowing for productive G protein coupling and recruitment of β -arrestin. We therefore suggest that G_{q/11}-coupled receptors, for example AT₁R, serve as mechanosensors mediating pressure-dependent, TRPC-mediated membrane depolarization in cerebral and renal arteries, which is at least in part responsible for myogenic vasoconstriction. Although our data indicate that AT₁Rs have a paramount function in resistance arteries, we suggest that pressure activation of other G_{q/11}-coupled receptors, for

example H₁Rs and ET_ARs, may exhibit similar and synergistic effects in other myogenic arteries, thereby determining tissue-specificity of myogenic arteries.

Conceptually, the most straightforward way to explain the reported involvement of TRPC6 in the myogenic response (Welsh *et al*, 2002) would be to conjecture that TRPC6 is a stretch-activated cation channel mediating depolarization of VSMCs. In this vein, other TRP channels including TRPV2 (Muraki *et al*, 2003), TRPM4 (Earley *et al*, 2004), TRPM7 (Numata *et al*, 2007) and TRPC1 (Maroto *et al*, 2005) have also been implicated as potential molecular correlates of stretch-activated cation channels.

In isolated VSMCs from resistance arteries, a positive pipette pressure of 10 cm H₂O in the whole-cell configuration is sufficient to evoke stretch-activated cation currents (Setoguchi *et al*, 1997). However, in isolated inside-out and cell-attached membrane patches from TRPC6-expressing HEK293 cells, such pressure values failed to elicit cation currents, although stretch-activated Cl⁻ channels were promptly activated. Increasing the negative pipette pressure up to $-70 \text{ cm H}_2\text{O}$ (corresponding to 50 mmHg) did not result in the rapid opening of cation channels after application of the suction pulse, thus contrasting with the typical behaviour of bona fide stretch-activated ion channels (Hamill, 2006). Moreover, our single channel recordings in the cell-attached configuration showed enhanced channel activity in response to a negative pipette pressure of 10 cm H₂O only when TRPC6 channels were co-expressed with G_{q/11}-coupled receptors, but not when TRPC6 channels were expressed alone. These results provide compelling support for the notion that TRPC channels are mechanically activated through GPCRs.

Our conclusions are in line with a recent study (Gottlieb *et al*, 2008) but contrast with a report about the direct activation of recombinant TRPC6 by stretch in isolated inside-out membrane patches (Spasova *et al*, 2006). In the latter study, the threshold pressure level needed to detect TRPC6 activation was found to be higher than 80 mmHg (Spasova *et al*, 2006). Thus, it would appear that TRPC6 activation requires higher pressures than those needed for the large conductance *Escherichia coli* mechanosensitive ion channel, which displays pressure values for half-maximal activation of about 80 mmHg and functions as a last-resort safety valve (Levina *et al*, 1999). However, mechanosensitive channels in mammalian cells should be tuned to lower-pressure set points to be able to participate in the dynamic regulation of physiological processes.

It has been reported that in cardiomyocytes, mechanical stress can activate the AT₁R independently of AII (Zou *et al*, 2004). In contrast to the latter report, the formyl peptide receptor in leukocytes analysed by a dynamic FRET technique was found to be inhibited by mechanical stress, thereby decreasing its constitutive activity and reducing pseudopod formation of leukocytes (Makino *et al*, 2006). Thus, it is an unresolved issue whether mechanical stress activates or inhibits GPCRs and whether such events are pertinent to physiological regulation.

Using a BRET approach, we show that membrane stretch allows the receptor to assume an active conformation, which is recognized by arrestins as avidly as that stabilized by agonist. We can only speculate about possible events leading to receptor activation by membrane stretch. A mechanically induced change of the lateral pressure profile exerted by the

lipid bilayer of the plasma membrane (Kung, 2005) or mechanical activation of proteins directly connected to the receptor may result in a shift in the distribution between active and inactive conformations, which have recently been characterized depending on mechanical stretch in the case of the AT₁R (Yasuda *et al*, 2008).

Taken together, these results impose a considerable revision of our present views of myogenic constriction (Bayliss effect), in particular, and G protein-coupled receptor function and regulation, in general. Our results suggest that G_{q/11}-coupled receptors function as sensors of membrane stretch in VSMCs and contribute to myogenic vasoconstriction of renal and cerebral arteries, emphasizing the novel concept that activation of TRPC channels by mechanical stimuli occurs through activation of GPCRs. GPCRs appear to be characterized by polymodal activation mechanisms and may serve as sensors for external chemical signals and transmembrane potential (Ben-Chaim *et al*, 2006). Recent data on orphan GPCRs (Levoye *et al*, 2006) and our findings suggest physiologically relevant, ligand-independent functions for GPCRs. Future investigations on ligand-independent signalling of GPCRs in other organs will be enlightening in this respect.

Materials and methods

Analysis of myogenic tone in cerebral arteries

After decapitation of anesthetized male Sprague-Dawley rats, brains were removed and transferred to cold (4°C), oxygenated (95% O₂ and 5% CO₂) physiological salt solution (PSS). Distal posterior cerebral and distal anterior cerebellar arteries were cannulated, allowing for application of intravascular pressure (for details, see Gollasch *et al*, 1998; Lohn *et al*, 2002).

Analysis of myogenic tone in isolated perfused kidneys

Isolated kidneys of male wild-type and RGS2^{-/-} mice were perfused in an organ chamber using a peristaltic pump at constant flow (0.3–1.9 ml min⁻¹) of oxygenated (95% O₂ and 5% CO₂) PSS. Perfusion pressure was determined by a pressure transducer after an equilibration period of 60–90 min. Data were recorded and analysed using a Powerlab acquisition system (Fesus *et al*, 2007).

Bioluminescence resonance energy transfer assay

To detect β-arrestin-2 recruitment by the human AT₁R, the BRET¹ technique was performed using a Fluostar Optima (BMG Lab-Technologies, Offenburg, Germany) as described previously (Charrest and Bouvier, 2003). Briefly, COS-7 cells were co-transfected with the cDNA encoding an AT₁R-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-Rluc) using the FuGENE6 reagent. Both cDNAs were kindly provided by the laboratory of Dr Bouvier, Montréal. Forty-eight hours post-transfection, approximately 75 000 cells were seeded into a 96-well microplate in 100 μl of a bath solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose and 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) AII. Hypo-osmotic stress was induced by adding 10 μl pure H₂O (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 two-fold concentrated bath solution. Effects of the AT₁R antagonist losartan were determined by adding 1 μM (final) of the antagonist 4 min before agonist or hypoosmotic stimulation. The Rluc substrate coelenterazine H (5 μM) was added 25 min 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 min) were calculated.

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 (H₁R), rat type-5 muscarinic acetylcholine receptor (M₅R), mouse angiotensin II AT_{1A} (AT₁R) and the enhanced green fluorescent protein (eGFP) reporter plasmid using the FuGENE6 reagent. A7r5 cells were transfected with the AT₁R in pIRES2-EGFP (Clontech, Palo Alto, CA, USA). Conventional whole-cell patch-clamp recordings were carried out at 23°C at 48 h after transfection. The bath solution contained 110 mM NaCl, 5 mM CsCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose and 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 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 a positive pressure of 8–10 cm H₂O 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, CA, USA) or were cotransfected with TRPC6 and 0.5 μg cDNA coding for the H₁R using 6 μl or 7.5 μl of the FuGENE6 reagent, respectively. For inside-out experiments, the cells were seeded on culture dishes treated with poly-L-lysine. Cell-attached and excised inside-out patch-clamp recordings were carried out at room temperature (23°C) 72 h after transfection. The bath and pipette solution for inside-out experiments contained 130 mM caesium methane-sulfonate, 102 μM CsCl, 1 mM MgCl₂, 3.949 mM CaCl₂, 10 mM BAPTA (100 nM free Ca²⁺) and 10 mM HEPES (pH 7.2 with CsOH), resulting in an osmolality of 294 mOsm kg⁻¹. For cell-attached measurements, the same pipette solution was used and the bath solution contained 130 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES and 10 mM glucose (pH 7.4 with KOH), resulting in an osmolality of 294 mOsm kg⁻¹ to define the membrane potential. The bath solutions also contained 50 μM NPPB. Patch pipettes made of borosilicate glass (Science Products, Hofheim, Germany) were coated with R-6101 (Dow Chemical, Midland, MI, 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 or cell-attached recordings was performed by applying negative pressure of 10 or 70 cm H₂O to the patch pipette through a water manometer. Finally, in the inside-out configuration, 10 μM 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.

Non-enzymatic isolation of renal VSMCs

Kidneys from 9- to 14-day-old mice were decapsulated and longitudinally dissected and the medullae were removed. Kidney halves were pressed through a series of stainless sieves with decreasing pore sizes (420 and 300 μm). Renal arteries were collected on a 100-μm sieve and transferred to collagen-coated coverslips and cultured with Dulbecco's modified Eagle medium and Ham's F12 (1:1) supplemented with 10% fetal calf serum and penicillin (100 U ml⁻¹) and streptomycin (0.1 mg ml⁻¹) for up to 6 days (37°C and 7% CO₂).

Statistical analysis

Data are presented as means ± standard error of the mean (s.e.m.). Unless stated otherwise, data were compared by a paired or unpaired Student's *t*-test, if a Gaussian distribution was confirmed by applying a Shapiro-Wilk (normality) test, and significance was accepted at *P* < 0.05. For multiple comparisons, the one-way analysis of variance with Scheffé *post hoc* means comparison was applied. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s. *P* ≥ 0.05.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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