Synergistic Activation of Vascular TRPC6 Channel by Receptor and Mechanical Stimulation via Phospholipase C/Diacylglycerol and Phospholipase A₂/ω-Hydroxylase/20-HETE Pathways

Ryuji Inoue,* Lars J. Jensen,* Zhong Jian, Juan Shi, Lin Hai, Andrew I. Lurie, Freja H. Henriksen, Max Salomonsson, Hiromitsu Morita, Yasuhiro Kawarabayashi, Masayuki Mori, Yasuo Mori, Yushi Ito

Abstract—TRPC6 is a non–voltage-gated Ca⁡²⁺ entry/depolarization channel associated with vascular tone regulation and remodeling. Expressed TRPC6 channel responds to both neurohormonal and mechanical stimuli, the mechanism for which remains controversial. In this study, we examined the possible interactions of receptor and mechanical stimulations in activating this channel using the patch clamp technique. In HEK293 cells expressing TRPC6, application of mechanical stimuli (hypotonicity, shear, 2,4,6-trinitrophenol) caused, albeit not effective by themselves, a prominent potentiation of cationic currents (I_{TRPC6}) induced by a muscarinic receptor agonist carbachol. This effect was insensitive to a tarantula toxin GsMTx-4 (5 μmol/L). A similar extent of mechanical potentiation was observed after activation of I_{TRPC6} by GTPγS or a diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (OAG). Single TRPC6 channel activity evoked by carbachol was also enhanced by a negative pressure added in the patch pipette. Mechanical potentiation of carbachol- or OAG-induced I_{TRPC6} was abolished by small interfering RNA knockdown of cytosolic phospholipase A₂ or pharmacological inhibition of ω-hydroxylation of arachidonic acid into 20-HETE (20-hydroxyeicosatetraenoic acid). Conversely, direct application of 20-HETE enhanced both OAG-induced macroscopic and single channel TRPC6 currents. Essentially the same results were obtained for TRPC6-like cation channel in A7r5 myocytes, where its activation by noradrenaline or Arg₈ vasopressin was greatly enhanced by mechanical stimuli via 20-HETE production. Furthermore, myogenic response of pressurized mesenteric artery was significantly enhanced by weak receptor stimulation dependently on 20-HETE production. These results collectively suggest that simultaneous operation of receptor and mechanical stimulations may synergistically amplify transmembrane Ca²⁺ mobilization through TRPC6 activation, thereby enhancing the vascular tone via phospholipase C/diacylglycerol and phospholipase A₂/ω-hydroxylase/20-HETE pathways. (Circ Res. 2009;104:1399-1409.)

Key Words: mechanotransduction G₉/q₁₁ protein–coupled receptor Ca²⁺ entry channel

It has widely been recognized that cellular functions are elaborately controlled by both neurohormonal activities and mechanical stresses.¹,² For example, in the cardiovascular system, the contractile status of arteries is not only regulated by autonomic nerve activities, circulating vasoactive hormones, and paracrinely released autacoids but is also greatly influenced by mechanical stresses such as blood flow and pressure.³–⁵ These 2 mechanisms are expected to operate simultaneously in vivo, but little is known about how they could interact mutually.

A member of the canonical transient receptor potential (TRP) protein family TRPC6 (the sixth canonical subfamily member of TRP protein) is ubiquitously expressed in vascular smooth muscle cells (VSMCs) and implicated in the regulation of vascular tone and remodeling.⁶ Like many other TRP isoforms, activation of TRPC6 channel...
has been reported to occur in a polymodal fashion. In several different types of blood vessels, stimulation of phospholipase (PL)C-coupled receptors has been shown to activate this channel via generation of diacylglycerol (DAG). In a few arteries in which the pressure-dependent autoregulation of blood flow is prominent (eg, cerebral artery), an elevation of intravascular pressure itself is thought to cause the activation of TRPC6 channel, thereby eliciting a vasoconstriction referred to as “myogenic response.”

The mechanism underlying the mechanical activation of TRPC6 channel is controversial. It was initially explained that DAG generated through mechanical activation of PL(C)8 secondarily activates TRPC6 channel.7 In contrast, a subsequent study proposed that TRPC6 channel can be directly activated by mechanical deformation of the cell membrane via a lipid bilayer-dependent mechanism.8 The latter proposal was however opposed by the finding that the observed mechanosensitive cation channel in TRPC6-expressing cell may reflect an endogenous mechanosensitive cation channel activity in the expression system.10 Furthermore, recent independent lines of evidence have provided an alternative view that Gq/11 protein/PLC-coupled receptors may themselves be activated by mechanical deformation of the cell membrane inside-out (I/O) current recordings and data analyses have been performed.46,7

The purpose of the present study, therefore, was to explore a possible explanation about the discrepant findings described above on the mechanosensitivity of TRPC6 channels and seek its physiological significance in native vascular tissues. As the result, we have found that TRPC6 channel exhibits mechanosensitivity only after its receptor-mediated activation, in which a metabolite of arachidonic acid (AA), 20-HETE (20-hydroxyeicosatetraenoic acid), a reportedly major vasoconstrictive lipid mediator,13 likely plays a pivotal role. As the result, we have found that TRPC6 channel may activate in response to mechanical deformation of the cell membrane via a lipid bilayer-dependent mechanism.8 The latter proposal was however opposed by the finding that the observed mechanosensitive cation channel in TRPC6-expressing cell may reflect an endogenous mechanosensitive cation channel activity in the expression system.10 Furthermore, recent independent lines of evidence have provided an alternative view that Gq/11 protein/PLC-coupled receptors may themselves be mechanically activated,11 and their increased cell surface density may allow TRPC6 channel to activate in response to mechanical stimuli.12

The purpose of the present study, therefore, was to explore a possible explanation about the discrepant findings described above on the mechanosensitivity of TRPC6 channels and seek its physiological significance in native vascular tissues. As the result, we have found that TRPC6 channel exhibits mechanosensitivity only after its receptor-mediated activation, in which a metabolite of arachidonic acid (AA), 20-HETE (20-hydroxyeicosatetraenoic acid), a reportedly major vasoconstrictive lipid mediator,13 likely plays a pivotal role. Furthermore, additional evidence has suggested that this mechanism may also contribute to the activation of vasoconstrictor-activated cation channels in A7r5 myocytes and operate in an intact mesenteric artery under receptor stimulation that is otherwise poorly responsive to increased intravascular pressure.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture and Transfection

Human embryonic cells HEK293 and embryonic aortic smooth muscle myocytes A7r5 (American Type Culture Collection, Manassas, Va) were maintained in 10% FBS-containing DMEM. HEK cells were used to cotransfect TRPC6 and CD8 cDNA (used as the expression marker) plasmids with a transfection reagent SuperFect (Qiagen, Hilden, Germany). Functional evaluation was made 48 to 72 hours after transfection. TRPC6-expressing cells were selected with bead-conjugated anti-CD8 antibody (Dynabeads M-450 CD8, Dynal Biotech).

Stealth Small Interfering RNA was transfected to HEK293 and A7r5 cells according to the instructions of the manufacturer.

Electrophysiology

The details of macroscopic and single channel [cell-attached (C/A), inside-out (I/O)] current recordings and data analyses have been described by Shi et al.14 To minimize variations arising from different cell size, the magnitude of current is normalized by dividing by cell capacitance.

Shear force (~10 dyn cm⁻²; see the online data supplement) was applied as a continuous laminar flow (~1 μL·s⁻¹) out of a Y-tube, which was initially placed at a distance of ~500 μm and quickly moved within 20 μm from a voltage-clamped cell.

Negative pressure was applied manually via suction via patch pipette, the magnitude of which was scaled and monitored by using a DMP-1B pneumatic transducer tester (BioTek Instruments, Winooski, VT).

All experiments were performed at room temperature (22°C to 26°C).

Video Microscopic Diameter Measurement

Arterial segments (second or third branches) of mesenteric artery obtained from male Sprague–Dawley rats (Charles River, Japan) were cannulated at both ends with fine-tipped glass capillaries with nylon threads. Intraluminal pressure was controlled using a pressure myograph.15

All procedures used for animal experiments were approved by local animal ethics committees of Fukuoka University and University of Copenhagen.

Solutions

The composition of solutions used in the present study was the same as described previously.15 Hypotonic solutions (HTSs) were made to give a desired hypotonicity by partially replacing external NaCl with sucrose or mannitol. Na⁺-free external solution was made by equimolar substitution of monovalent cations with N-methyl-D-glucamine (NMDG).

Chemicals

HET0016 was kindly provided by Taisho Pharmaceuticals (Tokyo, Japan).

Statistics

All data are expressed as means±SEM (indicated by columns/symbols and bars in each figure). Two-tailed paired and unpaired Student’s t tests were used to evaluate statistical significance: P<0.05 (*) or P<0.01 (**). For multiple comparisons, ANOVA followed by Bonferroni’s t test was used.

For more details of the methods, see the online data supplement.

Results

Four Distinct Mechanical Stimuli Potenti ate Receptor-Activated TRPC6 Currents

We first investigated whether TRPC6 channels expressed in HEK293 cells can be directly activated by HTS.9 However, 1 minute of exposure to HTS (~75 mOsM) only occasionally activated a small and slowly developing inward current in TRPC6-expressing HEK cells (Figure 1A). This current was strongly suppressed by pretreatment with DIDS (300 μmol/L) but unchanged on substitution of external Na⁺ and K⁺ with NMDG (Figure 1A and Online Figure I, A and B), thus likely reflecting a noncationic conductance. Stimulation of intrinsic muscarinic receptors in HEK cells with carbachol (CCh) (1 to 100 μmol/L) elicited a much noisier nonselective cationic current (reversal potential, 0.5±0.9 mV; n=8) showing weak inward suppression and outward rectification (Figure 1B), the hallmarks of TRPC6 channel. This current (hereafter designated as I_TRPC6) was insensitive to 300 μmol/L DIDS (Figure 1A and Online Figure I, C) or 5 μmol/L GsMTx-4 (Figure 2A and 2B and Online Figure II), a tarantula toxin reportedly inhibiting HTS-induced inward currents in TRPC6-expressing cells.9 In the rest of the present
study, to eliminate possible contribution of a slowly developing HTS-induced noncaticonic current, we added 300 μmol/L DIDS into the external solution.

In the next step, to examine whether mechanical stress affects the receptor-mediated activation of TRPC6, we observed the effects of HTS on CCh-induced I_{TRPC6} after the administration of CCh. Strikingly, the CCh-induced I_{TRPC6} was reversibly enhanced in response to HTS (Figure 1A) without significant change in the reversal potential (1.2 ± 0.9 mV, n = 8; Figure 1B; not significantly different with unpaired t test from the control above). In nontransfected HEK cells, however, little current was induced by CCh without discernible effects of HTS (Figure 1A, inset). The extent of enhancement of the CCh-induced I_{TRPC6} became more pronounced with increasing hypotonicity (Figure 1C) or weaker preceding receptor stimulation (Figure 1D) but remained unchanged in the presence of 5 μmol/L GsMTx-4 (Figure 2B, b). Similar HTS-induced enhancement of I_{TRPC6} was also observed when it was activated by intracellular perfusion of a nonhydrolyzable GTP analog GTPγS (10 μmol/L) or diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) (10 μmol/L) via a patch pipette. *Statistically significant with paired t test (in E).

In another series of experiments, we used other means to generate mechanical forces, shear force and a bulge-forming amphipathic agent 2,4,6-trinitrophenol (TNP). Both shear force (~10 dyn/cm²) and TNP (500 μmol/L), which were ineffective in the absence of receptor stimulation (insets in Figure 2C, a and b), markedly enhanced the magnitude of I_{TRPC6} activated by CCh (1 μmol/L) or intracellularly perfused GTPγS (10 μmol/L) (Figure 2C, a and b, and 2D).

The mechanical enhancement of receptor-activated I_{TRPC6} could also be reproduced at the single channel level. As
demonstrated and summarized in Figure 3, virtually no currents were evoked by a negative pressure up to −60 mm Hg (applied in the patch pipette: Figure 3A, a, and left columns in Figure 3B). However, after receptor activation (CCh 10 μmol/L in the pipette), the activity of 30 pS single cation channels was increased in a graded fashion depending on the intensity of negative pressure (−20 to −60 mm Hg; Figure 3A, b, and right columns in Figure 3B). The enhancing effect of negative pressure on CCh-induced single TRPC6 channel activity showed some delay in onset and outlasted the period of pressure application. In addition, regardless of TRPC6 protein expression, application of a strong negative pressure (≥−80 mm Hg) often led to irreversible development of irregular inward currents (Online Figure III).

**The Mechanism Underlying the Mechanical Enhancement of CCh-Induced \( I_{\text{TRPC6}} \)**

The time course of mechanical enhancement of receptor-activated \( I_{\text{TRPC6}} \) (time to 50% activation and deactivation with −75 mOsm HTS: 11.8±1.4 and 13.3±1.7 seconds, respec-
tively; n=15) was much slower than that expected for a “direct” bilayer-dependent activation. We thus reasoned that lipid mediators, especially the metabolites of AA, may be involved in this enhancement, as was previously reported for a distinct TRP family member TRPV4. Consistent with this speculation, pharmacological inhibition of PLA2 with AA-COCF3 (10 μmol/L) or pBPB (200 μmol/L) (pretreated for 5 to 10 minutes; Figure 4A, b), or specific small interfering (si)RNA knockdown of cytosolic PLA2 (Figure 4A, b) almost completely abolished HTS- or TNP-induced enhancement of receptor-activated I\textsubscript{TRPC6}. A similar extent of inhibition was also observed with 17-ODYA and HET0016 (pretreated for 5 to 10 minutes), the inhibitors for ω-ω'-monohydroxylase, which metabolizes AA into 20-HETE (Figure 4A, a and b). These
inhibitors were without effects on \(I_{\text{TRPC6}}\) when applied acutely. In contrast, the inhibitions of cyclooxygenase, lipoxygenase, and epoxyxygenase by indomethacin, NDGA, and miconazole (each 10 \(\mu\)mol/L), respectively, were all ineffective (Figure 4A, b). These results suggest the involvement of 20-HETE in mechanical potentiation of receptor-activated \(I_{\text{TRPC6}}\). To further corroborate this idea, we next applied 20-HETE exogenously. As shown in Figure 4B and 4C, direct application of 20-HETE (100 \(\mu\)mol/L) significantly enhanced both macroscopic \(I_{\text{TRPC6}}\) and single TRPC6 channel currents induced by OAG (10 \(\mu\)mol/L), although application of 20-HETE alone failed to induce a detectable current up to 10 \(\mu\)mol/L (left-most column in Figure 4C, b; not shown for macroscopic current). Furthermore, the enhancing effects of 20-HETE were not significantly affected by the PLC inhibitor U73122 (1 \(\mu\)mol/L) (Figure 4B, b, and 4C, b), thus excluding the involvement of PLC therein.

These results together suggest that activation of the cytosolic PLA\(_2\)/\(\omega\)-hydroxylase cascade and consequent production of 20-HETE is essential for the mechanical enhancement of receptor-activated TRPC6 current/channel activity.

**Vasoconstrictor-Activated Cation Channels in A7r5 Myocytes Become Susceptible to Mechanical Stimuli After Receptor Activation**

To explore the physiological significance of above-mentioned observations, we repeated the same experiments as above in a native VSMC model A7r5 myocyte. In this myocyte, vasoconstrictors such as noradrenaline (Norad), vasopressin, angiotensin, and endothelin were found to activate TRPC6-like currents.\(^{17-19}\) Consistent with these previous findings, abundant expression of TRPC6 was detected by the RT-PCR technique, and specific siRNA knockdown of TRPC6 almost abolished Norad- or Arg8-vasopressin (AVP)-induced cation current in parallel with reduced TRPC6 protein expression (Online Figure IV).\(^{19}\)

TRPC6-like currents evoked by Norad or AVP in A7r5 myocytes were greatly enhanced by mechanical stimuli (HTS, shear force), whether they were applied together with or after receptor stimulation (Figures 5A, b, through d, and 6A, a). These mechanical stimuli were however ineffective in the absence of receptor stimulation (300 \(\mu\)mol/L DIDS added; Figure 5A, a). The mechanical enhancement of TRPC6-like currents was more pronounced at lower concentrations of receptor agonists. As summarized in Figure 5B, a and b, application of shear force caused a more than 10-fold leftward shift in the dose–response relationship between agonist concentration and the density of evoked current (open versus filled circles).

Potentiating effects of mechanical stimuli were still observed when TRPC6-like currents were more directly activated by intracellular perfusion of GTP\(_\gamma\)S (10 \(\mu\)mol/L) or OAG (10 \(\mu\)mol/L) (Figure 6A, b, and 6C). As in the case of expressed TRPC6, this potentiation was greatly attenuated by pretreatment with AACOCF\(_3\) (10 \(\mu\)mol/L) or HET0016 (10 \(\mu\)mol/L) and siRNA knockdown of cytosolic PLA\(_2\) (Figure 6B through 6D). In contrast, neither GsMTx-4 (5 \(\mu\)mol/L) nor the PLC inhibitor U73122 (1 \(\mu\)mol/L) nor the protein kinase C blocker GF109203X (2 \(\mu\)mol/L), which was reported to inhibit the mechanical enhancement of endothelin-1–activated, GsMTx-4–inhibitable single cation channels in pulmonary and cerebral arterial myocytes,\(^{20}\) attenuated the mechanical potentiation of TRPC6-like currents (Online Figure V).

Consistent with the above macroscopic data, single TRPC6-like channel activity in A7r5 myocyte evoked by a low concentration of AVP (3 \(\mu\)mol/L) (reversal potential: \(-2.3\pm1.8\) mV, \(n=5\); unitary conductance: 32.6\(\pm\)1.1 pS, \(n=5\); Figure 7A, c) was also markedly enhanced by a negative pipette pressure in the range of \(-20\) to \(-80\) mm Hg (Figure 7A, a and b, 7C, and 7D). Furthermore, this enhancement diminished after pretreatment with HET0016 (10 \(\mu\)mol/L) (Figure 7C and 7D). In the absence of AVP, however, the negative pressure could induce virtually no single channel activities (Figure 7B and open circles in Figure 7C).

These results collectively suggest that a 20-HETE–mediated mechanism similar to that observed for expressed TRPC6 channel likely operates in synergistic activation of TRPC6-like channels in A7r5 myocytes by neurohormonal and mechanical stimulations.

**“Sensitized” Myogenic Response Is Mediated by 20-HETE Production**

Finally, to get more physiological insight into the above mechanism at the tissue level, we performed a videomicroscopic diameter measurement of pressurized rat mesenteric artery where expression of TRPC6 can be detected by RT-PCR (Figure 8A). Large (second or third) branches of this artery were reported to respond poorly to elevated intravascular pressure in the absence of receptor stimulation.\(^{21}\) As demonstrated and summarized in Figure 8, the sustained phase of pressure-induced diameter decrease (myogenic response) was marginal in the absence of agonist (filled triangles in Figure 8C). However, after partial vasoconstriction with the \(\alpha_1\)-adrenergic agonist phenylephrine (or noradrenaline) and neuropeptide Y, the magnitude of myogenic response was greatly enhanced (Figure 8B and filled circles in Figure 8C through 8E). This enhanced response was almost abolished by Gd\(^{3+}\) or SKF96365 at concentrations that effectively inhibit expressed TRPC6 channels (2 and 5 \(\mu\)mol/L, respectively; Figure 8E)\(^{22}\) but are ineffective for excess K\(^+\)-induced vasoconstriction (Figure 8F). Importantly, pretreatment with HET0016 (10 \(\mu\)mol/L) strongly counteracted this enhanced myogenic response (open circles in Figure 8C and 8D). These results suggest that 20-HETE likely mediates the sensitized myogenic response by \(\alpha_1\)-adrenergic receptor stimulation which involves the activation of TRPC6.

**Discussion**

The present results have clearly demonstrated that expressed TRPC6 channel is not primarily activated by mechanical stimuli. Instead, if once receptor-activated, it becomes mechano-sensitive via production of a PLA\(_2\)/\(\omega\)-hydroxylase metabolite 20-HETE. A similar mechanism seems also operative in a native VSMC model A7r5 myocyte, where simultaneous activation of PLC- and PLA\(_2\)-coupled signaling pathways...
synergistically enhances TRPC6-like channel activity. Furthermore, videomicroscopic data obtained from pressurized mesenteric arteries suggest that this synergism may be physiologically important to enhance myogenic response during neurohormonal stimulation. It is known from in vitro studies that arteries from circulations showing a prominent autoregulatory response (e.g., cerebral, coronary, and renal arteries) directly develop myogenic tone to pressurization. Thus, the mechanism identified in the present study may have more physiological significance in blood vessels, which are poorly responsive to increased intravascular pressure itself but show enhanced myogenic responsiveness after receptor stimulation. However, it cannot be ruled out that the myogenic tone in cerebral, coronary, and renal resistance-sized vessels in vivo is greatly enhanced by this synergistic mechanism because of the presence of sympathetic (noradrenergic) tone.

The mechanism for mechanical activation of TRPC6 channel is controversial. As a result of findings from a bacterial mechanosensitive cation channel model, MscL, an early study hypothesized that mechanical (stretch, hypotonicity) and chemical (formation of DAG) stimuli activate TRPC6 channel through a common lipid bilayer–dependent mechanism. The important rationale for this hypothesis was that GsMTX-4, an amphipathic venom peptide known to disturb the lipid-channel boundary, blocked both mechanical and receptor-mediated activation of the channel. However, the relevance of this hypothesis was soon questioned by the lack of reproducibility. An alternative mechanism has instead been proposed: activation of TRPC6 channel is the secondary consequence of mechanical activation of Gq/11/PLC-coupled receptors. More specifically, in both HEK293 and A7r5 cells, the increased cell surface density of angiotensin II receptor AT1 attributable to its overexpression rendered TRPC6 channel to be directly responsive to HTS, cell inflation/stretching and a physiological range of negative pressure, and this was almost completely prevented by the PLC inhibitor U73122 and antagonists-inverse agonists for AT1 receptor. Furthermore, myogenic response of cerebral

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**Figure 5.** Potentiating effects of shear force on vasoconstrictor-evoked TRPC6-like currents in A7r5 myocytes. A, Membrane currents recorded from A7r5 myocytes at −50 mV with no agonist (a), AVP (1 nmol/L [b], 100 nmol/L [c]), or noradrenaline (Norad) (100 nmol/L) (d). Shear force was applied simultaneously (b) or after receptor stimulation (c and d). B, Concentration–response curves for TRPC6-like current density with AVP (a) or Norad (b) in the absence (●) and presence (○) of shear force.
artery was profoundly inhibited by the inverse agonist of the AT₁ receptor, losartan. Although direct evidence is still lacking, these results have been interpreted to indicate that membrane stretch can directly cause an agonist-independent conformational change of the receptor thereby activating the downstream Gq/11/PLC/DAG/TRPC6 signaling cascade.

Our present results have confirmed the observation that TRPC6 channel per se is not mechanosensitive. Total insensitivity of receptor-activated and mechanically potentiated TRPC6 current to GsMTx-4 further supports that altered lipid bilayer mechanics would not be a primary mechanism to activate the channel. However, at an endogenous expression level of Gq/11/PLC-coupled receptors that we used, disparate observations were obtained. Firstly, receptor-mediated activation of TRPC6 channel is a prerequisite for its responsiveness to mechanical stimuli. Secondly, mechanical activation of TRPC6 channel likely involves the generation of a PLA2 metabolite 20-HETE rather than PLC metabolites. It has been reported that some G protein–coupled receptors (GPCRs) show a constitutive activity being capable of activating the downstream signaling cascades without binding of agonists, which is effectively decoupled by inverse agonists. If this were the case for the above study, the possibility must be taken into account that spontaneous activation of PLC, which would be greatly exaggerated by the overexpression of GPCRs, could produce a sufficient amount of DAG to activate TRPC6 channel. Indeed, our findings that even weak receptor activation could induce a prominent mechanosensitivity of TRPC6, and that TRPC6-expressing cells exhibiting an exclusively high spontaneous activity responded directly to mechanical stimuli (Online Figure VII), may support this speculation. In the above study using overexpression of GPCRs, however, the potential contribution of 20-HETE production was not tested. It is thus a remaining unresolved issue to clarify to what extent PLC- and PLA2-mediated signaling pathways respectively contribute to mechanical activation of TRPC6 channel in physiological settings.

20-HETE was previously reported to directly activate a small cationic current in TRPC6-overexpressing HEK cells (maximum current density: ≈2 pA/pF at −40 mV with 10 µmol/L). However, the concentration required to activate the current (EC_{50}=0.8 µmol/L) was much higher than that used in the present study (100 nmol/L), and we could record little discernible current at the latter concentration (Figure 4C, b). Furthermore, the observed properties of cationic current evoked by 20-HETE (linear current–voltage relationship; no sensitivity to flufenamate) are atypical of TRPC6 channel. These observations suggest that direct
The effects of 20-HETE, albeit present, would only marginally contribute to mechanical potentiation of TRPC6 channel observed in the present study. However, it is possible that the preceding activation of TRPC6 via a DAG-dependent mechanism may enhance the activating effect of 20-HETE or visa versa. This deserves further investigation.

It has been reported that reduced osmolarity or shear stress can activate a distinct TRP subfamily member TRPV4 via generation of a PLA2/epoxygenase metabolite 5,6-epoxyeicosatrienoic acid (EET). EET-mediated activation of TRPV4 in endothelial cell has been implicated in shear-induced vasodilation. Interestingly, a recent investigation revealed that the responsiveness of TRPV4 to hypoosmolarity was greatly enhanced (or “sensitized”) by concomitant activation of P2Y receptor by ATP. This sensitization could be assigned to physical interaction between TRPV4 channel and activated IP3 receptor via PLC-coupled receptor stimulation. Although the role of PLα2 is more primary in activating TRPV4 than TRPC6, the above finding raises the idea that the synergism of PLC and PLA2 may be a common biological strategy to amplify an otherwise marginal Ca2+ mobilization caused by either neurohormonal or mechanical stimulus alone.

In VSMCs, 20-HETE is thought to be the major AA product generated by cytochrome P450 CYP4A enzymes exhibiting \( \omega \)-hydroxylase activities, whereas vascular endothelial cells mainly produce EETs via CYP2C and CYP2J enzymes. EETs are suggested to mediate vasodilatory actions in both endothelium-dependent (see above) and -independent manners in part via TRPV channels. In human umbilical vein, 11,12-EET is also reported to facilitate the translocation of TRPC6 protein to caveolin-1-rich areas of the endothelial membrane, thereby prolonging membrane hyperpolarization. In contrast, 20-HETE has been shown to act as a potent vasoconstrictor independently of endothelial function and presumably involved in the release of vasoactive neuropeptides from sensory nerve terminals via TRPV1 activation. Part of these effects has been ascribed to increased voltage-dependent Ca\(^{2+} \) influx attributable to the inhibition of a large conductance Ca\(^{2+} \)-dependent K\(^+ \) channel (which leads to membrane depolarization) and potentiation of L-type voltage-dependent Ca\(^{2+} \) channel in VSMCs. 20-HETE is also reported to activate protein kinase C, thereby increasing the Ca\(^{2+} \) sensitivity of contractile machinery. Our present findings add a new important vascular target of 20-HETE to this repertoire, ie, TRPC6, which is expected to serve as “depolarization” channels enhancing Ca\(^{2+} \) influx in direct and indirect (via secondary activation of voltage-dependent Ca\(^{2+} \) channel or Na\(^+\)/Ca\(^{2+} \) exchanger) manners and hence increasing the vascular tone. Importantly, 20-HETE has been implicated in the pathogenesis of hypertension in rats. A similar correlation with increased blood pressure has also been suggested for the increased expression of TRPC3, the closest homolog of TRPC6, in human essential hypertensive patients. Furthermore, in the early stage of human essential hypertension, hyperactivity of sympathetic nervous system and excessive activation of renin–angiotensin–aldosterone system, both of which are expected to potentially activate vascular TRPC6 channels, are thought to play key roles. It may thus be worthy to investigate how these mechanisms would mutually interact and contribute to the pathological dysregulation of blood pressure, particularly...
with regard to the novel mechanism disclosed in this study, ie, the synergism of receptor and mechanical stimulations in TRPC6-mediated Ca$^{2+}$/H11001 mobilization.

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**Disclosures**

None.

**References**


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Online Fig.I

A

Na+-free

Current density (-50mV; pA/pF)

B

TRPC6-HEK

C

TRPC6-HEK

Online Fig.I
Online Fig.II
Online Fig. III
Online Fig.IV
Online Fig. V
Online Fig. VI
Online Fig. VII

A. TRPC6

B. TRPC6

C. TRPC6

D. TRPC6

Online Fig. VII
Supplement Material

Online Fig. I  Prolonged application of HTS induces a non-cationic current in HEK293 cells. A; a representative trace of HTS (-100mOsm)-induced inward current recorded at -50mV, and its mean current density 1 and 4 min after exposure to -25, -50, -75 and -100mOsm HTS. A rightmost filled column indicates the almost complete inhibition of HTS-induced current (1min exposure) by pretreatment with 300µmol/L DIDS for 2-5min. Columns and vertical bars indicate the mean ± sem (n=5-9). C; Receptor-activated TRPC6 current is insensitive to 300µmol/L DIDS but effectively blocked by 10µmol/L ruthenium red.

Online Fig. II  GsMTx-4 is unable to inhibit CCh- or GTPγS-induced TRPC6 current. Recorded at -50mV. A; a typical trace showing little effect of 5µmol/L GsMTx-4 on CCh- induced TRPC6 current (I_{TRPC6}). B; summary of the effect of 5µmol/L GsMTx-4 on CCh (100µmol/L)- or GTPγS (100µmol/L)-induced I_{TRPC6}. Relative current is calculated as the ratio of amplitudes 3min after to just before application of 5µmol/L GsMTx-4. n=4.

Online Fig. III  Outlasting effects of negative pressure on receptor-activated TRPC6 channel activity (A) and development of large irregular current by strong negative pressure (B). Representative traces in cell-attached recording.

Online Fig. IV  siRNA knockdown of TRPC6 protein abolishes Norad-induced cationic currents in A7r5 myocytes. A & B; an actual trace for Norad (100µmol/L)-induced cationic current (A) and its current voltage relationship in Na⁺-rich and -deficient (NMDG) conditions (B). C; mRNA expression profile of TRPC isoforms in A7r5 myocytes evaluated by RT-PCR. D; two stealth siRNAs greatly suppressed Norad (100µmol/L)-induced TRPC6-like cationic current with reduced expression of TRPC6 protein (inset: evaluated by immunoblot analysis with a TRPC6-specific antibody) and. Representative of three independent experiments.

Online Fig. V  GsMTx-4 is unable to suppress shear-induced potentiation of 1nM AVP-evoked TRPC6-like current in A7r5 myocytes. A; shear force potently enhanced a cationic current evoked by 1nM AVP. B; summary of
the enhancing effects of shear force on Norad (0.1µmol/L) and AVP (1nmol/L)-evoked cationic currents in the presence of GsMTx-4 (5µmol/L) or a PKC inhibitor GF109203X (GF; 2µmol/L). The definition of fold increase is the same as in Fig.1. No statistically significant difference is present among four different conditions for shear-induced increase in Norad- and AVP-evoked cation currents. n=5-9.

Online Fig.VI 20-HETE mediates the enhancement of myogenic response by phenylephrine in rat mesenteric artery.

A; relationships between the absolute diameter change (µmol/L) of cannulated rat mesenteric artery (2nd or 3rd branch) and intraluminal pressure (mmHg) in the absence or presence of 1µmol/L Phe or in Ca²⁺-free solution (n=8).  B; the same relationships as in A in the presence of 10µmol/L HET0016 (n=8).

Online Fig.VII Spontaneously active I_{TRPC6} is potentiated by mechanical stimulation. Over 200 whole-cell recordings, only 11 TRPC6-expressing HEK cells displayed direct mechanosensitivity. A; application of HTS (-75mOsm) itself caused a slow development of basal I_{TRPC6} showing a high spontaneous activity. B; summary of the effects of HTS on spontaneously active I_{TRPC6}. n=5 and 16 for low and high spontaneous activities, respectively. C; representative trace of shear force-induced potentiation of spontaneously active I_{TRPC6}. D; summary of the effects of shear force on spontaneously active I_{TRPC6}. n=6 and 12 for low and high spontaneous activities, respectively.

**Materials and Methods**

**Cell culture and transfection**

Human embryonic cell line HEK293 and embryonic aortic smooth muscle cell line A7r5 (purchased from ATCC; Manassas, VA, USA) were maintained in Dulbecco’s modified culture medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin, streptomycin; GIBCO), under 5% CO₂ with every 3-4 day passage with 0.0025% and 0.025% trypsin/EDTA (Invitrogen, USA). Great care was taken not to overdigest or damage the cells with trituration, which otherwise often impaired their mechanosensitivity.

Mouse TRPC6 DNA (Gene Accession No.: NM_013838) subcloned into the pCI-neo vector (Invitrogen, USA) was transfected together with pCI-neo-πH3-CD8 (cDNA of the T-cell antigen CD8) at the ratio of 5~10:1, to HEK cells of 50-80% confluency, with the aid of a transfection reagent SuperFect™ (Qiagen, Germany). CD8 was used as the
expression marker. Functional evaluation was made 48-72h after transfection, at the time point when the expression of TRPC6 proteins reached the peak. TRPC6-expressing cells were selected by using beads-conjugated anti-CD8 antibody (Dynabeads M-450 CD8, Dynal Biotech) as the expression marker. To knockdown the expression of TRPC6 protein, 40-50 pmole/ml stealth™/siRNA duplex (sense/antisense: 5’ to 3’); AAACCACCGUUGCAUAAGACC/ GGUCUUAUGCAACGCUGGUGUUGU was transfected to 30-50% confluent A7r5 myocytes (in the presence of 2.5µl/ml lipofectamine™2000) according to the manufacturer’s instruction (Invitrogen, USA). After 48-72h culture, almost no expression of TRPC6 protein was detectable by immunoblotting. For siRNA knockdown of cytosolic PLA₂ in HEK293 (NM_024420) and A7r5 cells (NM_133551), 100pmole/ml of the following stealth™/siRNA duplexes (Invitrogen, USA) were respectively used (sense/antisense: 5’ to 3’); AUUUCGUAUGGACUAUAUUCACCC/ GGGUUUGAAUUAGUCCAUAGCAA, and AUCCGAUGGACCAACUUGCUUGGU/ ACCAAGCAAGUUGGGUCAAUCCG. The efficiency of the siRNA was confirmed by immunoblotting with anti-PLA₂ antibody (4-4B-3C; Santa Cruz).

**Electrophysiology**

Patch electrodes (3-5MΩ) fabricated from 1.5mm borosilicate glass capillaries and heat-polished were used for whole-cell and single channel recordings, in conjunction with a high-impedance low-noise patch clamp amplifier (EPC9; HEKA Electronics, Germany) and an A/D, D/A-converter (Digidata 1200; Axon Instruments). Sampled data low-passed filtered at 1kHz and digitized at 5kHz were analyzed by using ‘Clampfit’ v.9.2 (Axon Instruments, USA). Longer time-frame recordings (e.g. whole-cell current traces in Fig. 1) were performed with PowerLab/400 (AD Instruments, Australia; sampling rate: 100Hz), and analyzed with the software Chart v3.6. The magnitude of recorded noisy currents was defined as their 5-10s averages over the time period of concern.

To minimize variations arising from different cell size, the magnitude of current is normalized by dividing by cell capacitance. Mean cell capacitance and resistance (measured between -100 and -50mV with Cs-internal solution) were 28.0±2.1pF and 7.8±1.4 GΩ for HEK293 cells (n=44), and 99.9±8.5pF and 3.8±0.3GΩ (n=43) for A7r5 myocytes, respectively.

For single channel recordings [cell-attached (C/A) and inside-out (I/O) patch
configurations], sampled data were low-pass filtered at 1kHz and stored on a computer hard disc after digitization at 5kHz. Single channel analysis was made using the software ‘Clampfit’ v.9.2 (Axon Instruments, USA). The mean single channel current (NP\text{a}) was calculated for each experiment just before and after each procedure by averaging the current amplitude over 5s with respect to the baseline as performed previously\textsuperscript{1}.

Shear force (~10dyn·cm\textsuperscript{-2}) was applied as a continuous laminar flow (~1µl·s\textsuperscript{-1}) out of a Y-tube, which was initially placed at a distance of ~ 500µmol/Lol/L and quickly moved within 20µmol/Lol/L from a voltage-clamped cell, and approximated using the equation: \(\tau=4\mu Q/\pi r^3\); \(\tau\), \(\mu\) and \(r\) denote shear force, the viscosity of solution (0.00797g·cm\textsuperscript{-1}·s\textsuperscript{-1}), and the inner radius of the tube (~100µmol/Lol/L), respectively. Bathing solution was constantly perfused at a rate of 0.8 - 1ml/min into a recording chamber, which would generate a shear force of ~0.5dyn·cm\textsuperscript{-2} according to the equation: \(\tau=3\mu Q/w h^2\) where \(w\) and \(h\) denote the width (10mm) and height of chamber flow (2mm), respectively.

Negative pressure was applied manually by suction via patch pipette, the magnitude of which was scaled and monitored by using a DMP-1B pneumatic transducer tester (BIO-TEK instrument, VT, USA).

All experiments were performed at room temperature (22-26 °C).

**Video microscopic diameter measurement**

Male Sprague Dawley rats weighing 200-300g (Charles River, Japan or Taconic, Denmark) were exsanguinated under anesthesia with intra-peritoneal injection of pentobarbital. After opening the abdominal cavity, arterial segments of 2\textsuperscript{nd} or 3\textsuperscript{rd} branches of mesenteric artery (300 –400µmol/L in the outer diameter recorded at 60 mm Hg) were excised, cleaned of attached fat and connective tissues, and cannulated at both ends with fine-tipped glass capillaries and fixed tightly with nylon threads, as performed previously\textsuperscript{2}. The lumen of the segments was perfused with Krebs’ solution (to which 10 mg BSA per ml had been added) under no-flow conditions, and intraluminal pressure was controlled using a pressure myograph system (Living Systems Instrumentation, Burlington, USA; or, Danish Myotechnology, Aarhus, Denmark). The experimental chamber (model CH/1, Living Systems Instrumentation; or DMT 120CP, Danish Myotechnology) accommodating an arterial segment was placed on a thermo-plate (Tokai Hit, Japan) and continuously superfused at a rate of ~10ml/min with Krebs solution aerated with 95%O\textsubscript{2} and 5%CO\textsubscript{2} (T=35-37°C; pH 7.40-7.50, both monitored in the experimental chamber), to which tested drugs were
added. The video images of arterial segments captured through a CCD camera under binocular microscopy (Stemi 200-C, Zeiss, Germany; or Olympus IX71, Japan) were measured for their diameter change every 100ms using a width analyzer (C3161, Hamamatsu Photonics; or Myoview, Danish Myotechnology). The results were stored on a computer hard disc after digitization (MacLab, AD Instruments, South Wales, Australia).

We tried to remove the endothelium completely with a wire/thread/hair inserted into the lumen of the blood vessel which was followed by the passage of air bubbles. However, the following confirmation by acetylcholine suggested that, in more than half of trials, albeit greatly reduced compared with control, slight vasorelaxation could still be induced. Thus, we cannot completely exclude the possible contamination of the contribution of endothelial cells in our myograph experiments.

The details of animal experiments were reviewed and approved by local animal ethics committees of Fukuoka University, Kyushu University and University of Copenhagen in advance, and all procedures used to sacrifice rats obeyed the guidelines set by the committees.

**Immunoblot**

Total lysate of A7r5 myocytes or HEK293 cells (about 10^6 cells) was prepared in sample buffer, and its protein concentration was determined by using the BCA protein assay kit (Pierce). 5% (v/v) 2-mercaptoethanol and 1% (w/v) bromophenol blue were added to the sample, and proteins were separated (30-40μg) on 10% (w/v) SDS-PAGE and blotted to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% (w/v) skim milk dissolved in Tween-PBS, the membrane was incubated with the anti-TRPC6 antibody (Alomone; see the above) (at 1:100-200 dilution) overnight. TRPC6 protein (~120kD) or PLA2 protein (~100kD) was visualized by chemiluminescence by incubating the PVDF membrane with the secondary antibody conjugated with horseradish peroxidase, and analyzed densitometrically.

**RT-PCR**

Single myocytes were dissociated from rat mesenteric arteries (2nd or 3rd branches) by enzymatic treatment [incubated in 2mg/ml collagenase (type I, Worthington, Lakewood, NJ, USA)-containing nominally Ca^{2+}-free external solution for 1h] and mechanical triturbation. Total RNA was extracted from single mesenteric arterial myocytes and A7r5 cells using a commercial kit (RNAeasy, Qiagen) according to the manufacturer’s instruction, and then subjected to reverse transcription polymerase reaction using a
protocol; preheating at 94°C for 1 min followed by 40 cycles (denaturation at 94°C for 10 seconds: annealing at 58-65°C for 30 seconds: extension at 72°C for 1 minute) and final extension at 72°C for 10 minutes. Annealing temperatures for each primer pair were determined using a gradient protocol.

Primer pairs used for respective rat TRP isoforms are as follows (forward/reverse: 5’ to 3’):

TRPC1 (DQ839447.1: nt. 236-710): GCG TAG ATG TGC TTG GGA GAA A /GCT CTC AGA ATT GGA TCC TCT CTC;
TRPC2 (AF136401.1: nt. 197-713) : CCA GGT GGT CCT CTG CGG AA/CAT CCT CAC TGG CCA GCG AGA;
TRPC3 (AB022331.1: nt. 474-948): GCTGGCCAAGCTGGCCAA/ GAACACAAGCAGCCAGGAAGA;
TRPC4 (AF421368.1: nt. 1151-1722): CCT CTC AGC ACA TCG ACA GGT /CCA AAT ATT GAC CAA AAC AGG GA;
TRPC5 (EF672039.1: nt. 987-1511): CAA GCT TCT AAC CTG CAT GAC CA/ CCT AAG TGG GAG TTG GCT GTG AA;
TRPC6 (A: nt. 327-1398): CAT CCC AGT GGT GCG GAA GA/ GCC TTC AAA TCT GTC AGC TGC A;
TRPC7 (XM225159.4: nt. 1907-2151): TCAACCTGTACTCCTACTAC/ GATTTCCTGATAGGAGTTGG.

**Solutions**

Pipette solution for whole-cell recording (in mM): 120 CsOH, 120 aspartate, 20 CsCl, 2 MgCl₂, 10 BAPTA/4 CaCl₂ (or 5 EGTA/1.5 CaCl₂), 10 HEPES, 2 ATP, 0.1 GTP, 10 glucose (adjusted to pH7.2 with Tris base); Extrinsic solution (in mmol/L): 140 NaCl, 5 KCl, 1 CaCl₂, 1.2 MgCl₂, 10 HEPES, 10 glucose (pH 7.4, adjusted with Tris base). Hypotonic solutions were made to give a desired hypotonicity (-25, -50, -75 and -100mOsm) by reducing the external NaCl concentration, to which equi-osmolar sucrose or mannitol was added to make the corresponding normotonic solutions. Na⁺ free external solution was made by equimolar substitution of monovalent cations with N-methyl,D-glucamine (NMDG).

Pipette solution for cell-attached (C/A) and inside-out (I/O) recordings (in mmol/L): 140 NaCl, 1 tetraethylammonium-Cl, 0.3 DIDS, 1.2 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose (pH 7.4, adjusted with Tris base).

Bathing solution for C/A recording (in mmol/L): 145 KCl, 2 MgCl₂, 1EGTA, 10 HEPES. Bathing solution for I/O recording (in mmol/L): 120 CsOH, 120 aspartate, 20
CsCl, 2 MgSO_4, 2 EGTA, 0.4 Ca, 10 HEPES, 2 ATP, 0.1 GTP (pH 7.2, adjusted with Tris base).

Drugs were topically applied using a fast solution exchange device ‘Y-tube’.

**Chemicals**

CCh, noradrenaline, GTPγS, indomethacin, p-bromophenacyl bromide (pBPB), miconazol, 17-octadecynoic acid (17-ODYA), and disodium 4,4′-disothiocyanatostilbene-2,2′-disulfonate (DIDS) were purchased from Sigma-Aldrich (USA), and AVP ([Arg^8]-vasopressin), AACOCF_3, nordihydroguaiaretic acid (NDGA), U73122, GF109203X from Calbiochem-Merck (USA), and OAG, 20-HETE and 2,4,6-trinitrophenol (TNP) from Cayman (UK), and GsMTx-4 from Peptides International. HET0016 was kindly provided by Taisho Pharmaceuticals (Japan).

Stock solutions for drugs were made by dissolving their powders in DMSO and used a more than 1000-fold dilution, the concentration of which did not affect the magnitude or kinetics of TRPC6 currents significantly.

**Statistics**

All data are expressed as means ± S.E.M (indicated by columns/symbols and bars in each figure). Two-tailed paired and unpaired Student’s *t*-tests were used to evaluate statistical significance with a criterion of *P* <0.05 (*) or *P*<0.01 (**). For multiple comparison, statistical significance was evaluated by ANOVA followed by Bonferroni’s *t*-test. ‘NS’ in figures: no statistically significant difference.

**References**
