TRPV2 Is a Component of Osmotically Sensitive Cation Channels in Murine Aortic Myocytes

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Abstract—Changes in membrane tension resulting from membrane stretch represent one of the key elements in blood flow regulation in vascular smooth muscle. However, the molecular mechanisms involved in the regulation of membrane stretch remain unclear. In this study, we provide evidence that a vanilloid receptor (TRPV) homologue, TRPV2 is expressed in vascular smooth muscle cells, and demonstrate that it can be activated by membrane stretch. Cell swelling caused by hypotonic solutions activated a nonspecific cation channel current (NSCC) and elevated intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]) in freshly isolated cells from mouse aorta. Both of these signals were blocked by ruthenium red, an effective blocker of TRPVs. The absence of external Ca\(^{2+}\) abolished this increase in [Ca\(^{2+}\)], caused by the hypotonic stimulation and reduced the activation of NSCC. Significant immunoreactivity to mouse TRPV2 protein was detected in single mouse aortic myocytes. Moreover, the expression of TRPV2 was found in mesenteric and basilar arterial myocytes. Treatment of mouse aorta with TRPV2 antisense oligonucleotides resulted in suppression of hypotonic stimulation-induced activation of NSCC and elevation of [Ca\(^{2+}\)], as well as marked inhibition of TRPV2 protein expression. In Chinese hamster ovary K1 (CHO) cells transfected with TRPV2 cDNA (TRPV2-CHO), application of membrane stretch through the recording pipette and hypotonic stimulation consistently activated single NSCC. Moreover, stretch of TRPV2-CHO cells cultured on an elastic silicon membrane significantly elevated [Ca\(^{2+}\)]. These results provide a strong basis for our purpose that endogenous TRPV2 in mouse vascular myocytes functions as a novel and important stretch sensor in vascular smooth muscles. (Circ Res. 2003;93:829-838.)

Key Words: TRPV2 ■ vanilloid receptor ■ mouse aorta ■ membrane stretch ■ vascular smooth muscle

Detection of mechanical stimuli is essential for diverse biological functions including audition, touch, and maintenance of vascular myogenic tone. In the latter, elevation of intravascular pressure depolarizes vascular smooth muscle cells via membrane stretch.\(^1\)\(^2\) This depolarization activates voltage-dependent L-type Ca\(^{2+}\) channels (VDCC) and increases [Ca\(^{2+}\)], resulting in vasoconstriction and/or myogenic tone.\(^3\) A large component of the elevation of [Ca\(^{2+}\)], by myogenic tone can be inhibited by blockers of VDCC. However, some components are resistant to these agents, and instead can be accounted for by a separate nonspecific cation channel that is permeable to Ca\(^{2+}\) and also is activated by the intravascular pressure.\(^4\)\(^5\) Because stretch-activated channels play obligatory roles in regulation of the myogenic tone, extensive studies have been performed to identify the molecular entity of these channels. Originally, a yeast \(MID1\) gene product (\(MID1\)) was shown to be a eukaryotic stretch-activated channel, because CHO cells expressing \(MID1\) responded to membrane stretch.\(^6\) A member of the transient receptor potential potential channels (TRP), specifically TRPC6, is sensitive to myogenic tone, possibly through the production of diacylglycerol derivatives (DAGs) by membrane stretch.\(^7\)

The vanilloid receptor (TRPV) family includes a cation selective channel. Two examples, TRPV1 and TRPV2, were first isolated from a cDNA library from rat sensory neurons.\(^8\)\(^9\) The TRPV family has substantial sequence homology to many other membrane proteins including all members of TRPs and \textit{Caenorhabditis elegans} (\textit{C elegans}) OSM-9 channel.\(^10\)\(^11\) Members of the TRP are activated by a diverse range of stimuli such as heat, protons, lipids, and/or change in extracellular osmolarity.\(^11\) Although TRPV1, a capsaicin-sensitive TRPV in nociceptors, is known to integrate response to noxious stimuli, this channel subtype also may participate in normal bladder function by mediating mechanically evoked purinergic signaling in the urothelium.\(^12\) TRPV4, a member of this family that is expressed in vascular endothelial cells as well as kidney, senses changes in cell volume and therefore is a sensor of osmotic pressure changes.\(^13\)\(^14\) TRPV4 is also modulated by DAGs and heat stimulation.\(^15\)\(^16\) More-
over, OSM-9 channels are essential in C elegans neurons for several forms of sensory transduction, including osmo- and mechanosensation. Although these findings suggest a fundamental relationship between TRPV and mechanosensors, it remains unclear whether TRPV can, in fact, function as stretch-activated channels, and their functional expression in vascular smooth muscles has not been demonstrated.

In the present experiments, we examined the possibility that TRPV2 (GRC/VRL1), a member of TRPVs, is involved in activation of NSCC by membrane stretch, in the setting of cell swelling in vascular smooth muscle cells. It is activated by heat and constitutively active in the presence of growth factors such as insulin growth factor I, platelet-derived growth factor, and serum. By using immunocytochemistry and an antisense strategy, we first demonstrated that TRPV2 is expressed in mouse vascular smooth muscle cells, and then showed that it is activated by hypotonic stimulation–induced cell swelling. Moreover, we used recombinant expression of TRPV2 in CHO cells to demonstrate that this channel can be activated by membrane stretch as well as hypotonic stimulation and is responsible for elevation of $[\text{Ca}^{2+}]_i$, by cell stretch.

Materials and Methods

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org. Briefly, CHO cells were transfected with the recombinant plasmids pIRES-TRPV2 and pIRES-GFP. Single cells isolated from aorta and mesenteric and basilar artery were obtained using a dispersion procedure involving collagenase and papain. Electrophysiological experiments and data analysis were done as described elsewhere. Stretch was applied to CHO cells using a chamber similar to that described by Naruse and Sokabe. RT-PCR and Western Blotting analyses for TRPV expression were performed as described previously. Oligodeoxynucleotides (ODNs) specific for mouse TRPV2 were designed and were introduced into intact aorta using a reversible permeabilization procedure. Immunostained cells with antibodies were observed under a confocal laser scanning microscope.

Results

Cell Swelling Induced by Hypotonic Stimulation Activates NSCC and Elevates $[\text{Ca}^{2+}]_i$ in Mouse Aortic Myocytes

To demonstrate NSCC activated by hypotonic stimulation–induced cell swelling, we applied a hypotonic osmotic solution to mouse aortic myocytes while measuring membrane currents and $[\text{Ca}^{2+}]_i$ (Figure 1). These myocytes were voltage-
clamped at −60 mV, and ramp voltage waveforms were applied every 5 seconds. The application of the hypotonic solution (reduction in osmolarity from 310 to 227 mOsm) caused activation of inward currents in 79% mouse aortic myocytes (15 out of 19 cells, Figure 1A). Just after the activation of the inward currents, the reversal potential of the currents in the current-voltage (I-V) relationship was −6.8±1.3 mV (n=6), which was close to the theoretical equilibrium potential of monovalent cations under this experimental condition (≈−9 mV, 1 and 2 arrows in Figure 1B). In 4 out of 6 cells, however, the I-V relationships were progressively shifted in the negative direction during this stimulation; the reversal potential was changed from −7.0±1.3 to −17.5±2.8 mV (n=4, P<0.05, 1 versus 3 arrows in Figure 1B). This shift of the I-V relationship is assumed to be caused by activation of Ca2+-activated Cl− currents (I_{Cl, Ca}) in mouse aortic myocytes, because removal of external Ca2+ abolished the shift of the I-V relationship of the current and the change in the equilibrium potential of Cl− reversed the shift (see the online data supplement).

Effects of hypotonic stimulation–induced cell swelling on [Ca2+]i, in mouse aortic myocytes are illustrated in Figure 1C. In 56% of the cells studied (71 of 127 cells), 227 mOsm hypotonic stimulation induced elevation of [Ca2+]i, which consisted of transient and sustained components. Neither Ca2+ influx through VDCC nor release of Ca2+ from Ca2+ storage sites was responsible for the elevation of [Ca2+]i, because pretreatment with 30 μmol/L diltiazem (diltz), 10 mmol/L caffeine (caff), 10 μmol/L tert-butylhydroquinone (BHQ), or the combination of these agents failed to affect the elevation of [Ca2+]i (Figures 1D and 1E).

To examine further this activation of NSCC and elevation of [Ca2+]i, due to hypotonic challenge, the osmotic stimuli were systematically changed, by superfusion with 310 (mannitol sol.), 278, 227, and 170 mOsm solution. Concentrations of cations and Cl− were kept constant during this protocol and equilibrium potential of monovalent cations, and Cl− was set at approximately −15 and +15 mV, respectively. Inward currents were activated by stepwise changes in tonicity in the presence or absence of external Ca2+ (Figures 2A and 2B). The I-V relationship of the inward currents revealed that only NSCC was activated in the absence of Ca2+ (see the online data supplement). Fluorescence signals due to changes in [Ca2+]i were measured in the same protocol (Figures 2C and 2D). In the presence of Ca2+, the hypotonic stimulation elevated [Ca2+]i, and the each response had both transient and sustained components (Figures 2C and 2F). Both components were abolished in the absence of Ca2+ (Figures 2D and 2F). Figure 2E summarizes the current density of NSCC and I_{Cl, Ca}, which were estimated at +15 and −15 mV, respectively. I_{Cl, Ca}
was induced only in the presence of external Ca$^{2+}$, suggesting that Ca$^{2+}$ entry through NSCC is required for the activation.

**TRPV Modulators on Cell Swelling Induced–NSCC and Ca$^{2+}$ Response**

Because ruthenium red (RuR) is defined as an effective blocker of TRPVs, we examined effects of RuR on hypotonic stimulation–induced NSCC and elevation of [Ca$^{2+}$] in mouse aortic myocytes (Figure 3). As shown in Figure 3A, 10 μmol/L RuR substantially inhibited inward currents activated by 227 mOsm solution, and the removal of RuR during the hypotonic stimulation resulted in recovery of these currents. Moreover, pretreatment of myocytes with 10 μmol/L RuR effectively inhibited the 227 mOsm hypotonic stimulation–induced inward currents (Figures 3B and 3Cb). In Figures 3D and 3E, the same protocols were used to examine the effect of RuR on elevation of [Ca$^{2+}$] by the hypotonic stimulation. The sustained component of the elevation of [Ca$^{2+}$] was effectively inhibited by application of 10 μmol/L RuR. In addition, the transient elevation was significantly inhibited by pretreatment with RuR. These inhibitory effects of RuR were not observed in any of the cells that were insensitive to the hypotonic stimulation (Figures 3Ca, 3D, and 3Fa).

TRPV4, which is activated by hypotonic stimulation and inhibited by RuR, is expressed in mouse aortic endothelial cells.\textsuperscript{15,24} TRPV4 is also sensitive to DAGs such as phorbol didecanoate (PDD) and 4α-PDD.\textsuperscript{15,24,25} Effects of 4α-PDD on [Ca$^{2+}$] were examined in mouse aortic myocytes; these results are summarized in Figure 3G. Application of 1 μmol/L 4α-PDD elevated [Ca$^{2+}$] in only 4 out of 31 myocytes; in contrast, all of the cells examined were sensitive to 227 mOsm hypotonic stimulation. Capsaicin can potently activate TRPV1.\textsuperscript{8} Although in two myocytes there was slight increase of [Ca$^{2+}$], by 1 μmol/L capsaicin (caps), this response was much smaller than that to 227 mOsm hypotonic stimulation (Figure 3G). These suggest that neither TRPV1 nor other TRPVs other than TRPV4 are involved in the hypotonic stimulation–induced NSCC and Ca$^{2+}$ elevation.
nor TRPV4 is involved in the NSCC in mouse aortic myocytes, which is activated by cell swelling through hypotonic stimulation and can be blocked by RuR.

TRPV2 Transcript and Protein Expression in Mouse Vascular Myocytes

In Figure 4A, we evaluated the expression of other TRPVs mRNA in an effort to find a candidate of NSCC activated by hypotonic stimulation–induced cell swelling. Total RNA isolated from acutely dispersed mouse aortic, mesenteric, and basilar arterial myocytes was subjected to RT-PCR. In mouse aortic myocytes, the transcripts of mRNA of TRPV2 and TRPV4 were present, whereas heat- and RuR-sensitive TRPV3 was not expressed (n=5 to 6). Expression of TRPV2 was also found in both mesenteric and basilar arterial myocytes (n=5 to 6). Immunocytochemistry using anti-TRPV2 antisera demonstrated that TRPV2 proteins were expressed in acutely dissociated mouse aortic myocytes (Figure 4B), whereas no immunoreactivity was detected in myocytes treated with Alexa488-conjugated secondary antibody alone (data not shown) or with preabsorption of anti-TRPV2 antibody by the immunizing peptide (IP, Figure 4B). Moreover, in mesenteric and basilar arterial myocytes, which were positively stained with anti-smooth muscle–specific α-actin antibody, expression of TRPV2 protein was confirmed by the immunocytochemical analysis.

TRPV2 Functions as Ca2+ Entry Channel Activated by Cell Swelling in Mouse Aortic Myocytes

To determine whether the endogenously expressed TRPV2 protein functions as NSCC and contributes to elevation of [Ca2+]i by hypotonic stimulation–induced cell swelling, we next utilized an antisense ODN designed to be specific for TRPV2. Mouse aortic strips were organ-cultured for 4 to 5 days after the treatment with sense and antisense ODNs. The expression of TRPV2 protein was significantly decreased in myocytes isolated from strips treated with the antisense ODN, whereas substantial TRPV2 protein remained in sense ODN-treated preparations (Figures 5A and 5B). Correspondingly, the density of NSCC activated by 227 mOsm hypotonic stimulation was markedly decreased with the antisense ODN compared with cells treated with the sense ODN (Figures 5C and 5E). We also examined the ability of TRPV2 to increase [Ca2+]i after hypotonic stimulation (Figures 5D and 5F). Treatment with the antisense ODN significantly decreased the elevation of [Ca2+]i, in response to 227 mOsm hypotonic stimulation. As a control, we tested the effects of the ODNs on ATP-induced membrane currents and elevation of [Ca2+]i, and observed no differences in cells from sense-versus antisense-ODN–treated aorta (Figures 5E and 5F).

Transient Expression of TRPV2 in CHO Cells

Based on these findings, the characteristics of NSCC activated by hypotonic stimulation in mouse aortic myocytes were compared with those due to the activation of mouse TRPV2, which was transiently expressed in CHO cells. As shown in Figure 6A, membrane currents recorded in CHO cells transfected with TRPV2 were strongly inhibited by 10 μmol/L RuR and this RuR-sensitive current reversed at −7.2±1.9 mV (n=4). In contrast, 10 μmol/L RuR had no effect on membrane currents recorded from native CHO cells (control-CHO, Figure 6C). Noisy channel events were consistently recorded when outside-out patches were obtained from TRPV2-CHO cells (Figure 6B, n=7). Application of 10 μmol/L RuR inhibited these events. This RuR-sensitive single channel current reversed at −3.0±1.1 mV (n=4) when 140 mmol/L K+ was included in the pipette solution. The replacement of 110 mmol/L Cl− with equimolar aspartate− and 70 mmol/L K+ with Cs+ did not change the reversal potential of this channel current (−5.7±1.7 mV, n=3).
Activation of TRPV2 by Membrane Stretch and Hypotonic Stimulation

We next examined whether TRPV2 can be activated by membrane stretch and hypotonic stimulation at the single channel level. As shown in Figure 7A, application of a negative pressure of 15 cm H$_2$O to the recording pipette did not elicit any channel activity, whereas that of 30 cm H$_2$O markedly activated channel currents. At $V_{H11002}$/H1100240 mV, reapplication of the negative pressure of 30 cm H$_2$O elicited channel currents that were inward. Even after the cessation of the membrane stretch, sporadic channel openings were still observed. Among GFP-positive TRPV2-CHO cells, 42% cells responded (Figure 7C, averaged current amplitude: 1.73 ± 0.47 pA at $V_{H11001}$/H1100130 mV; $P<0.05$ versus control). This pattern of channel openings was never observed in patches from control-CHO cells (Figures 7B and 7C, 7.7%, 0.08 ± 0.08 pA at +30 mV). In Figure 7D, a TRPV2-CHO cell was superfused with 227 mOsm hypotonic solution under the cell-attached patch-clamp configuration. The hypotonic stimulation activated channel currents in TRPV2-CHO cells, although not in control-CHO cells (Figure 7E).

Elevation of [Ca$^{2+}$]$_i$ in TRPV2-CHO Cells by Cell Stretch

Activation of TRPV2 in mouse aortic myocytes caused elevation of [Ca$^{2+}$]$_i$. To confirm that sarcolemmal stretch contributes to elevation of [Ca$^{2+}$]$_i$ through TRPV2, experimental protocols as shown in the schematic diagram in Figure 8 were performed in TRPV2- and control-CHO cells. Cells were voltage-clamped at $-30$ mV, where $I_{Cl-Ca}$ was almost nulled due to $E_{Cl}$. E, Averaged current density of 227 mOsm hypotonic stimulation–induced NSCC and Ca$^{2+}$ fluorescence ratio changes in a TRPV2 sense or antisense ODN-treated myocyte. Cells were voltage-clamped at $-30$ mV and 30 $\mu$mol/L ATP-induced membrane currents at $-60$ mV with TRPV2 sense and antisense ODNs. F, Summary of 227 mOsm hypotonic stimulation- and 30 $\mu$mol/L ATP-induced peak Ca$^{2+}$ fluorescence ratio changes with TRPV2 sense and antisense ODNs.$^*$ $P<0.05$, $**P<0.01$, statistically significant difference.
0.016±0.004). These findings suggest that cells expressing TRPV2 are more susceptible to the cell stretch than controls.

**Discussion**

Our study demonstrates that TRPV2, one of TRPVs, is expressed in mouse aortic myocytes and acts as an essential molecular component for nonselective cation channel (NSCC) activated by hypotonic stimulation–induced cell swelling, and shows that this occurs with elevation of [Ca²⁺]. This conclusion, that TRPV2 is an essential component of the hypotonic response of NSCC in murine vascular myocytes is based on the following lines of evidence. (1) Activation of NSCC and the elevation of [Ca²⁺], which were induced by hypotonic solutions, are both due to cation influx and are inhibited by RuR. (2) TRPV2-specific immunoreactivity was detected in myocytes from mouse mesenteric, basilar as well as aortic myocytes. (3) In mouse aortic myocytes, application of TRPV2 antisense ODN, but not that of sense ODN, substantially decreased TRPV2 protein expression and markedly reduced both the activation of NSCC and the elevation of [Ca²⁺], by hypotonic stimulation.

In the present study, single channel recordings established that TRPV2 is activated by hypotonic stimulation. Furthermore, TRPV2 is sensitive to membrane stretch induced by negative pressure through patch pipettes, and this involves a marked elevation of [Ca²⁺]. These results indicate that membrane stretch is a plausible activator of TRPV2. Although TRPV2 is activated by high temperature (>52°C) and growth factors, the present study provides the novel evidence that TRPV2 functions as a mechanosensor in various organs including vascular smooth muscles. CHO cells expressing MID1 responded to similar membrane stretch to that in the present study, 6 showing that MID1 is an eukaryotic stretch-activated channel. In addition, TRPC6 can be activated by change of membrane tension via myogenic tone in rat cerebral artery. 7 TRPV4 might also have a certain role in detecting mechanostimulation because TRPV4 is activated by hypotonic stimulation. However, mechanisms involved in opening of these channels by membrane stretch have not been defined. TRPVs, including TRPV2, have ankyrin repeats in the N-terminal region. Deletion of the ankyrin repeats abolished heat-activation of TRPV1 26 and TRPV4. 27 Because the ankyrin repeats interact

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**Figure 6.** Effects of RuR on channel currents in CHO cells that transiently express TRPV2. A, Inhibition by 10 μmol/L RuR of the membrane current recorded from a TRPV2-CHO cell. The ramp waveform command pulses were applied every 5 seconds at −10 mV. Original current traces (right, a and b) and the change in the current amplitude at 40 and −40 mV (left) are shown. B, Outside-out membrane patch was voltage-clamped at −30 mV, and ramp waveform command pulses were applied every 10 seconds. Pipette solution included 70 mmol/L K⁺ and Ca⁺ as cations and 110 mmol/L aspartate and 30 mmol/L Cl⁻ as anions, respectively. Right panels (a through c) show the original current traces obtained at the time denoted in the left. Dotted lines indicate the closed level of the channel. C, Summary of effects of RuR on the channel current. RuR-sensitive current amplitude was measured as difference before and after application of 10 μmol/L RuR. WC and OO indicate data obtained under the whole-cell and outside-out patch-clamp conditions, respectively. Numbers in parentheses indicate the number of cells studied. **Statistically significant difference (P<0.01).
with certain cytoskeletal proteins, this region of TRPV2 might be also important for acceptance of applied mechanical signals.

RT-PCR, immunocytochemical, and Western analyses revealed that mouse aortic myocytes express TRPV2. More interestingly and importantly, TRPV2 is present in basilar as well as mesenteric arterial myocytes. In rat cerebral artery, treatment with the antisense ODN specific for TRPC6 suppressed pressure-induced myogenic tone and depolarization, and abolished hypotonic stimulation–induced NSCC. Mouse vascular smooth muscles express TRPC1, TRPC3, TRPC4, and/or TRPC6.28,29 Although expression of TRPV2 and TRPC6 in the resistance arteries has not been quantitatively compared, the present finding demonstrates that TRPV2 may play a certain role in regulating vascular tones in these peripheral arteries. TRP channels can be activated/modulated by stimuli such as G protein–coupled receptor activation, store depletion, activated G protein, DAGs, inositol trisphosphate, and Ca²⁺.9,30–32 In principle, a number of different TRPs could be also sensitive to membrane stretch and/or hypotonic stimulation. Although our results clearly demonstrate that TRPV2 in vascular myocytes is one of essential component of NSCC activated by membrane stretch and hypotonic stimulation–induced cell swelling, experiments using intact arteries will develop total understanding of the physiological significance of TRPV2 for smooth muscle regulation, in particular, in resistance arteries.

Even though TRPC6 is sensitive to myogenic tone, this may arise from DAGs produced by activation of phospholipase C, after cell swelling. Thus, changes in myogenic tone can play an obligatory role in the activation of TRPC6.7 Moreover, activation of TRPV4 by hypotonic stimulation is mediated by phosphorylation of the tyrosine residue by Src family tyrosine kinase,31 suggesting that hypotonic stimulation releases a ligand that activates this kinase. Although the

Figure 7. Activation of channel currents by membrane stretch and hypotonic stimulation in TRPV2-CHO cells. In the cell-attached patch-clamp configuration, membrane stretch [generated by 15 (A) and 30 cm H₂O negative pressure (A through C)] was applied for 15 seconds through a recording pipette. Membrane potential of each cell was adjusted to 0 mV by superfusion with 140 mmol/L KCl. Pipette was filled with Na⁺-rich solution (140 mmol/L). A, Original current trace recorded at +40 and −40 mV in a TRPV2-CHO cell. In bottom panels, time scale has been expanded. Membrane stretch was applied 3 times during the recording. B, No effect of the membrane stretch on channel currents in a control-CHO cell. Cell was voltage-clamped at −30 mV. C, Summary of effects of the membrane stretch on channel activity in TRPV2- and control-CHO cells clamped at +30 mV. Individual channel activity was calculated and then averaged over 10 seconds (see the online data supplement). D, Original current trace at +30 mV in a TRPV2-CHO cell superfused with 227 mOsm hypotonic solution. In bottom panels, time scale has been expanded. E, Summary of effects of the hypotonic stimulation on channel activity in TRPV2- and control-CHO cells at +30 mV. Numbers in parentheses indicate the number of cell studied. Pooled data are averaged and the means are shown as cross symbols. *Statistically significant difference (P<0.05).
tyrosine residue is not conserved in TRPV2 and stimulation of the kinase by hypotonic stimulation has not been confirmed in mouse aorta, the possibility that cell swelling produces an endogenous ligand that activates TRPV2 cannot be ruled out. Local change of membrane tension produced by membrane stretch could affect channels, receptors, lipids, and/or enzymes, and the locally produced ligand, in particular, a certain lipid, may modulate the neighboring channels. Activity of TRPV1 and TRPM7 is regulated by inositol lipids that are closely localized with these channels. Taken together, the mechanism underlying the activation of TRPV2 by membrane stretch is remained to be determined.

RT-PCR analysis revealed that mouse aortic myocytes express TRPV4 as well as TRPV2, but not TRPV3. However, 4α-PDD, an activator of TRPV4, did not affect [Ca\(^{2+}\)]i in mouse aortic myocytes. Thus, TRPV4 appears not to serve as a major component of NSCC activated by hypotonic stimulation and is unlikely to contribute to the elevation of [Ca\(^{2+}\)]i in mouse aorta under the present experimental conditions. Definition of the functional roles of TRPV4 in vascular smooth muscles is required for understanding of TRPVs in vascular beds where it is expressed.

In conclusion, we have shown that TRPV2 is a major component of native Ca\(^{2+}\)-permeable cation channels, which respond to membrane stretch in mouse aortic myocytes, and that the activation of TRPV2 is responsible for mechanosensitive membrane depolarization.

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The TRPV2 is a component of osmotically-sensitive cation channels in murine aortic myocytes

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Materials and Methods

Recombinant expression in CHO-K1 cells

CHO-K1 (CHO) cells were grown in Dulbecco’s modified Eagle Medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 1 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidity controlled incubator equilibrated with 5% CO₂. Partially confluent CHO cells (40-60%) were co-transfected with two plasmid DNAs, pIRES-TRPV2 and pIRES-GFP, with a molar ration of 5:1, using jetPEI (Qbiogene, Inc). The expression level of TRPV2 was substantial in TRPV2-CHO cells; 40-60% cells were transfected 48-96 hr after the procedure. In this study, a batch of cells was discarded when the expression level was less than 20%. As a control test, only GFP was transiently expressed (control-CHO). All experiments were performed within 96 h after transfection.

Cell dispersion

Male ddY strain mice (Japan SLC Inc) weighing 30-60 g were anesthetized with ether, and then killed by exsanguination. After opening the chest, an approximately 1.5 cm long segment of thoracic aorta was excised. After removing connective tissues and rubbing inner
wall of the vessel with cotton pad to remove endothelium, strips approx. 0.7 cm long of the aorta were incubated in nominally Ca\(^{2+}\) and Mg\(^{2+}\) free Hanks solution for 5 min and then one containing 2 mg/ml collagenase (Amano) and 1 mg/ml papain (Sigma) for 45 min. Thereafter, the enzyme-treated strips were mechanically agitated in fresh Ca\(^{2+}\) and Mg\(^{2+}\) free Hanks solution which did not contain digestion enzymes. Dissociated cells were used within 6 h after cell dispersion. Ca\(^{2+}\) and Mg\(^{2+}\) free Hanks solution for cell-dispersion contained (mM) NaCl 137, KCl 5.4, Na\(_2\)HPO\(_4\) 0.168, KH\(_2\)PO\(_4\) 0.44, glucose 5.55 and NaHCO\(_3\) 4.17 (pH 7.45). Mesenteric and basilar arterial myocytes, which were dispersed with the similar procedure, were also used for immunocytochemical and RT-PCR analyses. Purity of smooth muscle cells was confirmed by staining with anti-smooth muscle specific a-actin (SM a-actin) antibody. Most round-shape cells (contracted cells) as well as elongated cells were positively stained with the SM a-actin antibody.

**Oligodeoxynucleotide sequence and reverse permeabilization**

Sense and antisense oligodeoxynucleotides (ODNs) specific for mouse TRPV2 were designed as follows: antisense; 5’-GGGTTGGAGGCTGAAGTCAT-3’; sense: 5’-ATGACTTCAGCCTCCAACCC-3’. All bases were phosphorothioated to limit ODN degradation. To assess cellular localization, fluorescein-isothiocyanate (FITC) was also conjugated to the 3’ end in some experiments. ODNs were synthesized and HPLC-purified commercially (Nippon Gene Research Labs). Sense and antisense ODNs were introduced into intact aortic strips using a reversible permeabilization procedure\(^ {1,2}\). To permeabilize the aorta, an approximately 0.7 cm aorta segment was first incubated for 30 min at 4 °C in the following solution (mM): KCl 120; MgCl\(_2\) 2, EGTA 10, Na\(_2\)ATP 5 TES 20, pH 6.8. Thereafter, the segment was placed in the solution containing ODNs (10 µM) for 90 min at 4
°C and then in the ODN containing solution with 10 mM MgCl$_2$. Permeabilization was reversed by placing the aorta for 30 min at 22 °C in a MOPS buffered physiological solution containing (mM): NaCl 140, KCl 5, MgCl$_2$ 10, glucose 5, MOPS 2 (pH 7.1). Finally, Ca$^{2+}$ was gradually increased over a 45 min period from nominally Ca$^{2+}$ free to 0.01, 0.1 and 1.8 mM. Following the reversible permeabilization procedures, the aorta was organ-cultured for 4-5 days in DMEM with 2% FCS. After 2.5 days, the culture medium was refreshed. For cell-dispersion, the same procedure to obtain acutely dissociated cells was used.

**Immunocytochemistry**

Polyclonal anti-mouse TRPV2 was raised by immunizing rabbits with GST fusion proteins containing aa 643-756 of mouse TRPV2 protein and the antibody was affinity-purified. A monoclonal antibody directed against N-terminus domain in mouse smooth muscle specific a-actin (SM a-actin, Neomarkers) was also used. Myocytes dissociated from mouse aorta with and without organ culture and from mouse mesenteric and basilar artery were settled down on coverslipes precoated with poly-L-lysin (Matsunami). These myocytes were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton/phosphate-buffered saline (PBS) for 15 min at room temperature. After rinsing in PBS containing 1% normal goat serum (NGS), the myocytes were pre-incubated for 1 hr with 10% NGS/PBS to prevent non-specific binding of antibodies, and incubated successively with 1:100 diluted TRPV2 or a-actin antiserum for 1 hr, with Alex 488-conjugated anti-rabbit IgG goat and Alex 546-conjugated anti-mouse IgG goat antiserum (Molecular Probe) for 1 hr after washing with 1% NGS/PBS. Pre-absorption of anti-TRPV2 antibody by the immunizing peptide (IP, aa. 643-756) was performed by adding equimolar amount of IP to anti-TRPV2 antibody. Immunostained cells were observed under a confocal laser
scanning microscope (BioRad Radiance 2100) equipped with an argon laser source. A single-wavelength of 488 nm and 543 nm was used for excitation of Alex 488 and 546, respectively, and the emitted fluorescence at 505 nm was collected through an objective lens with a 60 times magnification (Plan-Apo), and an optical section of 0.9 - 1.07 µm was projected on a single plane.

**Western Blotting Analysis**

A whole segment of mouse aorta treated with sense and antisense ODNs for 4 days was fixed with 10% trichloracetic acid for 1 h and homogenized. Samples were then centrifuged at 15000rpm (5 min) and the pellet was resuspended in the solution containing 9M urea/2% Triton-X/1% DTT. The 40 µl aliquot added to 10 µl LiDS (10%) was titrated with 1 M Tris-HCl and separated on a 10% polyacrylamide gel. Proteins were then transferred to a PVDF membrane and blocked for 12 h with Tris-buffered saline (TBS) containing 1% BSA and 0.2% Tween-20. The PVDF membrane was then exposed to TRPV2 polyclonal antibody (anti-rabbit, 1:2000 dilution) for 24 h. Following a 1 h washing period, anti-rabbit IgG-HRP (1:10000) was added to the PVDF membrane. Western blots were washed again (1 h) and detection reagents (Amersham Biosciences) were added to generate a chemi-luminescence product. To determine the relative quantities of TRPV2 protein with sense- and antisence ODNs against SM α-actin in each sample, the PVDF membrane was exposed to SM α-actin monoclonal antibody (anti-mouse, 1:2000 dilution) after removal of antibodies of TRPV2 and anti-rabbit IgG-HRP with Re-Blot Plus (Chemicon). Gels were scanned on a densitometer and signals specific for the TRPV2 against the SM α-actin bands in the same lane on the gel were calculated as a ratio to define the effects of TRPV2 ODNs.
Antibody specificity

We examined the specificity of the TRPV2 polyclonal antibody used in this study to prepare the protein of TRPV2-CHO and control-CHO cells. As shown in Supplement figure 1, we found the specific signal positive to TRPV2 antibody only in TRPV2-CHO cells.

Reverse Transcription (RT)-PCR amplification

RT- and non RT-PCR amplification for TRPV expression was performed as described previously\textsuperscript{3}. Total RNAs were extracted from freshly dissociated mouse aortic myocytes by the acid guanidium thiocyanate-phenol method following digestion with RNase-free DNase, and RT was performed according to the manufacture’s instructions. For total RNA extraction from mouse mesenteric and basilar arterial myocytes, we used RNeasy mini kit (Qiagen Inc). Oligonucleotide sequences of primers specific for TRPV2, TRPV3, and TRPV4 of the mouse (sense and antisense; 5’ to 3’) were ACCGCATGGTGTTTTAGAG and CTACAGCAAAGCAGAAAAGG for TRPV2, GCAAGGCTGAGATCCTGAAG and GGCATCTGACAGGATGGACT for TRPV3\textsuperscript{4}, CCCCATCCTCAAAGTCTTCA and GGTGTTCTCTCGGGTGTGTTGT for TRPV4. These primers yield 552, 506 and 589-bp TRPV2, TRPV3 and TRPV4 PCR products, respectively. The thermal cycler program used for PCR amplification included a 0.5 min denaturation step at 94°C, a 0.5 min annealing step at 55°C and a 0.5 min primer extension step at 72 °C for 35 cycles (GeneAmp 2400, Perkin Elmer ABI). Amplified products were separated on 1.5% agarose gels in Tris acetate/EDTA buffer, visualized with 1 µg/ml ethidium bromide, and documented on FAS 1000 (TOYOBO).

Electrophysiology
The resistance of microelectrodes filled with pipette solution was in the range of 3-5 MΩ. Membrane currents and voltage signals were monitored on a storage oscilloscope (VC-6041, Hitachi) and stored on videotape after being digitized by pulse code modulation (PCM) recording system (modified to acquire a DC signal, PCM 501ES, SONY). Data on tape were later downloaded into a computer (IBM-AT compatible) using an analogue-digital converter (Data Translation, DT2801A). Data-acquisition and analysis for whole-cell and single channel currents were carried out using AQ/Cell-soft, developed in the laboratory of Dr. Wayne Giles, University of Calgary, and single channel current analysis program V7.0C (PAT) developed by Dr. John Dempster, University of Strathclyde, respectively. In some experiments, ramp waveforms were applied as a voltage-clamp command using multi-pulse generators (WF1943 and FS-1915; NF Electronics). All current records were filtered at 1 kHz (4-pole Bessel filter, NF Electronics). For analysis of single channel currents, the mean current was calculated as the time integral of current for every 1000 data points and then divided by the duration used for the integration (0.25 s). Channel activities were expressed by temporal plots of the mean currents in 0.25 s bins for representative patches and by average±SEM of the mean currents of 10 s recordings from indicated numbers of patches. Cell capacitance was measured with the capacitance cancellation circuitry in the voltage-clamp amplifiers (EPC7, List; SEN2400, Nihon Kohden). The liquid junction potential between pipette and bath solutions (~10 mV) was corrected when aspartate-rich pipette solution was used.

Standard 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES) buffered solution of the following composition was used (mM): NaCl 137, KCl 5.9, CaCl₂ 2.2, MgCl₂ 1.2, glucose 14, HEPES 10 (pH 7.4). The bathing solution for the cell-attached patch clamp recording had the following composition (mM): KCl 140, CaCl₂ 2.2, MgCl₂ 1.2, HEPES 10
(pH 7.4). To apply cell swelling using hypotonic stimulation, cells were first superfused with solution containing (mM): NaCl 91.3, KCl 5.9, CaCl$_2$ 2.2, MgCl$_2$ 1.2, glucose 14, HEPES 10, mannitol 91, pH 7.4 (mannitol sol., 310 mOsm), and then with the solution without mannitol (227 mOsm). The pipette solution for the conventional whole-cell recording contained (mM): Cs-aspartate 110, CsCl 30, MgCl$_2$ 2, HEPES 10, EGTA 1 or 10, ATP-2Na 2 (pH 7.2). In the experiments shown in Figure 6, ATP-2Na was removed from this solution. For the amphotericin B-perforated recording (Figures 1, 3 and 5), EGTA and ATP-2Na were omitted from and 300 µg/ml amphotericin B was added to the pipette solution. For outside-out patch recording, 70 mM K$^+$ or 110 mM Cs-aspartate was replaced with equimolar Cs$^+$ or KCl, respectively. For the cell-attached patch clamp recording, the pipette solution contained (mM): NaCl 140, tetraethylammonium-Cl 10, MgCl$_2$ 1.2, glucose 14, HEPES 10 (pH 7.4). The relationship between hypotonic osmotic challenge and activation of NSCC or elevation of [Ca$^{2+}$]$_i$ was obtained by changing the concentration of mannitol in a stepwise manner from 137 (mannitol sol., 310 mosmol/l (mOsm)) to 278, 227 and 170 mOsm. The composition (except mannitol, in mM) was: NaCl 68.5, KCl 5.9, CaCl$_2$ 2.2, MgCl$_2$ 1.2, Glucose 14, HEPES 10 (pH 7.4). The pipette solution in these experiments (Figure 2 and Figure suppl-2) contained (mM): 140 CsCl, 10 HEPES, 300 µg/ml amphotericin B (pH 7.2). The TRPV2 openings in the cell-attached patch recording mode were obtained by applying negative pressure (suction) to the patch according to the procedure described previously$^6$. The suction was monitored as negative hydrostatic pressure, which was calibrated with a Hg-based pressure gauge (mercury/water=13.3). All experiments were performed at 25±1 °C.

Measurement of Ca$^{2+}$ fluorescence ratio
CHO and aortic smooth muscle cells were loaded with 2.5 µM Fura-2 acetoxyethyl ester (Fura-2) in standard HEPES solution for 45 min at room temperature. Measurement of Fura-2 fluorescence signals was performed using Argus/HisCa imaging system (Hamamatsu Photonics). The frequency of image acquisition was selected to be 0.2 Hz, unless otherwise specified.

**Application of cell-stretch to CHO cells in a silicon chamber**

Stretch was applied to CHO cells using a chamber similar to that described by Naruse and Sokabe\(^7\) (see the inset in Figure 8). CHO cells were cultured in a gelatin-coated chamber with a thin elastic silicon membrane (Sukaratek) at 37 °C in 5% CO\(_2\). After confirming cell-growth in this chamber, the recombinant expression plasmids with TRPV2 and GFP, were transfected. Both TRPV2- and control-CHO cells were used within 96 hrs after the transfection. The silicon chamber was placed on the stage of a fluorescence microscope (Nikon, TMD-300) after loading of cells with 2.5 µM Fura-2AM. To reduce vibration of the elastic silicon membrane after application of the cell-stretch and to prevent interaction of immersion oil for an objective lens with the silicon membrane, a glass plate which thickness was less than 80 µm was placed between the objective lens and the membrane (see the inset in Figure 8). The top of the chamber was covered with a plastic cap equipped with a silicon tubing connected to a syringe. A negative pressure was applied for 2 s through the tubing to expand the elastic silicon membrane. Because cells were small and firmly attached to the membrane, two dimensional area change of the cells were assumed\(^7\). For the calibration, the distance of two marked points which were located at the center of the membrane was measured before and during the application of the negative pressure. According to this measurement, it was estimated that cells were elongated by 17.7±4.0% (n=3) of the control.
during the cell-stretch. In the present experiments, cells attached close to the marked points were used for the measurement. The sampling frequency of fluorescence signals in this experiment was 1 Hz.

Data are expressed as mean±S.E.M. Statistical significance between two and among multiple groups was examined using Student’s t-test and one-way ANOVA, respectively. Statistical significance at P values of 0.05 and 0.01 is indicated in figures and text by * and **, respectively.

Supporting Results
In the presence of external Ca\(^{2+}\) (2.2 mM), the reversal potential of the inward currents activated by cell swelling following hypotonic stimulation shifted during the stimulation (Figure 1B, ‘1’ vs. ‘3’). To confirm that Ca\(^{2+}\) activated Cl\(^{-}\) current (I\(_{\text{Cl-Ca}}\)) is responsible, I-V relationships of 170 mOsm hypotonic stimulation-induced inward currents were studied with and without external Ca\(^{2+}\). In these experiments, equilibrium potential of monovalent cations (E\(_{\text{NSCC}}\)) and Cl\(^{-}\) (E\(_{\text{Cl}}\)) was set at approximately -15 and +15 mV, respectively. As shown in Figure suppl-2A, the reversal potential of the inward currents, which were induced by the hypotonic stimulation in the presence of Ca\(^{2+}\), was initiated near -15 mV (‘1’ vs. ‘2’), and it then changed to be more positive (‘1’ vs. ‘3’). The crossover of these I-V relationships (‘2’ and ‘3’, \(~+15\ \text{mV}\) revealed that activation of Cl\(^{-}\) current is responsible for the inward current and the shift of the reversal potential. In contrast, only NSCC was activated by the hypotonic stimulation in the absence of Ca\(^{2+}\) (Figure suppl-2B) because all I-V relationships had the same reversal potential, which was at the value expected from E\(_{\text{NSCC}}\). Taken together, in the presence of Ca\(^{2+}\), I\(_{\text{Cl-Ca}}\) is activated following NSCC.
**Figure legend**

**Figure suppl-1.** Protein was prepared from TRPV2- and control-CHO cells. Extracted proteins were loaded onto a 10% polyacrylamide gel and transferred onto nitrocellulose paper, blocked with 1% BSA in TBS+0.2% Tween-20, and proved with anti-TRPV2 antibody. Detection was made by generating a chemi-luminescence product.

**Figure suppl-2.** A and B, I-V relationships obtained during 170 mOsm hypotonic stimulation in the presence (A) and absence (B) of external Ca\(^{2+}\). \(E_{NSCC}\) and \(E_{Cl}\) indicate the theoretical equilibrium potential of monovalent cations and Cl\(^-\) under these experimental conditions. These I-V relationships were recorded in cells shown in Figures 2A and 2B.


Figure suppl-1/R1
Figure suppl-2/R1

A
170 mOsm in 2.2 mM Ca

B
170 mOsm in 0 mM Ca