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# Mechanosensitive Cation Channels in Arterial Smooth Muscle Cells Are Activated by Diacylglycerol and Inhibited by Phospholipase C Inhibitor

Kyoungh Sun Park, Yangmi Kim, Young-Ho Lee, Yung E. Earm, Won-Kyung Ho

**Abstract**—Mechanosensitive cation channels may be involved in the development of the myogenic tone of arteries. The molecular identity of these channels is not clear, but transient receptor potential channels (TRPCs) are good candidates. In the present study, we searched for mechanosensitive channels at the single-channel level in arterial smooth muscle cells using the patch-clamp technique and investigated the channel properties in the light of properties of TRPCs. With 140 mmol/L CsCl in the pipette solution, application of negative pressures to the back of the pipette induced the activation of channels the open probability of which increased with the amount of negative pressure. The current-voltage relationship was linear in symmetrical ionic conditions, and the single-channel conductances for Cs<sup>+</sup>, K<sup>+</sup>, and Na<sup>+</sup> were 30, 36, and 27 pS, respectively. When NMDG<sup>+</sup> was substituted for Cs<sup>+</sup> in the pipette solution, inward currents were abolished, whereas outward currents remained active, indicating that the channels were nonselective to cations. The channel activity was blocked by intracellular Gd<sup>3+</sup> and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid and increased by diacylglycerol and by cyclopiazonic acid. Phospholipase C inhibitor (U73122) inhibited not only channel activity but also the development of myogenic tone induced by stretching of the basilar arteries. These results suggest that the ion channel responsible for the development of myogenic tone is the 30-pS mechanosensitive cation channel that exhibits properties similar to those of TRPCs. (*Circ Res.* 2003;93:557-564.)

**Key Words:** mechanosensitive cation channels ■ transient receptor potential channel (TRPC)  
■ myogenic tone ■ phospholipase C

The arterial wall is continuously exposed to mechanical stimulation such as shear stress and luminal pressure. It is well known that such mechanical stimulation causes various biological responses in the vascular wall.<sup>1-4</sup> One of the important responses is the development of myogenic tone: a state of partial contraction that is dependent on the level of intraluminal pressure. This phenomenon was first described by Bayliss<sup>5</sup> 100 years ago, but the exact signal transduction pathways are still unclear.

Pressure-induced depolarization and the increase in intracellular Ca<sup>2+</sup> concentration are associated with the development of myogenic tone when the transmural pressure is elevated to 40 to 60 mm Hg.<sup>6</sup> Therefore, mechanosensitive ion channels, especially nonselective cation (NSC) channels, are thought to transduce the change in transmural pressure into membrane depolarization, and the activation of whole-cell cation currents in response to longitudinal stretch of isolated smooth muscle cells has been demonstrated by several authors.<sup>2,3,7</sup> However, the identity of the mechanosensitive NSC channels in smooth muscle cells remains unclear.

Transient receptor potential channels (TRPCs) are good candidates for this role in arterial smooth muscle, because they are expressed in vascular smooth muscle cells and they exhibit many of the biological properties of vascular cation currents.<sup>8-11</sup> This hypothesis was tested recently by Welsh et al,<sup>8</sup> who showed that myogenic tone and smooth muscle depolarization elicited by elevated pressure in intact cerebral arteries was attenuated by antisense oligodeoxynucleotides to TRPC6. Considering that smooth muscle TRPCs are often linked to phospholipase C (PLC), the inhibition of myogenic tone by a PLC inhibitor, as shown by a recent study, may also provide evidence for the involvement of TRPCs.<sup>12</sup> For further confirmation of this, it is necessary to identify the mechanosensitive NSC at the single-channel level in native smooth muscle cells and to investigate the channel properties in the light of known properties of TRPCs.

In the present study, we searched for single mechanosensitive NSC channels in isolated arterial smooth muscle cells using the patch-clamp technique and found that 30-pS cation channels are activated by applying negative pressure to the

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back of the pipette. We then demonstrated that these channels share properties common to TRPCs: they are activated by diacylglycerol (DAG) and by cyclopiazonic acid (CPA) and are inhibited by a PLC inhibitor. The PLC inhibitor also inhibited the development of myogenic tone induced by stretching of the basilar arteries. These results suggest that the ion channel responsible for the development of myogenic tone is the 30-pS mechanosensitive NSC channel belonging to the TRPC family.

## Materials and Methods

### Cell and Tissue Preparation

Rabbits (1.0 to 2.0 kg) of either sex were anesthetized with sodium pentobarbitone (50 mg/kg) and injected with heparin (100 U/kg) at the same time. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. Pulmonary arteries (third or fourth branches of the intralobar pulmonary arteries of a lower lobe), left anterior coronary arteries, and basilar arteries were used for the isolation of vascular smooth muscles. After the arteries were dissected out under the dissecting microscope and cut into small pieces, they were incubated in 3 mL of the first digestion medium (Ca<sup>2+</sup>-free Tyrode's solution containing papain 1 mg/mL) for 20 minutes and in the second digestion medium (Ca<sup>2+</sup>-free Tyrode's solution containing collagenase 1 mg/mL) for 5 minutes. Both of the two digestion media contained serum albumin (2 mg/mL) and dithiothreitol (1 mg/mL). Arterial strips were then transferred to the high-K<sup>+</sup> and low-Cl<sup>-</sup> storage medium, and cells were isolated from the strips by gentle agitation with a fire-polished glass pipette. The isolated cells were kept in the storage medium at 4°C until use. The procedures have been described previously in further detail.<sup>13</sup>

For myogenic tone measurement, basilar arteries with 0.3- to 0.5-mm outside diameter were made into helical strips in a Krebs-Henseleit (KH) solution. To avoid the possible influences of endothelium-derived factors, the endothelium of the strip was removed by gentle rubbing of the endothelial surface.

### Solutions and Drugs

#### Solutions

Ca<sup>2+</sup>-free normal Tyrode's solution contained (in mmol/L) NaCl 143, KCl 5.4, MgCl<sub>2</sub> 1, glucose 5.5, and HEPES 5, with pH adjusted to 7.4 with NaOH. The high-K<sup>+</sup> and low-Cl<sup>-</sup> storage medium contained (in mmol/L) KCl 50, L-glutamate 50, KH<sub>2</sub>PO<sub>4</sub> 20, taurine 20, MgCl<sub>2</sub> 3, glucose 20, HEPES 10, and EGTA 0.5, with pH adjusted to 7.3 by KOH. The bath and pipette solutions for single-channel recording contained (in mmol/L) CsCl 140, HEPES 10, EGTA 2, and MgCl<sub>2</sub> 1, with pH adjusted to 7.4 with CsOH. Cs<sup>+</sup> was replaced with Na<sup>+</sup> or K<sup>+</sup> in the ion substitution experiment shown in Figure 3. KH solution contained (in mmol/L) NaCl 119, KCl 4.6, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.5, NaHCO<sub>3</sub> 25, and glucose 11. The KH solution was aerated continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. All of the above chemicals were purchased from Sigma.

#### Drugs

The stock solutions of the DIDS, DOG, CPA, thapsigargin, U73122, and U73343 were made up in dimethylsulfoxide and were then diluted in external solution to the final concentration at volumes of ≤0.01%. All the drugs were purchased from Sigma. Solutions containing DOG were sonicated for 10 minutes before use.

### Electrophysiological Recording

Electrophysiological recording was performed in the cell-attach and inside-out patch configurations with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Inc). Gigaseals were formed with Sylgard-coated thin-walled borosilicate capillaries (Clark Electromedical Instruments). All recordings were performed at room tem-

perature (22°C to 24°C). The recorded signal was filtered at 1 kHz and transferred to a computer using the Digidata 1200 interface (Axon Instruments) at a sampling rate of 5 KHz. Continuous single-channel currents were analyzed with the pCLAMP program (version 6.0.3, Axon Instruments).

### Measurement of Myogenic Tone

Basilar strips were held horizontally in a temperature-controlled, 5-mL organ chamber. The KH solution was maintained at 37°C and was aerated continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After 30 minutes of washing in KH solution, one end of the muscle strip was connected to a force-displacement transducer to monitor the muscle contraction under a resting force of 5 mN and equilibrated for 60 minutes. We measured the magnitude of the response to KH solution containing 50.5 mmol/L KCl (K<sup>+</sup> substitution for Na<sup>+</sup>, high-K<sup>+</sup> solution) at different passive lengths. At a length that produces maximal K<sup>+</sup> contractions, we consistently measured a passive force of 5 mN. We made efforts to prepare strips of identical size, and the weight of the tissue was consistent. During equilibration for 60 minutes, KH solution containing 50.5 mmol/L KCl was applied repeatedly until the sustained force became reproducible. After equilibration for 60 minutes in normal KH solution, strips were stretched passively to the optimal length by imposing a stretch of 50% of resting length. After strips were stretched, changes in tension were recorded continuously. All experiments were conducted in phentolamine- and timolol-treated strips to eliminate possible α-adrenoceptor and β-adrenoceptor responses to endogenously released norepinephrine.

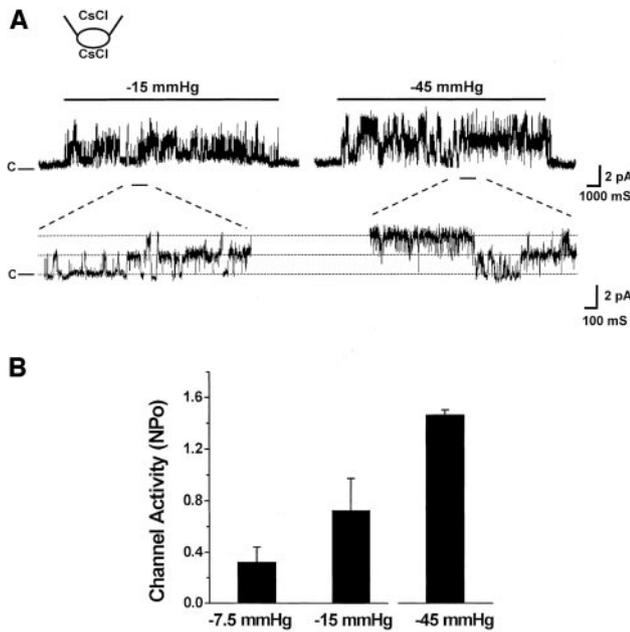
### Statistical Analysis

Channel activities were presented as NP<sub>o</sub>, where N is the number of channels and P<sub>o</sub> is the open probability. Results were expressed as mean ± SEM. Statistical significance was evaluated by paired Student's *t* test.

## Results

### Effects of Stretch on Single-Channel Currents

We recorded single-channel currents in pulmonary arterial smooth muscle cells using the patch-clamp technique. To exclude contamination by K<sup>+</sup> channels, K<sup>+</sup>-free CsCl (140 mmol/L) solutions were used both in the pipette and in the bath. Under this condition, little spontaneous channel opening was observed in cell-attached patches. When we applied negative pressure to the patch pipettes to identify mechanosensitive channels, channel opening was induced in ≈5% of patches (from >2000 trials). Figure 1A shows a representative result showing the effect of stretch on the channel activity when the pipette potential was held at -40 mV. We regarded the membrane potential of the cell as 0 mV in this condition (because the same solution was used in the bath and pipette), so the holding potential corresponded to 40 mV. The effect of stretch was immediate and reversible: channel opening was turned on and off by applying and releasing pressure, without a noticeable delay. When the magnitude of negative pressure was increased, channel opening became more frequent and the number of open channels increased. The amplitude of the single-channel current was not affected by negative pressure. The open probabilities obtained at various pressures in four patches were averaged and are shown in Figure 1B. NP<sub>o</sub> at 40 mV was 0.32 ± 0.12 at -7.5 mm Hg and increased to 0.73 ± 0.25 and 1.47 ± 0.04 at -15 mm Hg and -45 mm Hg, respectively (n=4). Channel activities could be maintained after patches were excised to the inside-out configuration, but the success rate was very



**Figure 1.** Effects of stretch on single-channel currents in pulmonary arterial smooth muscle cells. **A**, Current traces recorded in cell-attached patches at the pipette potential ( $V_{\text{pipette}}$ ) of  $-40$  mV with  $140$  mmol/L CsCl solutions in the bath and the pipette solutions. Bars above the current traces indicate the period of application of negative pressure ( $-15$  mmHg,  $-45$  mmHg) to the patch membrane. Note that channel activation by stretch is immediate and reversible. Current records during the period indicated with the bars below the current traces are expanded at bottom. The letter “c” represents the closed-channel level and dashed lines represent the open-channel level for up to 2 active channels. **B**, The average of open probability ( $\text{NP}_o$ ) obtained at various pressures in 4 patches.

low. Mechanosensitive channels with similar properties were also detected in smooth muscles isolated from coronary arteries and basilar arteries. In subsequent experiments, we used primarily cell-attached patches from pulmonary arterial smooth muscles and applied mild pressure (usually  $7.5$  to  $15$  mmHg) to activate mechanosensitive channels; this provided stable recordings for a period long enough to perform a series of experiments.

### Biophysical Properties of the Single-Channel Currents of Mechanosensitive Cation Channels

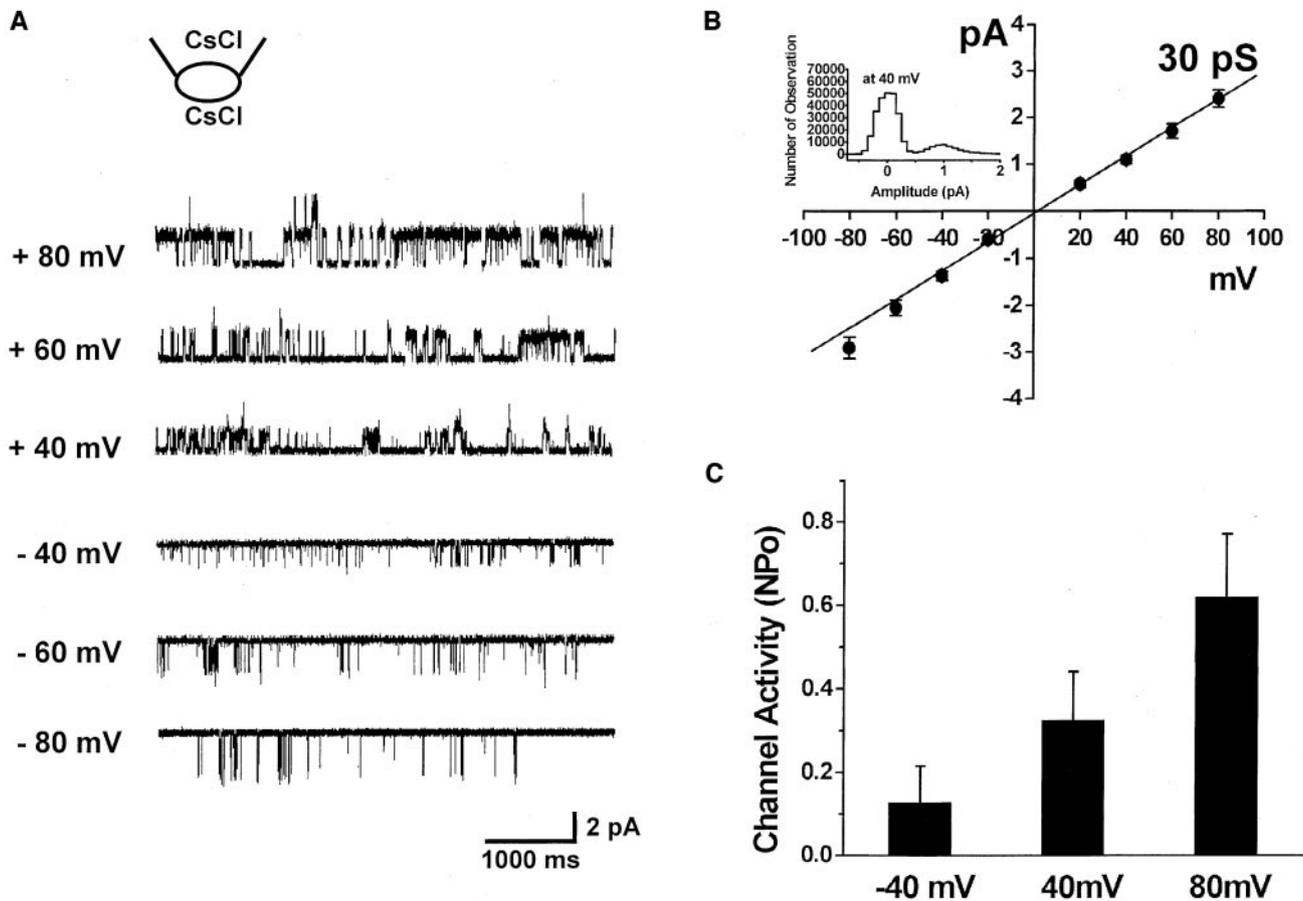
To determine the current-voltage ( $I$ - $V$ ) relationships of the mechanosensitive channels, the holding potential was changed from  $-80$  to  $+80$  mV in  $20$ -mV steps after the cell-attached configuration was formed, whereas single-channel activities were recorded in symmetrical  $140$  mmol/L  $\text{Cs}^+$  solution. Figure 2A shows representative single-channel current traces at various holding potentials. The amplitude of the single-channel current at each membrane potential was obtained from the amplitude histogram. The mean amplitudes averaged from 8 patches are plotted as a function of the membrane potential in Figure 2B. The  $I$ - $V$  relationship was almost linear, with a reversal potential of  $0$  mV, and the single-channel slope conductance was  $30$  pS. Figure 2C shows the voltage dependency of the  $\text{NP}_o$  of the mechanosensitive channel. The  $\text{NP}_o$  increased as the membrane

potential became more positive and decreased as it became more negative. This result suggests that whole-cell current associated with these mechanosensitive channels exhibits an outward-rectifying  $I$ - $V$  relationship. The dwell time histogram was obtained when only one channel opening was found. The mean open times of the channel at  $+40$  and  $-40$  mV were  $1.31 \pm 0.17$  and  $1.36 \pm 0.10$  ms ( $n=3$ ), respectively.

To determine the ionic selectivity of the mechanosensitive channel, single-channel activities were recorded in cell-attached patches with pipette and bath solutions of various ionic compositions. When external  $\text{Cl}^-$  in the pipette solution was replaced with aspartate, no significant change in the channel activity was observed (Figure 3A). The amplitude of inward current at  $-40$  mV ( $-0.96 \pm 0.09$  pA,  $n=5$ ) was not significantly different from that of outward current at  $+40$  mV ( $+0.97 \pm 0.07$  pA,  $n=5$ ), indicating that  $\text{Cl}^-$  does not contribute to ion current through the mechanosensitive channel. When  $\text{Cs}^+$  in the pipette solution was replaced by  $\text{NMDG}^+$ , outward currents recorded at positive potentials were unchanged, whereas no detectable inward currents were observed at membrane negative potentials (Figure 3B; same observation in two other cells). To test whether  $\text{Ca}^{2+}$  is permeable to this channel, the  $\text{CsCl}$  bath solution was replaced with isotonic  $\text{CaCl}_2$  solution when inside-out patch recording succeeded ( $n=3$ ). A representative trace is shown in Figure 3C. The amplitudes of outward currents recorded at  $+40$  mV before and after the replacement were  $1.05 \pm 0.03$  and  $1.20 \pm 0.13$  pA, respectively, indicating that the  $\text{Ca}^{2+}$  permeability is  $\approx 1.14$ -fold larger than the Cs permeability. To obtain the conductance of the channel for  $\text{K}^+$ , single-channel currents were recorded in cell-attached patches with pipette and bath solutions containing isotonic KCl solution. Opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels ( $\approx 280$  pS) were frequently observed even before the negative pressure was applied. In response to the application of negative pressure, opening of a channel with an amplitude similar to that observed in the  $\text{Cs}^+$  condition appeared (Figure 3D, top). When  $\text{Na}^+$  was used in both the pipette and bath solutions, the activity of the mechanosensitive channel was similarly observed, but the amplitude of single-channel current was slightly smaller than that observed with  $\text{K}^+$  (Figure 3D, bottom). The  $I$ - $V$  relationships of the single-channel currents in  $\text{K}^+$  and  $\text{Na}^+$  fitted well to straight lines, and the slope conductances were  $36$  and  $27$  pS, respectively (Figure 3D). These results indicate that the mechanosensitive channel activated by applying negative pressure to the membrane of arterial smooth muscle cells is an NSC channel. Hereafter, we refer to this channel as an  $\text{NSC}_{\text{MS}}$  channel.

### Pharmacological Characteristics of the $\text{NSC}_{\text{MS}}$ Channel

$\text{Gd}^{3+}$  has been used to specifically block mechanosensitive channels in a variety of tissues, including smooth muscle cells.<sup>14</sup> Thus, we investigated whether  $\text{Gd}^{3+}$  could block the  $\text{NSC}_{\text{MS}}$  channel recorded in arterial smooth muscle cells.  $\text{NSC}_{\text{MS}}$  channel activity was still observed with  $\text{Gd}^{3+}$  ( $30$   $\mu\text{mol/L}$ ) in the pipette solution (Figure 4A, left,  $n=3$ ). To test the effect of intracellular  $\text{Gd}^{3+}$ , single-channel currents of  $\text{NSC}_{\text{MS}}$  channels were recorded in the inside-out configura-



**Figure 2.** Current-voltage relationship and voltage dependency of mechanosensitive currents. A, Current traces recorded at patch potentials between  $-80$  and  $+80$  mV in cell-attached patches with  $140$  mmol/L CsCl solutions in the bath and the pipette solutions. A mild negative pressure ( $7.5$  mm Hg) was constantly applied to the patch pipette. The membrane potential of the cell was regarded as  $0$  mV, and the  $-V_{\text{pipette}}$  was regarded as the patch potential ( $V_m$ ; indicated by the numbers at left of current traces). B, Mean single-channel current-voltage relationship. Each point represents the average of single-channel amplitudes obtained from 8 cells. The points have been fitted by a straight line, and the slope gives an estimated mean conductance of  $30$  pS. Amplitude of the single-channel currents at each membrane potential was obtained from the amplitude histogram (inset). C, Voltage dependency of the  $NP_o$  of the mechanosensitive channel. Channel opening was increased by depolarization.

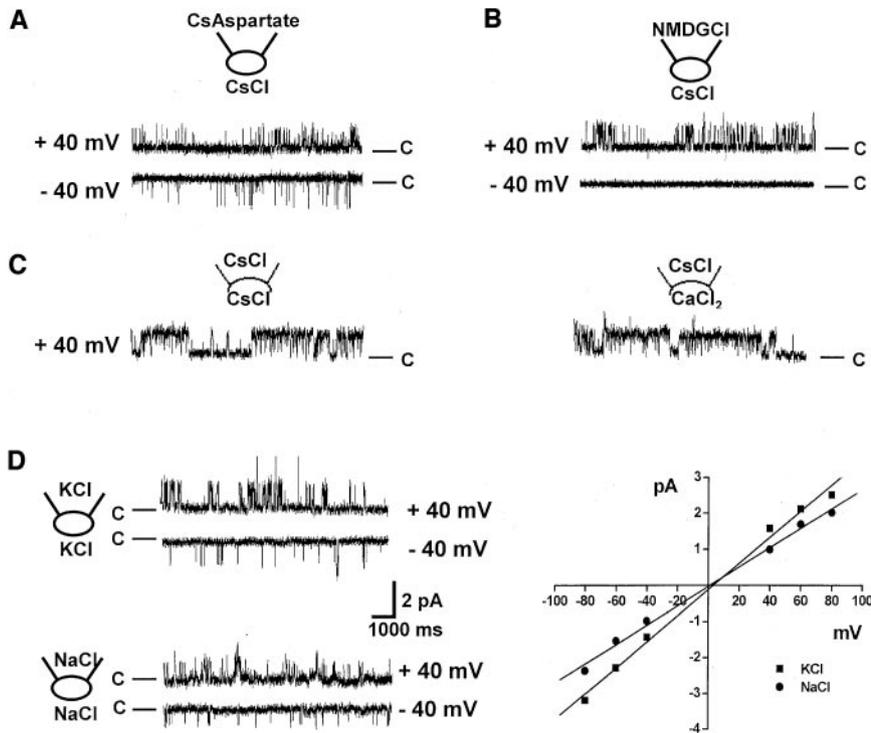
tion, and  $Gd^{3+}$  was applied to the bath solution. This blocked the  $NSC_{MS}$  channel almost completely (Figure 4A, right,  $n=4$ ). DIDS, generally known as a  $Cl^-$  channel blocker, has been shown to inhibit swelling-activated NSC currents in vascular smooth muscles.<sup>4</sup> Because DIDS was shown to block single-channel currents when it was applied to either side of the membrane,<sup>15–17</sup> the effect of DIDS on  $NSC_{MS}$  channels was tested while it was applied to the bath solution during channel recording in the cell-attached configuration. Interestingly, DIDS ( $300$   $\mu\text{mol/L}$ ), a known  $Cl^-$  channel blocker, also blocked the  $NSC_{MS}$  channel (Figure 4B, right,  $n=2$ ). We also tested the effect of other  $Cl^-$  channel blockers (niflumic acid,  $10$   $\mu\text{mol/L}$ ; NPPB,  $10$   $\mu\text{mol/L}$ ; and tamoxifen,  $10$   $\mu\text{mol/L}$ ), but all of them had no effect (data not shown). Figure 4C summarizes the effects of  $Gd^{3+}$  and DIDS on the activity ( $NP_o$ ) of the  $NSC_{MS}$  channel.

CPA and thapsigargin, which inhibit sarcoplasmic reticulum  $Ca^{2+}$ -ATPase, are shown to activate store-operated channels in various types of cells,<sup>18–20</sup> including vascular smooth muscles.<sup>21,22</sup> We studied the effect of CPA and thapsigargin recorded at  $+40$  mV while pipette pressure was maintained at

$-15$  mm Hg, and the change in channel activity by CPA was tested. Bath application of CPA ( $20$   $\mu\text{mol/L}$ ) induced a marked increase in channel opening within 1 to 2 minutes, and the activity was well maintained during the course of the cell-attached patch recordings (up to 10 minutes). However, the  $NSC_{MS}$  activity disappeared when the pressure was released, even in the continuous presence of CPA. The current traces shown on an expanded time scale in Figure 5A, bottom, confirm that the amplitude of the single-channel current was not changed by CPA. The effect of thapsigargin was tested, showing the marked increase in  $NSC_{MS}$  channel activity (Figure 5B). The increases in  $NP_o$  by CPA and thapsigargin ( $NP_o/NP_{o,\text{control}}$ ) are summarized in Figure 5C.

### **$NSC_{MS}$ Channel Is Activated by DAG and Inhibited by PLC Inhibitor**

In smooth muscle cells, TRPCs are suggested to encode NSC channels activated by PLC-linked receptors.<sup>9</sup> How PLC-linked receptors activate TRPCs is still unclear. A direct action of DAG is suggested for the activation of TRPC3 and TRPC6.<sup>23</sup> The role of PLC in  $NSC_{MS}$  activation was tested.



**Figure 3.** Ionic selectivity of the mechanosensitive cation channel. Current traces recorded in cell-attached patches (A, B, and D) and inside-out patches (C). Ionic compositions in the pipette and bath solutions were illustrated with current traces. A, Representative current traces recorded at +40 and -40 mV when the pipette solution contained isotonic Cs aspartate. B, Representative current traces recorded at +40 and -40 mV when the pipette solution contained isotonic NMDG-Cl. C, Representative current traces recorded at +40 mV before (left) and after (right) the bath solution was changed from 140 mmol/L Cs<sup>+</sup> to 100 mmol/L Ca<sup>2+</sup>. D, Current traces recorded with 140 mmol/L KCl (top left) and 140 mmol/L NaCl (bottom left) in the bath and pipette solutions. Pipette pressure was maintained at -7.5 mm Hg at each patch. Bottom, Single-channel current-voltage relationships obtained from each ionic conditions. The points have been fitted by straight lines, and the estimated conductances with K<sup>+</sup> and Na<sup>+</sup> pipette solutions are 36 and 27 pS, respectively.

The NSC<sub>MS</sub> channel activity was almost completely abolished by an inhibitor of PLC, U73122 (10  $\mu$ mol/L), whereas the biologically inactive analogue U73343 (10  $\mu$ mol/L) had no inhibitory effect (Figure 6A; n=3). In the presence of U73122, increasing the negative pressure failed to initiate channel opening. To test whether the NSC<sub>MS</sub> channel is regulated directly by DAG, we applied the membrane-permeable DAG analogue DOG (100  $\mu$ mol/L) to the bath solution during cell-attached patch recording (Figure 6A, second panel) or during inside-out patch recording (Figure 6A, first panel) of NSC<sub>MS</sub> current. In both cases, DOG increased the activity of the NSC<sub>MS</sub> channel significantly. Taken together, these results indicate that the activation of NSC<sub>MS</sub> channel by stretch requires PLC activity. Figure 6B summarizes the effects of DOG, U73122, and U73343 on NSC<sub>MS</sub> channel activity.

### Myogenic Tone Is Inhibited by PLC Inhibitor

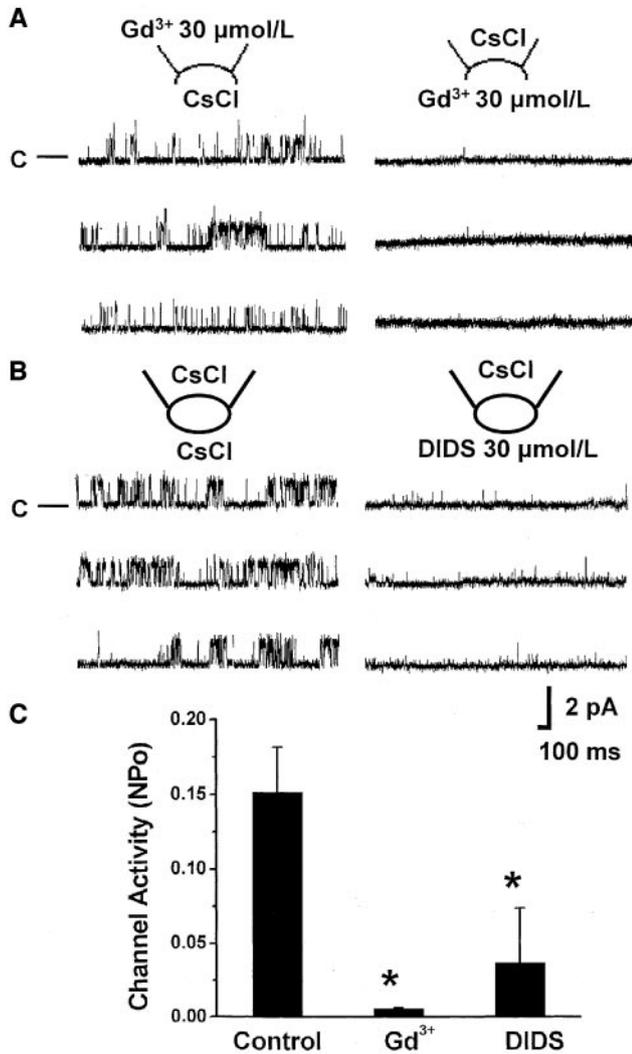
To test the possibility that the NSC<sub>MS</sub> channel is involved in the development of myogenic tone, the effect of the PLC inhibitor on myogenic tone was investigated in isolated basilar arteries. As shown in Figure 7A, stretching evoked a rapid rise in passive tension, followed by a passive stress relaxation response, as was observed in other studies.<sup>1,24</sup> The secondary increase in tension was regarded as a myogenic contraction. The magnitude of this myogenic contraction was  $32.08 \pm 8.02\%$  (n=13) of the tension produced by 50.5 mmol/L KCl. When stretch was applied again to the same tissue after pretreatment with U73122, the myogenic contraction was significantly inhibited and was reduced to  $21.63 \pm 5.29\%$  of the control. In contrast, U73343, the inactive analogue for PLC inhibition, did not affect myogenic contraction (Figure 7B). The effects of Gd<sup>3+</sup> (30  $\mu$ mol/L) and DIDS (300  $\mu$ mol/L) were examined in the same way, and the

results were summarized in Figure 7C. Gd<sup>3+</sup> did not affect the myogenic tone when applied in the bath solutions ( $105.7 \pm 16.4\%$  of control, n=14), whereas DIDS significantly inhibited the myogenic tone ( $49.3 \pm 7.7\%$  of the control, n=12).

### Discussion

In the present study, we identified that the NSC channels in arterial smooth muscle cells with a conductance of 30 pS are mechanosensitive channels. In addition to mechanosensitivity, the channels were found to be sensitive to DAG and CPA and were inhibited by a PLC inhibitor. The pharmacological characteristics of the channel were rather unique, in that they were inhibited by DIDS and intracellular Gd<sup>3+</sup> but not by extracellular Gd<sup>3+</sup>. Furthermore, the effects of PLC inhibitor, DIDS, and extracellular Gd<sup>3+</sup> on the myogenic tone were consistent with the effects of these agents on NSC<sub>MS</sub> channels. To the best of our knowledge, this article is the first to provide comprehensive information about the properties of mechanosensitive channels in native arterial smooth muscle cells at the single-channel level.

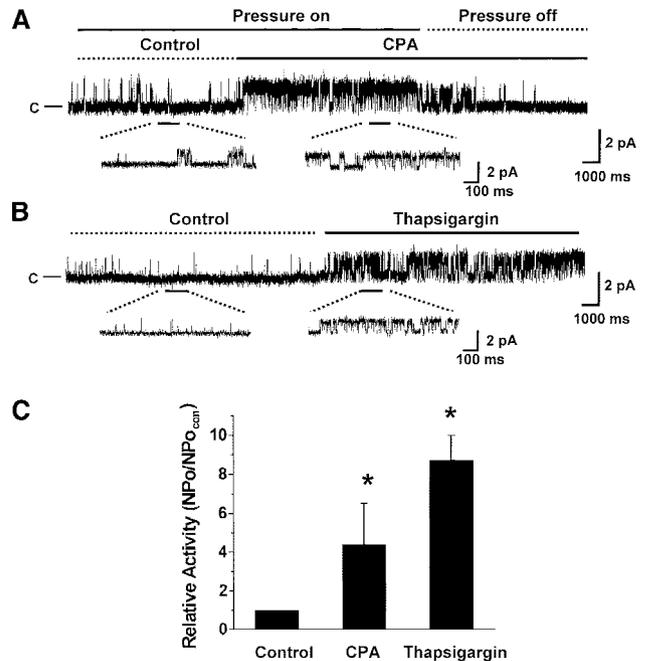
Only a few studies have described mechanosensitive currents in vascular smooth muscle cells at the single-channel level. Davis et al<sup>25</sup> demonstrated in porcine coronary arterial smooth muscles that patch pipette suction activated NSC channels that were permeable to K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> with conductances of 36, 23, and 11 pS, respectively. These conductance values are similar to the values reported here (Figure 3: 36 pS for K<sup>+</sup> and 27 pS for Na<sup>+</sup>). The activation pattern in response to patch pipette suction is also very similar between the two studies (compare Figure 1 in both articles). We found that the channel activities with similar properties were recorded in coronary, pulmonary, and basilar arteries of the rabbit. It appears that the NSC<sub>MS</sub> channels



**Figure 4.** Pharmacological characteristics of the mechanosensitive channel. A, Current traces recorded at +40 mV in inside-out patches in the presence of 30  $\mu\text{mol/L}$   $\text{Gd}^{3+}$  in the pipette (left) and the bath (right). There were 140 mmol/L CsCl solutions in the bath and pipette solutions. B, Current traces recorded at +40 mV in cell-attached patches before (left) and after (right) the application of 300  $\mu\text{mol/L}$  DIDS. There were 140 mmol/L CsCl solutions in the bath and pipette solutions. Patch configurations are illustrated above the current traces. Pipette pressure was maintained constant at  $-7.5$  mm Hg. C, Graph showing effects of  $\text{Gd}^{3+}$  and DIDS on the  $\text{NP}_o$ . Data are presented as mean  $\pm$  SEM. \* $P < 0.01$  vs control, determined by paired  $t$  test ( $n = 4$  for  $\text{Gd}^{3+}$  and  $n = 2$  for DIDS).

observed in the present study are distributed among different species and different arteries.

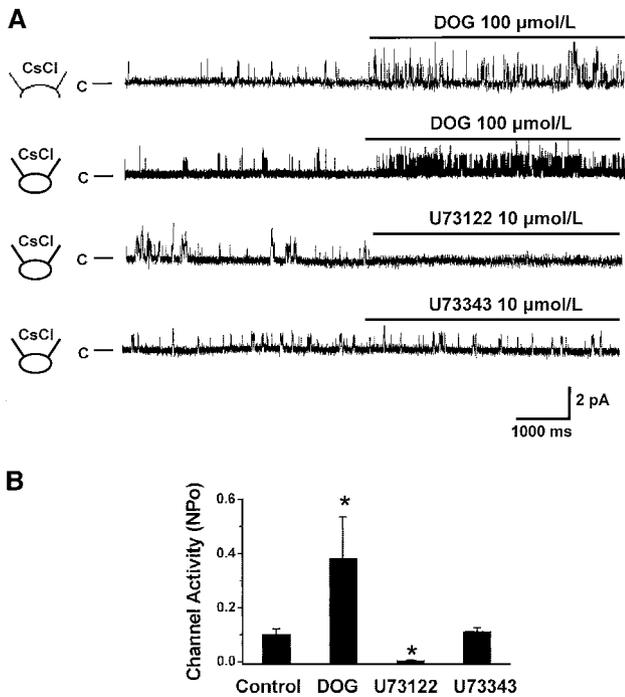
We have shown that  $\text{NSC}_{\text{MS}}$  channels are greatly potentiated by DAG and inhibited by the PLC inhibitor U73122, suggesting that  $\text{NSC}_{\text{MS}}$  channels in native arterial cells are related with PLC-linked cation channels. In vascular smooth muscle cells, TRPC6 was proposed as a candidate channel involved in receptor-stimulated cation currents.<sup>9,26</sup> Furthermore, antisense to TRPC6 caused the loss of myogenic tone and inhibition of cation current, suggesting that TRPC6 is a candidate channel involved in mechanosensitive cation currents.<sup>8</sup> TRPC6 has also been presented as an NSC channel



**Figure 5.** Effect of CPA and thapsigargin on the mechanosensitive cation channel. A, Current traces recorded in a cell-attached patch with 140 mmol/L CsCl solutions in the bath and pipette during application of CPA. Bars above the current traces indicate the period of application of negative pressure (15 mm Hg) and CPA.  $V_m = +40$  mV. The letter “c” represents the closed-channel level. Recordings during the period indicated with short bars are shown in an expanded time scale at bottom. B, Current traces recorded in a cell-attached patch with 140 mmol/L CsCl solutions in the bath and pipette during the application of thapsigargin. The sequence of applying pressure and thapsigargin is indicated with bars above the typical current traces obtained in each experimental condition.  $V_m = +40$  mV. C, Graph showing the effects of CPA and thapsigargin on relative channel activity ( $\text{NP}_o/\text{NP}_{o,\text{control}}$ ). \* $P < 0.001$  vs control, determined by paired  $t$  test ( $n = 6$  for CPA and  $n = 3$  for thapsigargin).

activated by DAG, independently of store depletion.<sup>23</sup> Single-channel properties of the TRPC6 current expressed in CHO-K1 cells showed a linear  $I$ - $V$  relationship, with a conductance of 35 pS in symmetrical  $\text{Cs}^+$  solution,<sup>23</sup> which properties are similar to those of the  $\text{NSC}_{\text{MS}}$  channel investigated in the present study. Taken together, TRPC6 is one of the most probable molecular components of the mechanosensitive NSC channel in native arterial smooth muscle cells.

We showed that CPA and thapsigargin potentiate  $\text{NSC}_{\text{MS}}$  channels greatly (Figure 5). CPA and thapsigargin are specific inhibitors of sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  ATPase, and their application results in a relatively rapid passive depletion of intracellular  $\text{Ca}^{2+}$  store.<sup>20</sup> Activation of store-operated channels by CPA and thapsigargin have been shown in various types of cells,<sup>18–20</sup> including vascular smooth muscles.<sup>21,22</sup> Whether the effect of CPA and thapsigargin on  $\text{NSC}_{\text{MS}}$  channels represents the relationship between  $\text{NSC}_{\text{MS}}$  channels and store depletion is not yet clear and needs to be elucidated in future studies. Considering that TRPC3 and TRPC1 have been proposed as subunits of store-operated channels<sup>27,28</sup> and can also be directly activated by DAG,<sup>23,29</sup> involvement of TRPC3 and TRPC1 in forming  $\text{NSC}_{\text{MS}}$

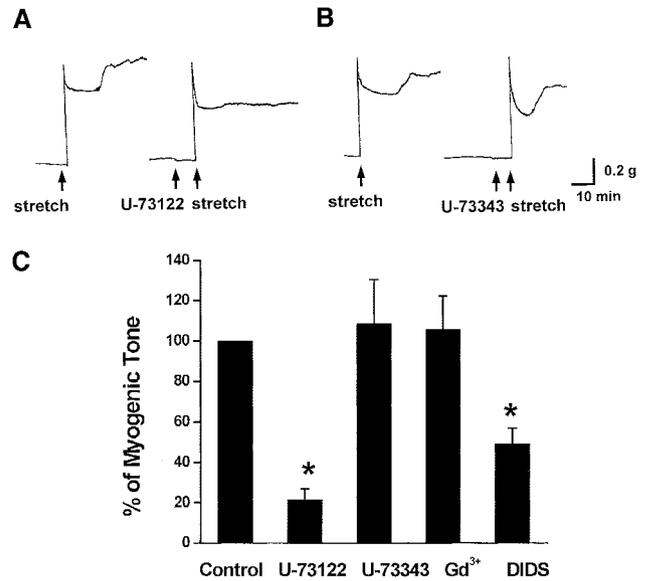


**Figure 6.** Effect of PLC inhibitor on the mechanosensitive cation channel. A, Current traces recorded with symmetric 140 mmol/L CsCl solutions during bath application of 100  $\mu\text{mol/L}$  DOG (first and second panels), 10  $\mu\text{mol/L}$  U73122 (third panel), and 10  $\mu\text{mol/L}$  U73343 (bottom panel). Configurations of patch recordings are illustrated with current traces. Bars above the current traces indicate the period of application of each chemical to the bath solutions.  $V_m = +40$  mV. Pipette pressure was maintained constant at  $-7.5$  mm Hg. B, Graph showing effects of DOG, U73122, and U73343 on  $\text{NP}_o$ . \* $P < 0.01$  vs control, determined by paired  $t$  test ( $n = 6$  for DOG,  $n = 3$  for U73122 and  $n = 3$  for U73343).

channels should be considered. Single-channel conductances of TRPC3 and TRPC1 were reported to be 66 pS<sup>30</sup> and 20 pS,<sup>19</sup> respectively. These values can be considered to be comparable to the single-channel conductance of the  $\text{NSC}_{\text{MS}}$  channel.

Even though the characteristics of  $\text{NSC}_{\text{MS}}$  channels appeared to share common properties with TRPCs, they do not correspond to those of any type of TRPCs reported previously.  $\text{NSC}_{\text{MS}}$  channels are sensitive to DAG or CPA, but either of them was essential in channel activity. Channel activity disappeared even in the presence of DAG or CPA when negative pressure was released, indicating that mechanical stimulation is crucial for the channel activation. However, mammalian TRPCs with such mechanosensitivity have not yet been reported. This may imply that the  $\text{NSC}_{\text{MS}}$  channel is formed by channel proteins that are not yet identified. Another possibility is that a channel with a new property is formed by a coassembly of different subunits of TRPCs. This is supported by the observation that hetero-oligomerization of different TRPC species resulted in the formation of a hetero-multimeric cation channel with distinct biophysical and regulatory properties,<sup>29</sup> and this possibility should be tested in future studies.

Whether the  $\text{NSC}_{\text{MS}}$  channel reported here is indeed the channel involved in the development of myogenic tone still



**Figure 7.** Effect of PLC inhibitor on myogenic tone of basilar arteries. Muscle tension curves recorded in response to the application of stretch in the absence (left) and in presence (right) of U73122 (A) and U73343 (B). The secondary increase in tension after the initial relaxation response on stretch was regarded as a myogenic contraction. Arrows indicate time points of application of stretch or drugs. C, Relative magnitude of myogenic tone in the presence of U73122, U73343,  $\text{Gd}^{3+}$ , and DIDS. Data are presented as mean  $\pm$  SEM. \* $P < 0.01$  vs control, determined by paired  $t$  test ( $n = 13$  for U73122,  $n = 5$  for U73343,  $n = 14$  for  $\text{Gd}^{3+}$ , and  $n = 12$  for DIDS).

needs further investigation. This hypothesis is supported by the result showing that U73122 inhibits both the  $\text{NSC}_{\text{MS}}$  channel and stretch-induced contraction. Inhibition of myogenic tone by U73122 was also reported recently by another group.<sup>12</sup> The abolition of myogenic tone in the preparation treated with TRPC6 antisense<sup>8</sup> and the resemblance between the  $\text{NSC}_{\text{MS}}$  channel and TRPC6 discussed above may support this hypothesis. The possibility of the involvement of capacitive  $\text{Ca}^{2+}$  entry in myogenic contraction was recently suggested,<sup>31</sup> but experimental evidence is lacking at present. Further investigations should clarify the molecular identity of  $\text{NSC}_{\text{MS}}$  channels in native arterial smooth muscle cells.

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