A protein kinase G-sensitive channel mediates flow-induced Ca^{2+} entry into vascular endothelial cells

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ABSTRACT **The hemodynamic force generated by blood flow is considered to be the physiologically most important stimulus for the release of nitric oxide (NO) and prostacyclin (PGI_2) from vascular endothelial cells (1). NO and PGI_2 then act on the underlying smooth muscle cells, causing vasodilation and thus lowering blood pressure (2, 3). One critical early event occurring in this flow-induced regulation of vascular tone is that blood flow induces Ca^{2+} entry into vascular endothelial cells, which in turn leads to the formation of NO (4, 5). Here we report a mechanosensitive Ca^{2+}-permeable channel in vascular endothelial cells. The activity of the channel was inhibited by 8-Br-cGMP, a membrane-permeant activator of protein kinase G (PKG), in cell-attached membrane patches. The inhibition could be reversed by PKG inhibitor KT5823 or H-8. A direct application of active PKG in inside-out patches blocked the channel activity. Gd^{3+}, Ni^{2+}, or SK&F-96365 also inhibited the channel activity. A study of fluorescent Ca^{2+} entry revealed a striking pharmacological similarity between the Ca^{2+} entry elicited by flow and the mechanosensitive Ca^{2+}-permeable channel we identified, suggesting that this channel is the primary pathway mediating flow-induced Ca^{2+} entry into vascular endothelial cells.—Yao, X., Kwan, H. Y., Chan, F. L., Chan, N. W. K., Huang, Y. A protein kinase G-sensitive channel mediates flow-induced Ca^{2+} entry into vascular endothelial cells. *FASEB J.* 14, 932–938 (2000)**

Key Words: blood flow · shear stress · nonselective cation channel · endothelium

VASCULAR ENDOTHELIAL CELLS are not merely a selective permeability barrier between blood and underlying tissues but actively play an important role in maintaining homeostasis of circulation and regulating vascular tone. This is achieved mainly by the release of autocoids, particularly nitric oxide (NO) and prostacyclin (PGI_2), which subsequently diffuse to the underlying smooth muscle cells and regulate the vascular tone (2, 3). Deficiencies in autocoids have been implicated in serious cardiovascular diseases including essential hypertension, coronary diseases, and chronic heart failure (4).

The majority of the autacoid-related studies have been confined to agonist (acetylcholine, bradykinin, histamine, ATP, etc.) -induced release of NO and PGI_2. However, there is little evidence as to the physiological significance of the agonist-induced NO and PGI_2 release *in vivo* (6, 7). On the other hand, shear stress generated by blood flow is considered to be the most important physiological factor triggering the release of NO and PGI_2 from endothelium (1, 6). Shear stress induces a transmembrane influx of extracellular Ca^{2+}, leading to the activation of a Ca^{2+}-sensitive constitutive form of nitric oxide synthase, which catalyzes the production of NO (4, 5). The elevation of $[Ca^{2+}]_i$ also stimulates the production and release of PGI_2 (8). However, the mechanism and regulation of flow-induced Ca^{2+} entry, a key early event in the flow-induced regulation of vascular tone, remain obscure. Some reports suggested that the shear stress associated with blood flow might induce changes in shape and conformation of endothelial cytoskeleton together with extracellular matrix (6). These changes were speculated to indirectly open K^+ channels, causing subsequent membrane hyperpolarization and Ca^{2+} entry (6, 9, 10). A mechanosensitive Ca^{2+}-permeable channel has also been detected in vascular endothelial cells (11). However, very little has been known about the physiological importance of these mechanisms in flow-induced Ca^{2+} influx and virtually nothing is known about the intracellular regulation of flow-induced Ca^{2+} entry.

In the present study, we identified for the first time a mechanosensitive Ca^{2+}-permeable cation channel that was sensitive to the regulation by protein kinase G. Inhibition of this channel abolished the rise of $[Ca^{2+}]_i$ elicited by flow, suggesting that this channel is the main pathway mediating flow-induced Ca^{2+} entry into vascular endothelial cells.

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MATERIALS AND METHODS

Materials

Active protein kinase G (PKG, a bovine recombinant isoform 1α), 8-Br-cGMP, cGMP, SK&F-96365, KT5823, and H-8 were obtained from Calbiochem (San Diego, Calif.). ECV304, an endothelial cell line derived from human umbilical veins, was purchased from ATCC (Rockville, Md.). Primary antibody against von Willebrand factor and FITC-labeled secondary antibody were from DAKO (Glostrup, Denmark). Culture media RPMI, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were supplied by Gibco-BRL (Grand Island, N.Y., Md.). Fluo3/acetoxymethyl ester (Fluo3/AM) and Pluronic F127 were obtained from Molecular Probes (Eugene, Oreg.). ATP, EGTA, EDTA, trypsin, $NiCl_2$, $GdCl_3$, flufenamic acid, DCDPC, and collagenase were purchased from Sigma (St. Louis, Mo.).

Preparation of aortic endothelial cells

Primary aortic endothelial cells were isolated from rat aorta and cultured as described elsewhere (12). Briefly, male Sprague-Dawley rats were decapitated. The thoracic aorta was removed and washed twice with sterile PBS. Fat and connective tissues were then trimmed off. The aorta was cut into small sheets and treated with 0.2% collagenase in PBS for 15 min at 37°C. The suspension after the enzyme digestion was centrifuged at 800 *g* for 5 min. The cells were resuspended in 90% RPMI and 10% FBS, then kept in an incubator at 37°C.

Cell culture

Both the isolated aortic endothelial cells and ECV304 cells were cultured in 90% RPMI 1640 and 10% FBS. The cells were incubated in T-25 tissue culture flasks in air with 5% CO_2 atmosphere at 37°C. Confluent cell monolayers were passaged using 0.25% trypsin containing 2.5 mM EDTA. For primarily isolated aortic endothelial cells, only cells from the first two passages were used for experiments.

Immunofluorescence

The identity of the primary cultured rat aortic endothelial cells was confirmed by immunostaining using an antibody against von Willebrand factor. The cultured cells were fixed in 4% formaldehyde in PBS for 2 h and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. The cells were stained with a polyclonal antibody against human von Willebrand factor (diluted 1:400 in PBS with 1% BSA) overnight at 4°C. The slides were then washed in PBS and incubated with FITC-labeled goat anti-rabbit IgG for 1 h. For controls, some slides were incubated in 1% BSA in PBS without the primary antibody. Some slides were counterstained with 0.00003% DAPI in 0.9% NaCl. After washing in PBS, the slides were mounted in glycerol and examined under a fluorescence microscope. The results showed that >98% of the cells were positively stained, indicating they were of endothelial origin.

Resting membrane potential

In the preliminary study, the resting membrane potentials were estimated in whole-cell configuration with the pipette solution containing KCl saline and the bath solution containing NaCl saline. The resting membrane potentials were -58 ± 4.5 mV (n=10) for primary aortic endothelial cells and -55 ± 5.6 mV (n=8) for ECV304 cells.

Single-channel recording

Single-channel currents were measured by standard methods (13, 14) with an EPC-9 patch clamp amplifier. The signal was sampled at 5.0 kHz and filtered at 1 kHz for data analysis. Data were analyzed by TAC and TAC-fit software. The probability of the channel being open (P_o) was estimated from the total time spent in open state divided by total time of the record. For cell-attached patches, a time period of 2 min was allowed for diffusion of chemicals such as 8-Br-cGMP and KT5823 into intracellular space. Thereafter, a continuous recording of 60 s was used to estimate P_o values. The negative pressure (suction) was applied to the patch pipette using a syringe. The pressure was monitored by an H_2O manometer.

Active PKG used was a bovine recombinant isoform 1α. Vehicle contained 10 μM cGMP, 10 μM ATP, and 7 mM $MgCl_2$, which were required for PKG activation (15). Vehicle was added prior to addition of active PKG. Ca^{2+} saline contained in mM: 100 $CaCl_2$, 10 HEPES, pH 7.4; Na^+-glutamate solution contained in mM: 142.5 Na^+-glutamate, 1 EGTA, 10 HEPES, pH 7.4; NaCl saline contained in mM: 140 NaCl, 2.5 KCl, 1 EGTA, 10 HEPES, pH 7.4; KCl saline contained in mM: 140 KCl, 2.5 NaCl, 1 $CaCl_2$, 10 HEPES, pH 7.4. The results were presented as mean ± SE (n). The significance of difference was tested by paired Student's t test. $P<0.05$ was regarded as statistically significant. All experiments were conducted at room temperature.

$[Ca^{2+}]_i$ measurement

Cells were prepared and loaded with fluorescence dye Fluo3/AM as described elsewhere (16). Briefly, the cells were grown in culture medium on circular discs overnight at 37°C. For loading of Fluo3/AM, cells were incubated for 1 h in the dark at room temperature with 10 μM membrane-permeant Fluo3/AM and 0.02% Pluronic F127 in normal physiological saline solution (N-PSS) containing 140 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES, pH 7.4. Flow was initiated by pumping N-PSS with or without 8-Br-cGMP, KT5823, H-8, Gd^{3+}, SK&F-96365, or Ni^{2+} to a specially designed chamber that resembled the one described in ref 4. We used a flow rate with shear stress of ~5 dyne/cm^2. A higher shear rate frequently led to the detachment of the cells from culture discs. The cells were treated with or without 8-Br-cGMP, KT5823, H-8, Gd^{3+}, SK&F-96365, or Ni^{2+} for 2 min before the start of the laminar flow. For the flow experiments in Ca^{2+}-free conditions, the cells were washed and flow was initiated in a Ca^{2+}-free physiological saline solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 2 mM EGTA, 5 mM HEPES, pH 7.4. Fluorescence signal was monitored and recorded by an MRC-1000 Laser Scanning Confocal Imaging System with MRC-1000 software. Data analysis was performed with Confocal Assistant and Metaflour.

RESULTS

A mechanosensitive Ca^{2+}-permeable channel sensitive to inhibition by PKG

With the use of a patch clamp technique, we identified a mechanosensitive Ca^{2+}-permeable cation

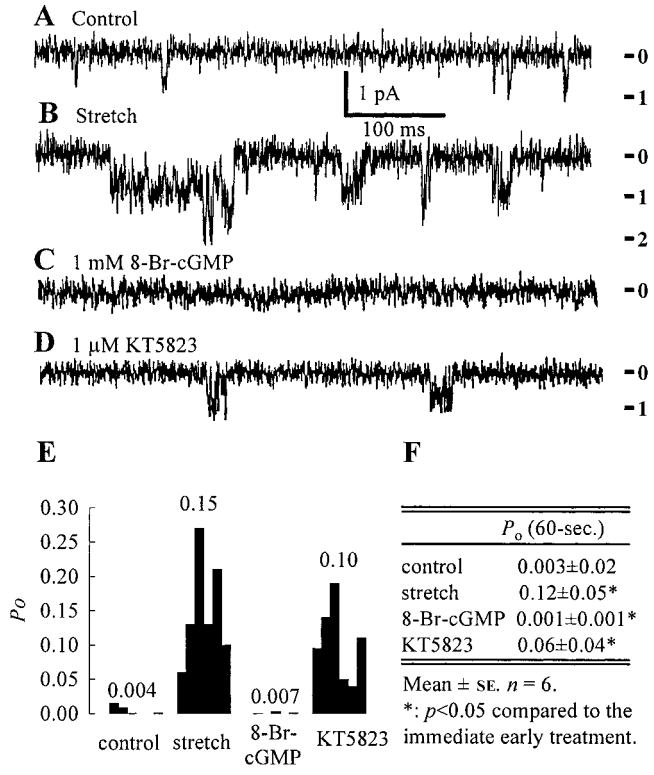


Figure 1. Effect of 8-Br-cGMP and KT5823 on the stretch-activated channel in cell-attached patch(es) of aortic endothelial cell(s). The patch membrane potential was displaced −40 mV from its resting potential. The pipette/bath contained Ca^{2+} saline/Na^{+}-glutamate solution. Panels *A–D* are single-channel current traces. *A)* Control. *B)* Suction (membrane stretch). *C)* 1 mM 8-Br-cGMP in the presence of suction. *D)* 1 μM KT5823 in the presence of 1 mM 8-Br-cGMP and suction. *E)* P_o values of the above patch in 10 s intervals for a 60 s period. The average P_o for a 60 s period is displayed on the top. *F)* P_o values calculated from different patches of aortic endothelial cells for a 60 s period. Each later treatment was placed in the presence of the previous treatment(s).

channel in isolated aortic endothelial cells and in cultured human umbilical vein endothelial cell line ECV304. **Figure 1*A–D*** shows the pattern of single-channel activity recorded from a typical cell-attached patch with Ca^{2+} saline in the pipette and Na^{+}-glutamate solution in the bath. The patch membrane potential was −40 mV displaced from its resting potential. A very low level of channel activity could be observed immediately after formation of a giga-ohm seal. The channel activity increased drastically when a negative pressure (suction) of 20 mmHg was applied into the pipette (Fig. 1*B, E*), suggesting that the channel was sensitive to mechanostress or membrane deformation. The channel activity diminished 2 min after addition of 1 mM 8-Br-cGMP (membrane-permeant) to the bath (Fig. 1*C, E*). For this patch, P_o values in 10 s intervals for 60 s period under different treatments are shown in Fig. 1*E*. Mean P_o values estimated from different inside-out patches are shown in Fig. 1*F*. 8-Br-cGMP decreased the P_o from 0.12 ± 0.05 to 0.001 ± 0.001 (n=6, P<0.05) for aortic endothelial cells (Fig. 1*F*) and from 0.09 ± 0.04 to 0.003 ± 0.002 (n=5, P<0.05) for ECV304 cells. 8-Br-cAMP at 1 mM had no effect on the channel activity (n=4).

In some patches, after the channel activation by suction, the pipettes were swiftly removed from the cells to form inside-out patches. A negative pressure of 20 mmHg was maintained throughout this procedure while the patch membrane potential was held at −80 mV. The inward single-channel currents persisted when the cytoplasmic side of the membrane patches was exposed to Na^{+}-glutamate solution while the pipette contained Ca^{2+} saline (100 mM $CaCl_2$). This result suggested that the observed inward channel current must be Ca^{2+} currents, since Ca^{2+} in the pipette was the only candidate that could generate inward currents under this condition. Unlike in cell-attached patches, 1 mM 8-Br-cGMP had no effect on the channel activity in inside-out membrane patches, suggesting that 8-Br-cGMP may not modulate the channel directly and that some cytoplasmic factor(s) may be needed for its action on the mechanosensitive channel. Since cGMP is an intracellular second messenger that activates PKG, we next examined a possible involvement of PKG. A potent and specific PKG inhibitor, KT5823 (1 μM) (17), was applied to the cell-attached patches, and it reversed the channel inhibition caused by 8-Br-cGMP with P_o increased from 0.001 ± 0.001 to 0.06 ± 0.04 (n=6, P<0.05) for aortic endothelial cells (Fig. 1*F*) and from 0.003 ± 0.002 to 0.10 ± 0.01 (n=3, P<0.05) for ECV304 cells. Another PKG inhibitor, H-8 (10 μM), also increased P_o from 0.002 ± 0.002 to 0.10 ± 0.05 (n=3, P<0.05) for aortic endothelial cells and from 0.003 ± 0.002 to 0.09 ± 0.03 (n=3, P<0.05) in ECV304 cells.

To further determine the role of PKG, we applied an active form of PKG directly to the cytoplasmic side of inside-out patches. **Figure 2*A–E*** illustrates a representative experiment showing current traces together with P_o values in 10 s intervals for a 60 s period with different treatments. Activation of PKG requires the presence of Mg^{2+}, cGMP, and ATP (15). Vehicle containing Mg^{2+} (7 mM), cGMP (10 μM), and ATP (10 μM) had no effect on the channel activity. Subsequent application of active exogenous PKG (10 nM) reduced P_o by 94 ± 6% (n=4, P<0.05) in the patches obtained from the aortic endothelial cells (Fig. 2*F*) and by 96 ± 4% (n=4, P<0.05) in the patches obtained from ECV304 cells. The inhibition apparently required cGMP and ATP, since PKG was ineffective when they were absent in the bath solution. The inhibitory effect of PKG was reversed by 1 μM KT5823, with P_o increased from 0.005 ± 0.005 to 0.09 ± 0.03 (n=4, P<0.05) for aortic endothelial cells (Fig. 2*F*) and from 0.004 ± 0.002 to 0.16 ± 0.03 (n=3, P<0.05) for ECV304 cells. H-8 at 10 μM also

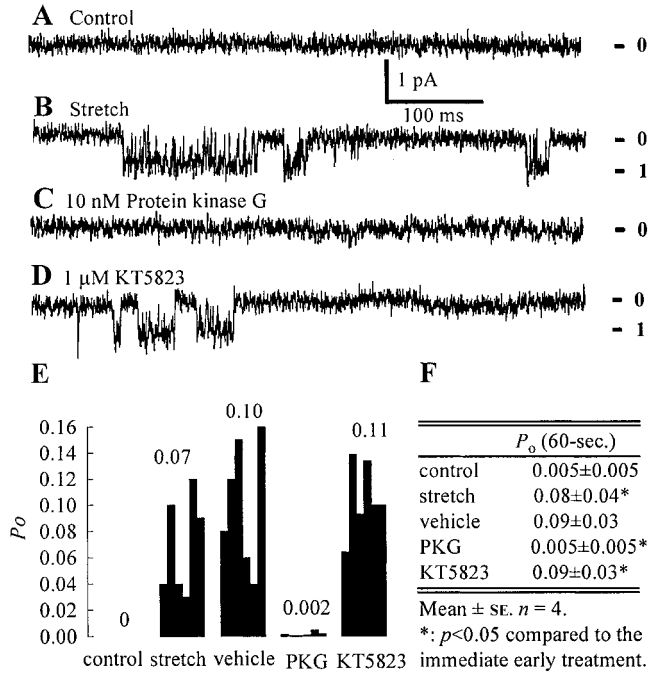


Figure 2. . Effect of protein kinase G and KT5823 on the stretch-activated channel in inside-out patch(es) of aortic endothelial cell(s). The patch membrane potential was held at −80 mV. The pipette/bath contained Ca^{2+} saline/Na^+-glutamate solution. Panels *A–D* are current traces. *A*) Control. *B*) Suction (membrane stretch) plus vehicle (vehicle had no significant effect on channel activity). *C*) 10 nM protein kinase G in the presence of suction and vehicle. *D*) 1 μM KT5823 in the presence of 10 nM PKG, suction, and vehicle. *E*) P_o values of the above patch in 10 s intervals for a 60 s period. The average P_o for a 60 s period is displayed on the top. *F*) P_o values calculated from different patches of aortic endothelial cells for a 60 s period. Each later treatment was placed in the presence of the previous treatment(s).

increased P_o from 0.004 ± 0.003 to 0.08 ± 0.05 (n=3, P<0.05) for aortic endothelial cells and from 0.005 ± 0.004 to 0.13 ± 0.03 (n=3, P<0.05) in ECV304 cells. Taken together, these results indicate that a mechanosensitive Ca^{2+}-permeable channel is present in both arterial and venous endothelial cells and that the activity of this channel is inhibited by PKG via a phosphorylation-dependent mechanism.

Relative permeability of the channel to different cations

Relative permeabilities of the mechanosensitive channel to Na^+, K^+, and Ca^{2+} were estimated. The channel was identified by its response to suction and its sensitivity to 8-Br-cGMP in cell-attached configuration. After achieving inside-out membrane patches, we constructed the single-channel I-V relationships (**Fig. 3**) and obtained the averaged slope conductance (g) and reversal potential (E_{Rev}). The channel appeared to be an inward rectifier (Fig. 3). In the pipette/bath solution of NaCl/KCl saline, g is calculated to be 32 ± 3 pS and E_{Rev} is 0 ± 0.1 mV (n=6). In the pipette/bath of Ca^{2+} saline/Na^+-glutamate solution, g is 9 ± 2 pS and E_{Rev} is 20 ± 4 mV (n=4). Calculation of relative permeabilities from reversal potential measurements with constant field equation (18, 19) yielded the permeability ratios of PCa:PNa:PK = 5:1:1. There was no measurable change either in conductance or in reversal potential when Cl^- in the bath was replaced by glutamate, suggesting that this channel was not permeable to Cl^-.

Effects of Ni^{2+}, Gd^{3+}, and SK&F-96365

The sensitivity of the channel activity to putative channel inhibitors was examined in outside-out membrane patches with Na^+-glutamate solution in the pipette and Ca^{2+} saline in the bath. The inhibitors were applied to the bath; therefore, they were in direct contact with the extracellular side of the membrane patches. Gd^{3+} (20 μM), an inhibitor of mechanosensitive channels (20, 21), inhibited the stretch-activated channel activity in aortic endothelial cells by reducing P_o from 0.08 ± 0.01 to 0.01 ± 0.01 (n=3, P<0.05). SK&F-96365 (50 μM), an inhibitor for receptor-mediated Ca^{2+} entry (22), decreased P_o from 0.34 ± 0.13 to 0.004 ± 0.004 (n=3, P<0.05). Ni^{2+} (3 mM), a blocker for Ca^{2+} entry that competes for Ca^{2+} binding site (23), almost completely suppressed the channel activity, with P_o reduced from 0.22 ± 0.04 to 0.001 ± 0.001 (n=3, P<0.05). A comparable inhibition was also observed in ECV304 cells. All inhibitions were reversible. Two other putative cation channel blockers, flufenamic acid (100 μM) (24) and DCDPC (10 μM) (24), had no effect on the channel activity (n=3).

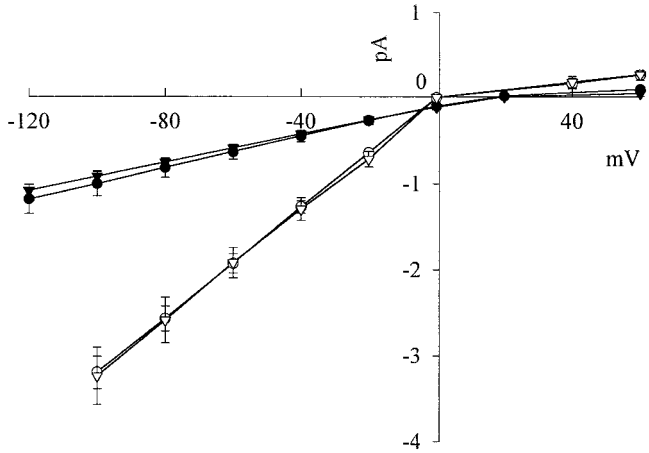


Figure 3. Single-channel current-voltage relationship under different ionic conditions. I-V relationship was obtained from inside-out patches of aortic endothelial cells (circles) or ECV304 cells (triangles). The pipette/bath contained: Ca^{2+} saline/Na^+-glutamate solution (filled circles or triangles) or NaCl/KCl saline (open circles or triangles). Mean ± SE (n=4–6).

Effects of 8-Br-cGMP, KT5823, and H-8 on the $[Ca^{2+}]_i$ increase elicited by flow

To study the role of this mechanosensitive channel in flow-induced Ca^{2+} entry, a laminar flow with shear stress of ~5 dyne/cm^2 was applied to the cultured endothelial cells. The shear stress of 5 dyne/cm^2 was in the low range of physiological shear stress (5–50 dyne/cm^2) acting on endothelial cells in arteries (25). Changes in $[Ca^{2+}]_i$ were monitored by fluorescence dye Fluo3/AM. The flow caused a transient increase in $[Ca^{2+}]_i$ that lasted for ~2 min in the aortic endothelial cells and for ~10 min in ECV 304 cells. This Ca^{2+} transient was related to Ca^{2+} influx since removal of extracellular Ca^{2+} abolished the transient. The rise in $[Ca^{2+}]_i$ was inhibited by 8-Br-cGMP in a dose-dependent manner, with an IC_{50} of 80 μM (n=13) for aortic endothelial cells and IC_{50} of 75 μM for ECV304 cells (n=35). 8-Br-cGMP at 2 mM completely blocked the $[Ca^{2+}]_i$ rise (n=7–20) (**Fig. 4*A, B***), whereas 8-Br-cAMP at 2 mM had no effect (n=10–30). The inhibitory effect of 8-Br-cGMP was reversed by 1 μM KT5823 or 10 μM H-8 (Fig. 4*A, B*), suggesting the involvement of PKG.

A comparison was made in **Fig. 5** regarding the potency of 8-Br-cGMP in its inhibitory action on the $[Ca^{2+}]_i$ increase elicited by flow and on the activity of the mechanosensitive channel. A close resemblance between two dose-dependent curves indicates that 8-Br-cGMP inhibits the $[Ca^{2+}]_i$ increase and the activity of mechanosensitive channel with similar potency.

Taken together, it is likely that an activation of PKG by 8-Br-cGMP closes the mechanosensitive Ca^{2+}-permeable channel, therefore blocking the Ca^{2+} entry induced by flow, whereas an inhibition of PKG by KT5823 or H-8 leads to opening of the channel, thus causing an increase in Ca^{2+} influx.

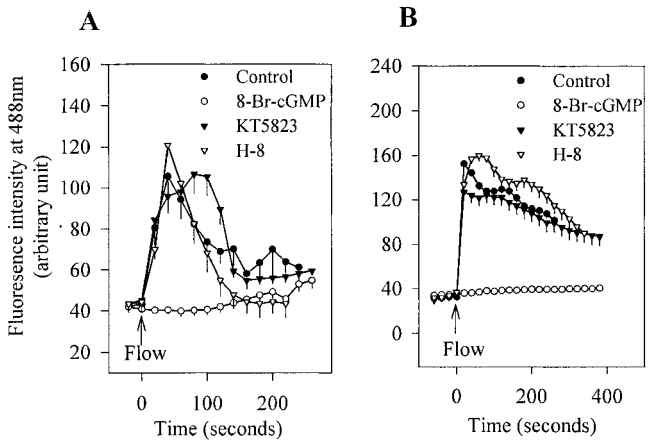


Figure 4. The effect of 8-Br-cGMP, KT5823, H-8 on flow-induced increase in $[Ca^{2+}]_i$. At the time indicated by the arrow, a laminar flow with the sheer stress of ~5 dyne/cm^2 was applied. Shear stress-induced increase in $[Ca^{2+}]_i$ was observed in control (filled circles), in the cells treated with 1 μM KT5823 plus 2 mM 8-Br-cGMP (filled triangles) and in the cells treated with 10 μM H-8 plus 2 mM 8-Br-cGMP (open triangles). No $[Ca^{2+}]_i$ elevation was observed in the cells treated with 2 mM 8-Br-cGMP alone (open circles). *A*) Aortic endothelial cells. *B*) ECV304 cells. Mean ± SE (n=7–20)

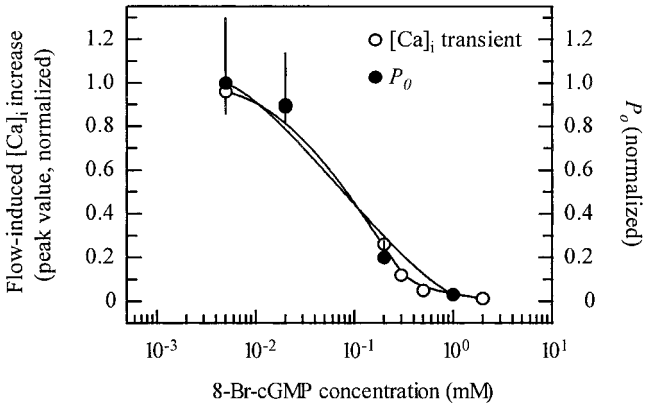
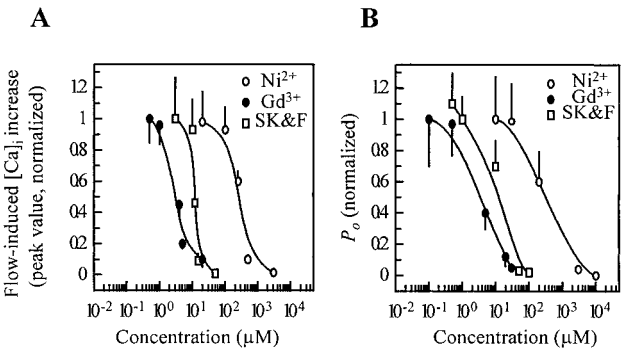


Figure 5. A concentration-dependent inhibition by 8-Br-cGMP on activity of the mechanosensitive Ca^{2+}-permeable channel and on flow-induced Ca^{2+} transient in aortic endothelial cells. P_o values during a 60 s period were calculated from cell-attached patches with the pipette/bath contained Ca^{2+} saline/Na^+-glutamate solution (n=3). The patch membrane potential was −40 mV displaced from its resting potential. The concentration of 8-Br-cGMP was increased stepwise in the same patch to generate a concentration-dependent response curve. P_o value in the absence of 8-Br-cGMP was normalized to 1. For the flow-induced Ca^{2+} transient (n=13), the peak amplitude of Ca^{2+} transient was plotted vs. 8-Br-cGMP concentrations and the peak value in the absence of 8-Br-cGMP was normalized to 1. Mean ± SE.

Effects of mechanosensitive channel blockers on the $[Ca^{2+}]_i$ increase elicited by flow

We tested the sensitivity of the $[Ca^{2+}]_i$ increase to putative channel blockers. Blockers of the mechanosensitive channel, Gd^{3+}, SK&F-96365, and Ni^{2+}, also inhibited the rise in $[Ca^{2+}]_i$ in a concentration-dependent manner with respective IC_{50} values of 2.5 μM, 12 μM, and 0.2 mM in isolated aortic endothelial cells (**Fig. 6*A, C***). The respective IC_{50} values were 10 μM, 25 μM, and 0.62 mM in ECV304 cells (Fig. 6*C*). For comparison, the potency of these three inhibitors in their actions on P_o values of the mechanosensitive cation channel is also presented in Fig. 6*B, C*. A remarkable similarity in IC_{50} was observed (Fig. 6*C*). In contrast, flufenamic acid (100 μM) and DCDPC (10 μM), which did not block the mechanosensitive channel in our experiments, had no effect on the shear stress-induced rise in $[Ca^{2+}]_i$. These data demonstrate a striking pharmacological similarity between the rise in $[Ca^{2+}]_i$ elicited by flow and the mechanosensitive Ca^{2+}-permeable channel.



C

Cell types	Primary aortic endothelial cells		ECV304 cells	
IC_{50}	IC_{50} (n) based on $[Ca]_i$ change	IC_{50} (n) based on P_o change	IC_{50} (n) based on $[Ca]_i$ change	IC_{50} (n) based on P_o change
Ni^{2+}	0.20 mM (17)	0.30 mM (3)	0.62 mM (21)	0.70mM (3)
SK&F-96365	12 μM (19)	17 μM (3)	25μM (30)	31 μM (3)
Gd^{3+}	2.5 μM (16)	3.5 μM (3)	10 μM (21)	14 μM (3)

Figure 6. A concentration-dependent inhibition by Ni^{2+}, Gd^{3+}, or SK&F-96365 on activity of the mechanosensitive Ca^{2+}-permeable channel and on flow-induced Ca^{2+} transient. P_o values during a 60 s period were calculated from outside-out patches with the pipette/bath containing Na^+-glutamate solution/Ca^{2+} saline (n=3). The patch membrane potential was held at −80 mV. The concentration of blockers was increased stepwise in the same patch to generate a concentration-dependent response. P_o value in the absence of blockers was normalized to 1. For the flow-induced Ca^{2+} transient (n=16–30), the peak amplitude of Ca^{2+} transient was plotted vs. blocker concentrations. The peak value in the absence of each blocker was normalized to 1. *A*) Flow-induced $[Ca^{2+}]_i$ transient in aortic endothelial cells. *B*) P_o in aortic endothelial cells. *C*) Comparison of IC_{50} (n=3–30).

DISCUSSION

Shear stress generated by blood flow is reported to induce Ca^{2+} influx in vascular endothelial cells, thus stimulating the production of NO and PGI_2, which cause subsequent vasodilation (4, 5, 8). The results described in this report suggest that, in both arterial and venous endothelial cells, shear stress may first activate a mechanosensitive Ca^{2+}-permeable channel in the plasma membrane, causing Ca^{2+} influx and an elevation of $[Ca^{2+}]_i$. This channel is a nonselective cation channel with relative permeability ratios of *P*Ca:*P*Na:*P*K = 5:1:1. The activity of the channel is inhibited by PKG. Blockage of this Ca^{2+}-permeable channel by 8-Br-cGMP, Ni^{2+}, Gd^{3+}, or SK&F-96365 abolishes the $[Ca^{2+}]_i$ rise induced by flow (Figs. 5, 6), suggesting that the channel is the main pathway mediating flow-induced Ca^{2+} entry into vascular endothelial cells. A striking pharmacological similarity between the $[Ca^{2+}]_i$ increase elicited by flow and the mechanosensitive Ca^{2+}-permeable channel as regards the sensitivity to inhibition by 8-Br-cGMP, Ni^{2+}, Gd^{3+}, or SK&F-96365 further substantiates the critical role of this channel in flow-induced Ca^{2+} entry.

Flow-induced rise in $[Ca^{2+}]_i$ can result from extracellular Ca^{2+} entry and intracellular Ca^{2+} release from internal stores (4, 26). In agreement with Kanai's report (4), we did not observe any flow-induced rise in $[Ca^{2+}]_i$ when Ca^{2+} was absent in extracellular medium. In addition, the chemicals that blocked the mechanosensitive Ca^{2+}-permeable channel in plasma membrane, including Ni^{2+}, Gd^{3+}, 8-Br-cGMP, and SK&F-96365, abolished the rise in $[Ca^{2+}]_i$ elicited by flow. Therefore, it is likely that Ca^{2+} influx through the mechanosensitive Ca^{2+}-permeable channel is the major contributor to the flow-induced $[Ca^{2+}]_i$ rise. The release of Ca^{2+} from intracellular store, if it exists, may require Ca^{2+} entry as a triggering signal.

Previous evidence suggested that flow may activate an inwardly rectifier K^+ channel (9) or a calcium-activated K^+ channel (10), therefore hyperpolarizing the endothelial cells and increasing the driving force for Ca^{2+} entry. However, it is unknown whether these K^+ channels are mechanosensitive. The mechanism by which shear stress elicits the activation of these channels is still unclear (9). The present study indicates that a Ca^{2+}-permeable channel is mechanosensitive, being able to open when mechanic force is generated by flow. Membrane hyperpolarization resulting from the opening of K^+ channels, therefore, may not be a prerequisite for flow-induced Ca^{2+} entry into vascular endothelial cells. Nevertheless, membrane hyperpolarization caused by the opening of K^+ channel will undoubtedly increase the driving force for Ca^{2+} entry and thus facilitate Ca^{2+} entry.

Vascular endothelial cells contain a cGMP-producing enzyme, guanylate cyclase, the activity of which is stimulated by NO (3). Flow-induced Ca^{2+} influx is known to stimulate the production of NO, which subsequently activates guanylate cyclase, leading to an elevation of cGMP in vascular endothelial cells. Increased productions of NO and cGMP have been detected under flow conditions in cultured endothelial cells and in isolated vascular segments (4, 27, 28). The data from our results suggest that the elevated cGMP may in turn inhibit Ca^{2+} entry via a PKG-dependent phosphorylation, therefore providing a feedback mechanism through which Ca^{2+} influx is finely regulated dependent on the production of NO and cGMP (**Fig. 7**). As shown in Fig. 7, this feedback model also allows the production of NO to be regulated by the amount of available NO in vascular endothelial cells. This model may also explain the puzzle of why the $[Ca^{2+}]_i$ rise induced by flow is transient (4). Flow-induced Ca^{2+} entry elevates NO and cGMP. The elevated cGMP will in turn inhibit Ca^{2+} influx. A combination of these processes may result in a transient rise of $[Ca^{2+}]_i$ in response to flow stimulation.

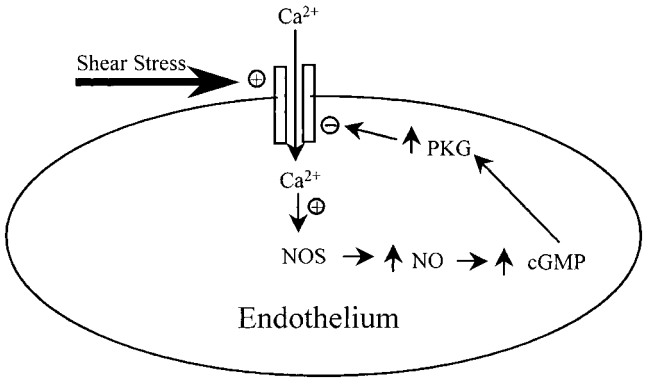


Figure 7. Proposed model for signal transduction following flow stimulation. A mechanosensitive nonselective cation channel (MS-NSC) is activated by flow stimulation. Extracellular Ca^{2+} entry activates nitric oxide synthase (NOS) to produce NO, which subsequently elevates intracellular cGMP. As a feedback regulation, the intracellular cGMP inhibits the Ca^{2+} entry by inhibiting the mechanosensitive Ca^{2+}-permeable channel.

In conclusion, a mechanosensitive Ca^{2+}-permeable cation channel is identified in vascular endothelial cells. The activity of this channel is regulated by PKG. It is likely that this channel is the main Ca^{2+} entry pathway mediating blood-flow induced Ca^{2+} influx in vascular endothelial cells. FJ

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