Depletion of Intracellular Ca^{2+} Stores Sensitizes the Flow-Induced Ca^{2+} Influx in Rat Endothelial Cells

Hiu-Yee Kwan, Pan-Cheung Leung, Yu Huang, Xiaoqiang Yao

Abstract—Hemodynamic shear stress elicits a rise in endothelial $[Ca^{2+}]_i$, which may serve as a key second messenger to regulate many flow-associated physiological and biochemical processes. In the present study, we used Mn^{2+} quenching of fluorescent dye Fluo3 as an assay to investigate the Ca^{2+} influx of rat aortic endothelial cells in response to flow. We found that the Ca^{2+} signaling in response to flow could be greatly influenced by the status of intracellular Ca^{2+} stores. Depletion of intracellular Ca^{2+} stores by thapsigargin (4 μmol/L) or cyclopiazonic acid (10 μmol/L) drastically sensitized the Ca^{2+} influx in response to flow. Ca^{2+}-mobilizing agonist bradykinin (100 nmol/L) or ATP (100 μmol/L) had similar sensitizing effect. The effect of bradykinin or ATP was blocked by *Xestospongin C* and U73122, suggesting that the sensitization was related to the IP_3-mediated store depletion. On the other hand, the Mn^{2+} quenching in response to flow was greatly reduced by ochratoxin A (100 nmol/L), an agent that could increase the filling state of intracellular Ca^{2+} stores. In addition, we found that depletion-sensitized Ca^{2+} influx in response to flow was mediated by a PKG-inhibitable cation channel and that the influx was affected by membrane potential and K^+ channel activity. In conclusion, the present study argues for a critical role of intracellular Ca^{2+} status in determining the Ca^{2+} signaling in response to flow and it provides a general mechanistic explanation for the stimulatory role of blood-borne agonists on flow-induced Ca^{2+} influx. **(*Circ Res*. 2003;92:286-292.)**

Key Words: flow shear stress ■ mechanotransduction ■ calcium ■ store depletion ■ ion channel

Shear stress generated by blood flow elicits diverse responses in vascular endothelium.[1–4] One of the early responses to flow is the elevation of endothelial cytosolic Ca^{2+} level.[5–8] An increase in intracellular Ca^{2+} level may lead to multiple shear stress–associated physiological and biochemical changes such as production of nitric oxide and PGI_2,[2] and activation of Src tyrosine kinase and C-kinase.[9]

The mechanism by which endothelial cells sense the hemodynamic flow and transform the mechanical signals of flow shear stress into the intracellular Ca^{2+} signals is still not well understood. Several putative mechanisms have been proposed: (1) shear force may transmit to endothelial cell by interconnecting actin cytoskeleton, thus activating signal transduction cascades without a specific stretch receptor[1]; (2) flow may cause membrane hyperpolarization, thereby increasing the driving force for extracellular Ca^{2+} entry[10,11]; (3) flow may increase ATP concentration in unstirred boundary layer at the cell surface; the elevated ATP may then stimulate Ca^{2+} influx by opening Ca^{2+}-permeable purinoceptor P_2X_4[1,7]; (4) flow shear stress may displace a layer of glycoproteins in extracellular space, thus modifying ion channels and receptors[12]; and (5) flow shear stress may activate mechanosensitive nonselective cation channels, resulting in increased Ca^{2+} influx across the plasmalemma.[8,13]

Besides flow shear stress, Ca^{2+} influx of endothelial cells can be stimulated by Ca^{2+}-mobilizing agonists such as bradykinin, histamine, and ATP. These agonists trigger IP_3 receptor–mediated Ca^{2+} release from intracellular Ca^{2+} stores, resulting in store depletion. The depletion of stores could then stimulate Ca^{2+} influx across the plasmalemma.[14] This type of Ca^{2+} entry, which has been termed the store-operated Ca^{2+} entry, is the predominant Ca^{2+} entry pathway in nonexcitable cells including vascular endothelial cells.[4,15,16] Up to the present, however, very little is known about the relationship between two crucial Ca^{2+} signaling pathways, ie, flow-induced Ca^{2+} entry and store-operated Ca^{2+} influx. It is not known whether flow-induced Ca^{2+} influx is in any way related to the status of intracellular Ca^{2+} stores in vascular endothelial cells.

In the present study, we examined the role of intracellular Ca^{2+} stores in regulating flow-induced Ca^{2+} influx. We found that there was interplay between the status of intracellular Ca^{2+} stores and flow-induced Ca^{2+} signaling. Depletion of intracellular Ca^{2+} stores greatly sensitized the Ca^{2+} influx elicited by flow. Attempts were also made to explore the role of Ca^{2+}-permeable channels and K^+ channels in flow-induced Ca^{2+} influx.

Materials and Methods

Preparation and Culture of Aortic Endothelial Cells

Primary endothelial cells were isolated from rat aortas and cultured as described elsewhere.[17] Briefly, male Sprague-Dawley rats of

Original received January 30, 2002; resubmission June 11, 2002; accepted December 18, 2002.

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Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000054625.24468.08**

about 250 to 300 g were decapitated. The thoracic aortas were removed and washed twice with sterile phosphate-buffered saline (PBS). The aortas were cut open into 4×4-mm strips and treated with 0.2% collagenase (type II from Sigma) in PBS for 15 minutes at 37°C with endothelial side facing up. The suspension after the enzyme digestion was centrifuged at 600*g* for 5 minutes. The cells were resuspended in 5 mL culture medium that contained 90% RPMI and 10% FBS with 100 U/mL penicillin and 100 μg/mL streptomycin. After 1 hour incubation at 37°C, the medium was replaced once to remove unattached cells. Attached endothelial cells were cultured in an incubator with 5% CO_2 at 37°C. Culture medium was changed every 3 days. To avoid possible loss of endothelial properties during culture condition, only those cells from the first two passages were used for experiments. The identity of the primary cultured rat aortic endothelial cells was examined by immunostaining using an antibody against von Willebrand factor and the results showed that >98% of the cells were of endothelial origin.[8] Animals were supplied by the Laboratory Animal Service Center, Chinese University of Hong Kong, Hong Kong. We followed the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Ca^{2+} or Mn^{2+} Influx Measurement

Cells were loaded with fluorescent dye Fluo3-acetoxymethyl ester (Fluo3-AM) as described elsewhere.[8] Briefly, cells were loaded with 10 μmol/L Fluo3-AM for 1 hour in the dark at room temperature with 0.02% Pluronic F127 in normal physiological saline solution (NPSS), which contained (in mmol/L) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 HEPES (pH 7.4). Flow was initiated by pumping 0Ca^{2+}-PSS or NPSS or Mn^{2+}-PSS containing appropriate inhibitors at a defined shear force to a specially designed parallel plate flow chamber.[8] For the flow experiments in Ca^{2+}-free condition, the cells were washed and then maintained for ≈5 minutes in 0Ca^{2+}-PSS before the initiation of flow. For depletion experiments, the cells were pretreated with 4 μmol/L thapsigargin or 10 μmol/L cyclopiazonic acid (CPA) or 100 nmol/L bradykinin or 100 μmol/L ATP for 15 to 20 minutes. When needed, cells were treated with or 100 nmol/L ochratoxin (OTA) for 10 minutes, or with 2 mmol/L 8-Br-cGMP, 1 μmol/L KT5823, and 10 μmol/L H-8 for 5 minutes before the start of flow. 0Ca^{2+}-PSS contained (in mmol/L) 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 2 EGTA, and 5 HEPES (pH 7.4). Mn^{2+}-PSS contained (in mmol/L) 140 NaCl, 5 KCl, 1 $MnCl_2$, 1 $MgCl_2$, 10 glucose, and 5 HEPES (pH 7.4). An excitation wavelength of 488 nm was provided by an MRC-1000 Laser Scanning Confocal Imaging System and fluorescence signals were collected using a 515 nm–long pass emission filter. Data analyses were performed with MetaFluor. The responses of Ca^{2+}-Mn^{2+} to flow were displayed as the percentage of fluorescence relative to the intensity before flow.

Western Blot

Western blot analyses of VASP (vasodilator stimulated phosphoprotein) were performed as described elsewhere.[18] Briefly, cells were treated with ATP and bradykinin for 5 minutes and then solubilized in ice-cold extraction buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton-X-100, and protease inhibitor cocktail (Roche). Protein (50 μg) was separated on 8% SDS/PAGE. Proteins were then blotted onto PVDF membrane (Millipore). Membranes were blocked with PBS and 5% nonfat dried milk at room temperature overnight. Immunoblotting was preformed with an appropriate primary antibody, either anti-VASP rabbit antiserum M4 (ImmunoGlobe) (diluted 1:1000) or mouse monoclonal P-VASP phosphoserine 239 antibody 16C2 (NanoTools) (diluted 1:1000). Immunodetection was accomplished with peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies (Amersham). Antibody binding was detected by ECL system (Amersham).

Materials

Fluo3-AM and Pluronic F127 were obtained from Molecular Probes, Inc. Tissue culture media were from Gibco/BRL. Bradykinin, 8-Br-cGMP, KT5823, H-8, thapsigargin, cyclopiazonic acid (CPA), PPADS (pyridoxalphophate-6-azophenyl-2′,4′-disulfonic acid), *Xestospongin C* (XeC), and ochratoxin A (OTA) were from Calbiochem. HOE140 (a synthetic peptide: H_2N-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH), U73122, U73343, reactive blue, suramin, $MnCl_2$, $CaCl_2$, ATP, HEPES, and EGTA were purchased from Sigma.

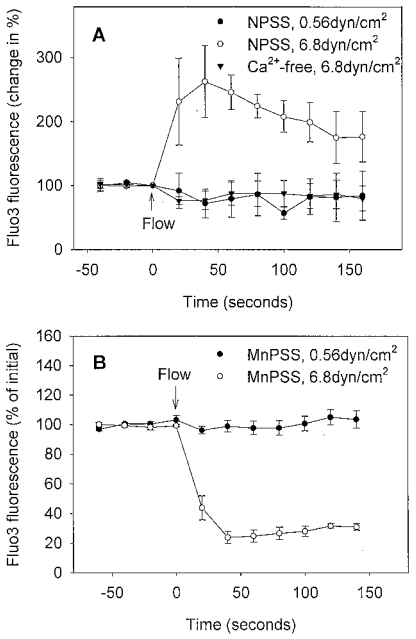

Figure 1. Dependence of Ca^{2+} influx or Mn^{2+} quenching on shear force. A, Time course of Ca^{2+} responses to the shear stress of 0.56 or 6.8 dyn/cm². Arrow indicates the point at which shear force was applied to the cells bathed in NPSS or 0Ca^{2+}-PSS. B, Time course of Mn^{2+} quenching in response to the shear stress of 0.56 or 6.8 dyn/cm². Shear stress was applied to the cells bathed in Mn^{2+}-PSS. Fluorescence intensity before the initiation of flow was normalized to 100%. Mean±SE (n=3 to 11 independent experiments, 5 to 20 cells per experiment).

Results

Flow-Induced Ca^{2+} and Mn^{2+} Influx

Flow caused a transient rise in $[Ca^{2+}]_i$ that was dependent on the magnitude of shear force (Figure 1A). At a low shear stress level of 0.6 dyn/cm², $[Ca^{2+}]_i$ remained constant. When the shear stress level was increased to 6.8 dyn/cm², flow triggered a marked increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ reached its peak in 40 to 60 seconds, then gradually declined.

Flow-induced rise in $[Ca^{2+}]_i$ could be caused by increased Ca^{2+} influx[5,8,14,19–21] or by stimulated Ca^{2+} release from IP_3- or arachidonic-sensitive intracellular Ca^{2+} stores.[4,14,22,23] In our experiments, the rise in $[Ca^{2+}]_i$ in response to flow was due to Ca^{2+} influx alone because chelation of extracellular Ca^{2+} with EGTA completely abolished it (Figure 1A). This is consistent with the results from many groups that reported that flow could induce Ca^{2+} influx without affecting intracellular Ca^{2+} release.[5,19,21] On the other hand, some other groups showed that flow could induce intracellular Ca^{2+} release. One possible reason for this discrepancy is that the shear stress we applied was not high enough to trigger intracellular Ca^{2+} release, because IP_3 production may need high shear stress

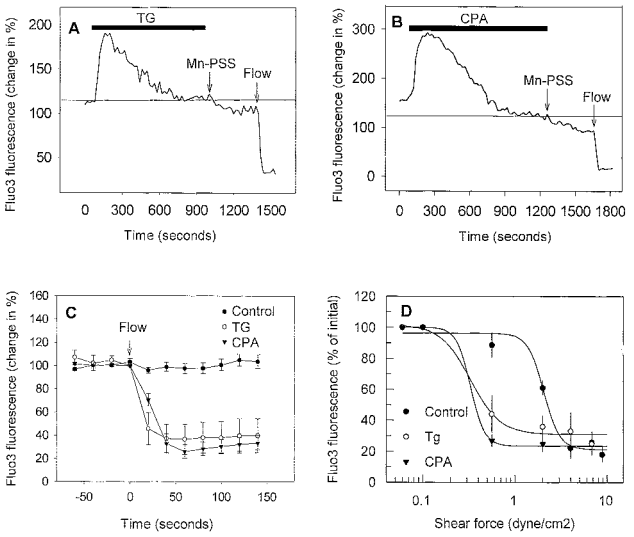

Figure 2. Sensitization of Mn^{2+} quenching in response to flow by thapsigargin or CPA. A and B, Fluorescence changes recorded from a representative cell treated by thapsigargin (A) or CPA (B) followed by flow shear stress; a reference line was added to better illustrate the Mn^{2+} quenching caused by substituting NPSS with Mn^{2+}-PSS. C and D, Portion of the experiments showing the flow-induced fluorescence quenching. C, Time course of fluorescence quenching in response to the shear stress of 0.56 dyn/cm^2. D, Depletion-induced shift in shear stress response curves. Peak values of flow-induced Mn^{2+} quenching were plotted. Cells were treated with 4 μmol/L thapsigargin or 10 μmol/L CPA for 15 to 20 minutes. Control had no treatment. Fluorescence intensity before the initiation of flow was normalized to 100%. Mean±SE (n=4 to 11 independent experiments, 5 to 20 cells per experiment).

($\approx$30 dyn/cm^2).[22] We did not test the effect of high shear stress because it frequently led to cell detachment.

Mn^{2+} is known to be a good substitute for Ca^{2+} in defining Ca^{2+} entry pathways.[20,24] Because there is no intracellular Mn^{2+} store, the quenching reflects the influx through the plasma membrane alone.[25] In agreement with the Ca^{2+} responses, low shear stress did not cause Mn^{2+} quenching, whereas high shear stress triggered rapid Mn^{2+} quenching (Figure 1B).

Effect of Store-Depletion on Flow-Induced Mn^{2+} Quenching

Application of thapsigargin (4 μmol/L) or CPA (10 μmol/L) caused an immediate rise in cytosolic Ca^{2+} (Figures 2A and 2B). The rise in cytosolic Ca^{2+} was mainly due to intracellular Ca^{2+} release because it could be recorded in cells bathed in $0Ca^{2+}$-PSS. After $[Ca^{2+}]_i$ level returned to its basal level and stabilized, bathing media were changed from Ca^{2+}-containing NPSS to Mn^{2+}-containing Mn^{2+}-PSS. The change of solutions resulted in fluorescence quenching due to store-operated Mn^{2+} influx. Very little quenching could be observed for the cells that were not subjected to thapsigargin or CPA pretreatment. Flow was applied $\approx$2 minutes later after the fluorescence signals stabilized. Flow shear stress triggered additional Mn^{2+} influx on top of the store-operated Mn^{2+} influx (Figures 2A and 2B). We focused our attention on this flow-induced Mn^{2+} influx and investigated the effect of store depletion on flow-induced Mn^{2+} quenching. Figures 2C and 2D displayed a portion of the experiments related to the flow-induced Mn^{2+}quenching. In both the control and thapsigargin- or CPA-pretreated cells, the magnitude of flow-induced Mn^{2+} quenching correlated with the intensity of flow shear force (Figure 2D). Thapsigargin or CPA pretreatment caused a marked shift of flow dose response curve to the left, decreasing SF50 values (the shear force that caused 50% Mn^{2+} quenching) from $\approx$2 to 0.35 dyn/cm^2; whereas it had no effect on the maximal Mn^{2+} quenching at relatively high shear stress levels of $>$4 dyn/cm^2 (Figure 2D). At the shear stress of 0.56 dyn/cm^2, fluid flow greatly increased Mn^{2+} influx in thapsigargin- or CPA-pretreated cells, whereas it had no effect in control cells (Figure 2C).

Effect of Bradykinin and ATP on Flow-Induced Mn^{2+} Quenching

Similar to what were observed in thapsigargin or CPA experiments, 100 nmol/L bradykinin or 100 μmol/L ATP pretreatment elicited an immediate rise in cytosolic Ca^{2+} (Figures 3A and 3D). Changes of bathing solutions from NPSS to Mn^{2+}-PSS caused fluorescence quenching due to the store-operated Mn^{2+} influx. This type of Mn^{2+} quenching was not obvious in cells without being subjected to the agonist

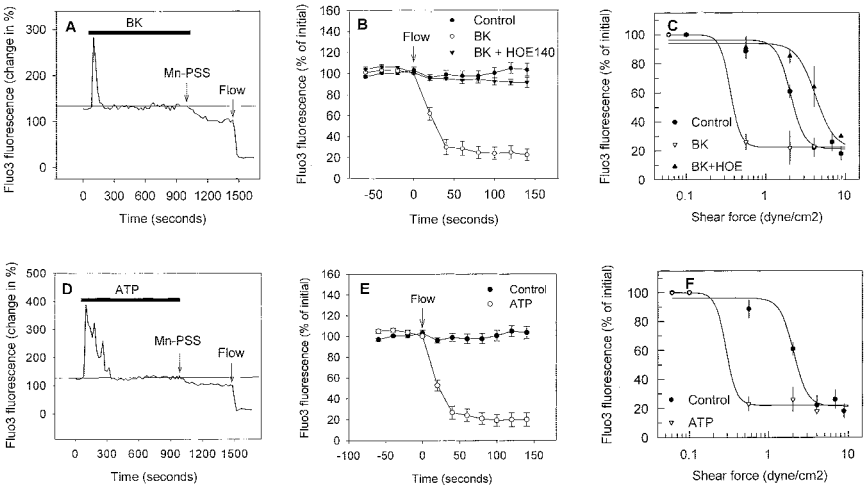

Figure 3. Sensitization of flow-induced Mn^{2+} quenching by bradykinin or ATP. A and D, Fluorescence changes recorded from a representative cell treated with bradykinin (A) or ATP (D) followed by flow shear stress. A reference line was added to better illustrate the Mn^{2+} quenching caused by substituting NPSS with Mn^{2+}-PSS. B, C, E, and F, Portion of the experiments showing the flow-induced fluorescence quenching. B and E, Time course of fluorescence quenching in response to the shear stress of 0.56 dyn/cm^2. C and F, Agonist-induced shift in shear stress response curves. Cells were pretreated with 100 nmol/L bradykinin with/without 1 μmol/L HOE140 or 100 μmol/L ATP for 15 to 20 minutes. Control had no treatment. Mean±SE (n=3 to 13 independent experiments, 5 to 20 cells per experiment).

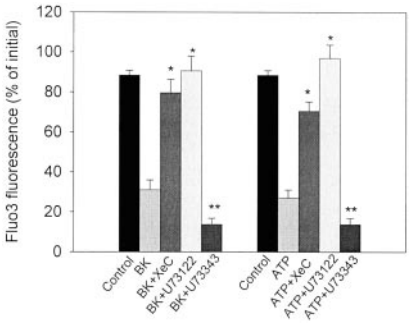

Figure 4. Effect of XeC and U73122 on the agonist-sensitized Mn^{2+} quenching in response to flow. Cells were pretreated with 100 nmol/L bradykinin or 100 μmol/L ATP for 15 to 20 minutes before the application of shear stress of 0.56 dyn/cm^2. In some experiments, agonist was applied together with 30 μmol/L XeC or 10 μmol/L U73122 or 10 μmol/L U73343. Control had no pretreatment. Fluorescence intensity before the initiation of flow was normalized to 100%. Mean±SE (n=4 to 20 independent experiments, 5 to 20 cells per experiment). *P<0.001 compared with BK or ATP treatment alone; **P<0.001 compared with U73122 treatment.

pretreatment. Shear stress triggered additional Mn^{2+} influx on top of the store-operated Mn^{2+} influx (Figures 3A and 3D). In most experiments, agonists were removed before the initiation of the flow to avoid possible direct effect by agonists. Nevertheless, we found that the inclusion of agonists in the flowing solution had no additional effect on flow-induced Ca^{2+} response. Figure 3B, 3C, 3E, and 3F showed a portion of the experiments related to the Mn^{2+} quenching in response to flow. Bradykinin and ATP pretreatment shifted the flow dose response curve to the left, whereas they had no significant effect on the maximal Mn^{2+} quenching (Figures 3C and 3F). Bradykinin pretreatment decreased the SF50 values from ≈2 to 0.35 dyn/cm^2, whereas ATP pretreatment reduced the SF50 values from ≈2 to 0.29 dyn/cm^2. Between the shear force values of ≈0.15 to 3 dyn/cm^2, the cells pretreated with agonists showed a greater degree of Mn^{2+} quenching than the cells without the pretreatment. The Mn^{2+} responses to the shear stress of 0.56 dyn/cm^2 were shown in Figure 3B and 3E for bradykinin and ATP-treated cells, respectively.

Attempts were made to determine the receptors that mediated the actions of bradykinin and ATP. As shown in Figure 3C and 3F, bradykinin-induced sensitization was blocked by an inhibitor of B_2 bradykinin receptor HOE140 (1 μmol/L), suggesting the involvement of B_2 receptor. Several putative purinoceptor antagonists including suramin, PPADS, and reactive blue failed to block ATP-stimulated intracellular Ca^{2+} release even under nonflow condition, suggesting that action of ATP was mediated by a receptor that was insensitive to these three putative inhibitors.

We also tested the effect of XeC, an IP_3 receptor blocker, and U73122, a phospholipase C inhibitor. Both XeC (30 μmol/L) and U73122 (10 μmol/L) inhibited bradykinin- or ATP-induced sensitization of Mn^{2+} response to flow, whereas U73343 (10 μmol/L), an inactive analog of U73122, had no effect (Figure 4). It should be noted that we treated the cells with high concentration of XeC (30 μmol/L) at 37°C on a shaker for 1.5 hours in order to overcome the poor permeability of the chemical.

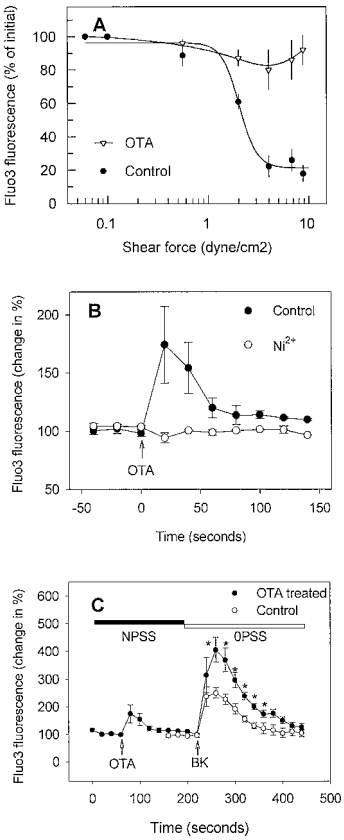

Figure 5. Effect of OTA on Ca^{2+} signaling. A, Effect of OTA on flow-induced Mn^{2+} quenching. Cells were pretreated with/without 100 nmol/L OTA for 10 minutes. Shear stress was applied to cells bathed in Mn^{2+}-PSS. Peak values of flow-induced Mn^{2+} quenching were plotted. B, Effect of OTA (200 nmol/L) on Ca^{2+} influx. Cells were bathed in NPSS. C, Effect of OTA on bradykinin-induced intracellular Ca^{2+} release. OTA (200 nmol/L) was applied to cells bathed in NPSS. Bathing solution was changed to 0Ca^{2+}-PSS before the application of bradykinin 100 nmol/L. Fluorescence intensity immediately before the initiation of flow (A) or application of OTA (B) or bradykinin (C) was normalized to 100%. Mean±SE (n=4 to 11 independent experiments, 5 to 20 cells per experiment). *P<0.05 compared with the control.

Effect of OTA on Flow-Induced Mn^{2+} Quenching

Because store depletion sensitized the flow-induced Mn^{2+} quenching, an increased filling of Ca^{2+} stores would presumably desensitize or reduce the flow-induced Mn^{2+} quenching. We used OTA to increase the filling state of intracellular Ca^{2+} stores.[26] Figure 5A showed that treatment of cells with 100 nmol/L OTA indeed diminished the flow-induced Mn^{2+} quenching in the shear stress range up to ≈9 dyn/cm^2 (Figure 5A). However, the present data could not differentiate the possibilities of whether OTA treatment actually abolished or only desensitized the flow response because we did not test the effect of OTA at a high shear stress range. As mentioned previously, high shear stress (>9 dyn/cm^2) led to the cell detachment.

An alternative interpretation for Figure 5A could be that OTA directly inhibited Ca^{2+} influx pathway. However, this is unlikely because OTA has previously been shown to stimulate Ca^{2+} influx by others.[26] This was confirmed in our

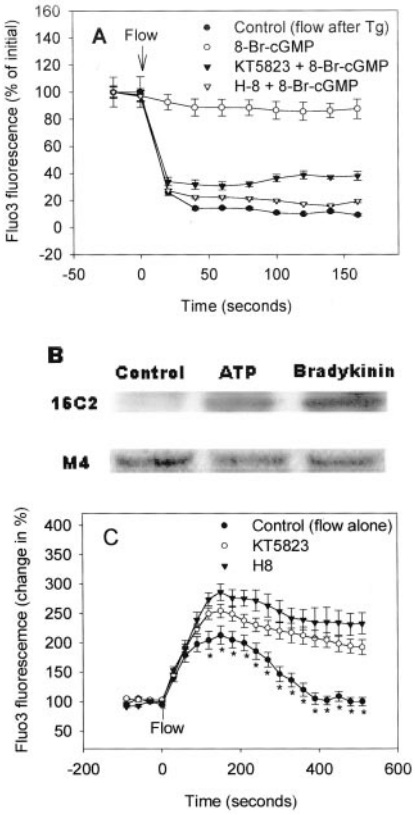

Figure 6. Role of a PKG-inhibitable channel. A, Effect of PKG activator or inhibitor on the depletion-sensitized Mn^{2+} quenching in response to flow. B, effect of Ca^{2+}-mobilizing agonists on PKG activity. Cells were treated with ATP (100 μmol/L) or bradykinin (100 nmol/L ATP) for 5 minutes. B, top, Western blot with antibody (16C2) against phosphorylated VASP; bottom, Western blot with antibody (M4) against total VASP. C, Effect of PKG inhibitor on flow-induced Ca^{2+} transients. A and C, Cells were pretreated with 4 μmol/L thapsigargin for 20 minutes. PKG activator (2 mmol/L 8-Br-cGMP) or inhibitor (1 μmol/L KT5823, 10 μmol/L H-8) was introduced 5 minutes before the initiation of flow. Mean±SE (n=4 to 10 independent experiments, 5 to 20 cells per experiment). *$P<0.05$ compared with KT5823 or H-8 treatment.

experiments. We found that application of OTA (100 or 200 nmol/L) elicited an immediate rise in cytosolic Ca^{2+} and this rise in $[Ca^{2+}]_i$ was due to Ca^{2+} influx because it could be blocked by Ni^{2+} (3 mmol/L) (Figure 5B).

We also compared the bradykinin-induced Ca^{2+} release in control cells and the cells exposed to OTA for 150 seconds. As shown in Figure 5C, the bradykinin-induced Ca^{2+} release was significantly higher in OTA-exposed cells than in control cells, suggesting that OTA indeed increased the filling state of intracellular Ca^{2+} stores.

The Channel That Mediates Depletion-Sensitized Mn^{2+} Influx

Our previous study[8] showed that a protein kinase G (PKG)–inhibitable cation channel mediated the flow-induced Ca^{2+} entry in rat vascular endothelial cells. In the present study, we explored the question of whether the depletion-sensitized Ca^{2+} response to flow was mediated by the same PKG-inhibitable cation channel. Figure 6A showed that thapsigargin sensitized the flow-induced Mn^{2+} quenching and the sensitization was completely abolished by a PKG activator 8-Br-cGMP (2 mmol/L) (Figure 6A). The action of 8-Br-cGMP was reversed by PKG inhibitor KT5823 (1 μmol/L) or H-8 (10 μmol/L) (Figure 6A). It should be mentioned that thapsigargin-induced store depletion process was completed in about 10 minutes after the addition of TG (Figure 2A), and 8-Br-cGMP was added after that; therefore, the addition of 8-Br-cGMP might not significantly affect the store depletion process. Taken together, these results suggest that, like the basal flow-induced Ca^{2+} influx, the depletion-sensitized Ca^{2+} influx in response to flow was also mediated by the same PKG-inhibitable cation channel.

We have previously proposed that a rise in $[Ca^{2+}]_i$ activates endothelial NO-cGMP-PKG pathway and the activated PKG can then inhibit a Ca^{2+}-influx channel, forming a negative feedback circuit to prevent the $[Ca^{2+}]_i$ from getting too high.[8,15] Previous studies demonstrated that PKG could inhibit a Ca^{2+}-influx channel.[8,15] In the present study, we tested whether an elevation of $[Ca^{2+}]_i$ by Ca^{2+}-mobilizing agonists would indeed activate PKG. Figure 6B showed that ATP and bradykinin treatment drastically increased the amount of phosphorylated VASP (50 kDa), whereas it had little effect on total VASP (50 kDa) expressed in isolated endothelial cells. Detection of phosphorylated VASP at S-239 is considered to be the best method for measuring PKG activation.[27] These results added more evidence to the negative feedback hypothesis.

Treatment of the cells by thapsigargin may elicit a rise in $[Ca^{2+}]_i$, which elevates cytosolic cGMP levels and activates PKG. If this pathway serves as a negative feedback mechanism to prevent $[Ca^{2+}]_i$ from getting too high, an inhibition of PKG is expected to augment the flow-induced Ca^{2+} response and, at the same time, result in a slower return of $[Ca^{2+}]_i$ to its resting levels. This is confirmed in Figure 6C, which showed that inhibition of PKG by KT5823 (1 μmol/L) or H-8 (10 μmol/L) increased the magnitude of flow-induced Ca^{2+} influx and slowed down the falling phase of $[Ca^{2+}]_i$ transients.

It should be stressed that, although PKG mechanism may serve to limit the $[Ca^{2+}]_i$ rise when $[Ca^{2+}]_i$ is high, the role of PKG mechanism at "resting" $[Ca^{2+}]_i$ levels is unclear. At the resting $[Ca^{2+}]_i$ levels, PKG activity is low. It is not known whether this low level of PKG activity could still inhibit Ca^{2+} influx to a certain degree. Further studies are needed to clarify the matter.

Possible Role of Membrane Hyperpolarization and K^+ Channels in Depletion-Sensitized Mn^{2+} Quenching

It is well documented that >25 mmol/L K^+ can effectively prevent membrane hyperpolarization in vascular endothelial cells.[28] As shown in Figure 7, clamping the membrane potential by 35 mmol/L K^+ caused a significant reduction in depletion-sensitized Mn^{2+} quenching in response to flow. Ba^{2+} (30 μmol/L), an inhibitor for inward rectifier K^+ channels, had similar inhibitory effect. Inhibition of Ca^{2+}-sensitive K^+ channels by iberiotoxin (10 nmol/L) or charybdotoxin plus apamin (50 nmol/L each) also reduced the Mn^{2+} quenching. These data suggest that the membrane hyperpo-

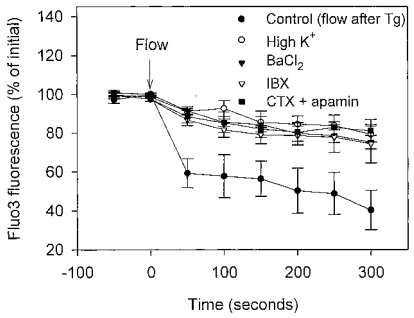

Figure 7. Effect of high K^+ concentration and K^+ channel inhibitors on the depletion-sensitized Mn^{2+} quenching in response to flow. Cells were pretreated with 4 μmol/L thapsigargin for 20 minutes. Flow was applied in the absence or presence of high KCl (35 mmol/L) or $BaCl_2$ (30 μmol/L) or iberiotoxin (IBX, 10 nmol/L) or charybdotoxin (CTX) plus apamin (50 nmol/L each). Mean±SE (n=3 to 4 independent experiments, 5 to 15 cells per experiment). All treatments caused significant reduction in flow-induced responses compared with the control ($P<0.05$).

larization mediated by inward rectifier K^+ channels and Ca^{2+}-sensitive K^+ channels may play a role in depletion-sensitized Mn^{2+} influx in response to flow.

Discussion

In the present study, we found that the Ca^{2+} signaling in response to flow could be greatly influenced by the status of intracellular Ca^{2+} stores. Depletion of intracellular Ca^{2+} stores by inhibitors of endoplasmic reticulum Ca^{2+}-ATPase thapsigargin and CPA drastically sensitized the Ca^{2+} influx in response to flow. Ca^{2+}-mobilizing agonists bradykinin and ATP had similar effect. The magnitude of sensitization induced by all these agents was similar. They reduced the SF50 values by 6- to 7-fold from ≈2 to 0.3 dyn/cm^2. Under the condition of store depletion, a relatively small shear force might trigger a large increase in Ca^{2+} influx. On the other hand, an increased filling of intracellular Ca^{2+} stores by OTA suppressed the flow-induced Ca^{2+} influx. Another line of evidence that supports the crucial role of store depletion came from XeC and U73122 experiments. XeC and U73122 treatment abolished the ATP- or bradykinin-induced flow sensitization, suggesting that the action of ATP and bradykinin was due to IP_3 receptor–mediated store depletion.

The finding that Ca^{2+}-mobilizing agonists may augment the flow-induced Ca^{2+} influx has extensive physiological implications. Vascular endothelial cells are exposed to circulating blood that contains numerous Ca^{2+}-mobilizing agents including metabolites, local paracrine agents, growth factors, and cytokines. Our data suggest that these endogenous substances may significantly sensitize the flow-induced Ca^{2+} signaling through their actions on store depletion. This type of sensitization may represent a general mechanism through which vascular endothelial cells integrate shear stress with other simultaneous stimuli to potentiate Ca^{2+} signaling, thereafter modulating diverse arrays of Ca^{2+}-dependent physiological processes.

It has long been recognized that shear stress may stimulate the rise of $[Ca^{2+}]_i$ in vascular endothelial cells.[5,6,8,19,29] Several groups demonstrated that the rise of endothelial $[Ca^{2+}]_i$ in response to flow required the presence of endothelial agonist ATP in flow media.[2,7] These authors suggest that flow may increase ATP concentration in the unstirred boundary layer at the cell surface. The elevated ATP may then stimulate Ca^{2+} influx by opening Ca^{2+}-permeable purinoceptor P_2X_4.[1,7] However, it should be noted that the Ca^{2+} influx stimulated by flow does not necessarily require the presence of ATP. Several groups have independently showed that flow could stimulate Ca^{2+} influx in the absence of any Ca^{2+} mobilizing agonists.[5,6,8,19,21,29] In our experimental condition, flow was able to trigger Ca^{2+} influx in the absence of ATP. However, the presence of ATP and bradykinin could greatly sensitize the flow-induced Ca^{2+} influx due to store depletion. Our results differ from what was reported by Yamamoto et al[7] who favored the direct participation of P_2X_4 receptors in $[Ca^{2+}]_i$ responses to flow. One possible explanation for this discrepancy is that the concentrations of ATP used were very different in these two studies. As reported by Yamamoto et al,[7] the involvement of P_2X_4 purinoceptor was only evident when ATP concentration was low (<250 nmol/L), whereas in the present investigation, we used a much higher ATP concentration of 100 μmol/L. It is possible that there exist two separate mechanisms; P_2X_4-mediated Ca^{2+} influx pathway may operate at low ATP concentration, whereas store depletion–mediated Ca^{2+} influx may become dominant when ATP concentration is high. At high ATP concentration, P_2X_4-mediated pathway may be masked by IP_3-mediated pathway. This is in agreement with the concept that P_2X_4 purinoceptor is more sensitive to ATP, whereas P_2Y is generally more sensitive to ADP.[30]

It should be noted that flow-induced Ca^{2+} influx was not consistently observed by all investigators. Several researchers failed to observe any flow-elicited $[Ca^{2+}]_i$ rise in endothelial cells under flow condition.[2,31,32] The causes of discrepancy are still unknown. From the present study, a mechanistic interpretation can be proposed: it is likely that different investigators have used the endothelial cells with different intracellular Ca^{2+} status. Numerous conditions/procedures may alter the filling status of intracellular Ca^{2+} stores. These include Ca^{2+} or agonist concentration in culture media. Under some conditions, the stores could be fully loaded with Ca^{2+}; therefore, flow may not be able to initiate a significant increase in Ca^{2+} influx. Under other conditions, the Ca^{2+} content in intracellular stores could be low; this would greatly augment the Ca^{2+} rise in response to flow.

A mechanosensitive Ca^{2+}-permeable cation channel has been suggested to mediate the flow-induced Ca^{2+} entry in vascular endothelial cells.[8,13] The activity of this stretch-activated cation channel is downregulated by protein kinase G. In the present study, we demonstrate that depletion of intracellular Ca^{2+} stores can sensitize the flow-induced Ca^{2+} influx, and furthermore, PKG is able to inhibit the flow-induced Ca^{2+} influx regardless of whether the cells are sensitized by store depletion or not. These results suggest that, like basal flow–induced Ca^{2+} influx, the depletion-sensitized Ca^{2+} influx in response to flow was mediated by the same PKG-inhibitable cation channel. However, the present data do not rule out the possibility that cGMP and PKG may modulate other proteins in addition to the PKG-

inhibitable cation channel. It should also be mentioned that, although the present data clearly demonstrate that depletion of stores sensitizes the flow-induced Ca^{2+} influx, the underlying mechanism of this sensitization remains unclear. Ca^{2+} influx is determined by the opening probability of Ca^{2+}-permeable channels. Store-depletion is known to stimulate Ca^{2+} influx via "physical coupling between endoplasmic reticulum components and plasma membrane channel,"[33,34] or Ca^{2+} influx factor,[33,35] or secretion-like vesicle fusion.[33,36] It is possible that one of these mechanisms may serve to sensitize the flow-induced Ca^{2+} influx. On the other hand, the $[Ca^{2+}]_i$ rise and subsequent PKG activation induced by store depletion may serve to limit the Ca^{2+} influx and prevent $[Ca^{2+}]_i$ from getting too high. The data from our results also suggest that the sensitization may involve K^+ channels and membrane hyperpolarization since inhibition of K^+ channels reduced the sensitization response.

In conclusion, the present study demonstrates that flow-induced Ca^{2+} signaling can be greatly influenced by the status of intracellular Ca^{2+} stores. Depletion of intracellular Ca^{2+} stores sensitizes the Ca^{2+} influx elicited by flow. This type of sensitization may represent a general mechanism by which blood-borne agonists potentiate the endothelial Ca^{2+} signaling in response to flow and thereby regulating many physiological and biochemical processes in vascular system.

Acknowledgments

This study was supported by Hong Kong Research Grant Council (CUHK4079/00 mol/L) and Chinese University Research Committee Funding.

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