Mechanical stress-induced Ca$^{2+}$ entry and Cl$^{-}$ current in cultured human aortic endothelial cells

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Nakao, Miki, Kyoichi Ono, Susumu Fujisawa, and Toshihiko Iijima. Mechanical stress-induced Ca$^{2+}$ entry and Cl$^{-}$ current in cultured human aortic endothelial cells. Am. J. Physiol. 276 (Cell Physiol. 45): C238–C249, 1999.—A fluid stream through a microtube was applied to cultured human aortic endothelial cells to investigate the endothelial responses of both the ionic currents and intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) to mechanical stimulation. The fluid stream induced an increase in [Ca$^{2+}$]i that was dependent on both the flow rate and the intracellular Ca$^{2+}$ concentration. GDP$\beta$S and niflumic acid inhibited the fluid stream-induced increase in [Ca$^{2+}$]i, whereas Ba$^{2+}$ and tetraethylammonium ion exhibited no effect. The fluid stream-induced [Ca$^{2+}$]i increase was accompanied by the activation of an inward current at −52.8 mV. The reversal potential of the fluid stream-induced current shifted to positive potentials when the external Cl$^{-}$ concentration was reduced but was not affected by variation of the external Na$^{+}$ concentration. During the exposure to the fluid stream, [Ca$^{2+}$]i was voltage dependent, i.e., depolarization decreased [Ca$^{2+}$]i. We therefore conclude that the fluid stream-induced current is largely carried by Cl$^{-}$ and that the Cl$^{-}$ current may thus play a role in modulating the Ca$^{2+}$ influx by altering the membrane potential of endothelial cells.

shear stress; stretch; calcium signaling

VASCULAR ENDOTHELIAL CELLS lining the surface of blood vessels are constantly exposed to various mechanical forces induced by the blood pressure and pulsatile flow. The endothelial cell membrane exhibits certain ion channel responses to mechanical forces, and the resulting changes in membrane potential and/or intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]i) are involved in modulating a number of second messenger pathways such as the production of endothelium-derived relaxing factor and PGI$_2$, the synthesis of various proteins, and the regulation of gene expression (1, 11, 24, 30).

Forces acting on endothelial cells in vivo are generally classified into at least three different categories (2, 17): shear stress acting parallel to the surface of endothelial cells, transmural force due to blood pressure, and membrane stretch as a consequence of pulsatile vessel expansion. The endothelial responses to these mechanical forces, particularly those of [Ca$^{2+}$]i, and ion channels, have been investigated based on the above classification. For example, a shear stress of only 0.7 dyn/cm$^2$ has been reported to activate a K$^+$ current in bovine aortic endothelial cells (BAEC) grown on the inner surface of glass capillary tubes (27). The activation of the K$^+$ current results in hyperpolarization that may facilitate Ca$^{2+}$ entry (12, 27). It remains controversial, however, as to whether or not shear stress causes an increase in [Ca$^{2+}$]; several groups have reported an obvious [Ca$^{2+}$]i elevation using a parallel-plate flow chamber, whereas others have been unable to obtain such responses (4, 31, 34). When a physiological solution was blown onto endothelial cells, a Ca$^{2+}$-permeable cation channel was activated and thus increased [Ca$^{2+}$]i (33). On the other hand, membrane stretch, applied as negative pressure from the patch pipette, activated a cation channel in the pig aorta (15) and porcine cerebral capillaries (29). Furthermore, the stretching of cellular membranes increased [Ca$^{2+}$]i in endothelial cells cultured on silicon membranes (21). The responses of ion channels and [Ca$^{2+}$]i are thus considered to differ depending on how mechanical forces are applied to endothelial cells. These findings indicate that endothelial responses may vary in the physiological environment, where endothelial cells are subjected to complex mechanical forces, depending on the regions of vasculature and hemodynamic conditions.

In this study, a stream of fluid was used to apply mechanical stress to cultured human aortic endothelial cells (HAEC). Although the method may not cause pure shear stress and also likely includes other mechanical forces, it enables us to monitor [Ca$^{2+}$]i and the concomitant changes in ionic currents simultaneously. It is thus a useful method for examining the cellular mechanisms underlying functional linkage between Ca$^{2+}$-transient and ionic currents. We show herein that the fluid stream facilitates the Ca$^{2+}$ influx and activates the Cl$^{-}$ current. In addition, the possible role of the Cl$^{-}$ current in regulating the Ca$^{2+}$ entry is also discussed.

MATERIALS AND METHODS

Culture of endothelial cells. HAEC at passage 3 or 4 were purchased from Clonetics (San Diego, CA) and Kurabo (Osaka, Japan). The cells from Clonetics were grown in culture medium (endothelial basal medium, Clonetics) supplemented with 2% fetal bovine serum, bovine brain extract protein contents (12 µg/ml), human recombinant epidermal growth factor (10 ng/ml), 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, and 50 ng/ml amphotericin B in 5% CO$_2$-containing air at 37°C. The culture medium used for the cells from Kurabo (Humedia-EB2, Kurabo) was similar to that described above, except that 5 ng/ml human recombinant fibroblast growth factor B and 10 µg/ml heparin were added instead of bovine brain extract protein contents. The culture medium was exchanged every 48 h until a subconfluent growth stage was obtained. The cells were detached by exposure to 0.025% trypsin in a Ca$^{2+}$- and Mg$^{2+}$-free solution containing 0.01% EDTA for ~180 s, diluted in the culture medium, and then reseeded on coverslips (9 × 9 mm) coated with fibronectin (Biomedical Technologies, Stoughton, MA) with a cell density of ~2,500 cells/cm$^2$. We kept the cells in culture for 1–4 days.
before use. No differences were found in the experimental results obtained by using these two different sources and types of culture media.

Solution and drugs. The HEPES-buffered saline (HBS) used as the standard bath solution contained (in mM) 136.9 NaCl, 5.4 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 11.1 glucose, and 5.0 HEPES. The pH was adjusted to 7.4 with NaOH. In some experiments, MgSO₄ was used instead of MgCl₂, although no significant difference was observed compared with the experiments using MgCl₂ in HBS. When the extracellular Ca²⁺ concentration ([Ca²⁺]) varied, CaCl₂ was either added to HBS or simply omitted from HBS. The low-CI⁻ solutions (17.4, 35, and 70 mM; see Figs. 5B, 9, and 10) were prepared by replacing NaCl with equimolar NaOH plus methanesulfonic acid. In some experiments, sodium isethionate was used as a substitute for NaCl. The external solution containing tetrathylenammonium ion (TEA) was prepared by adding 20 mM TEA chloride to HBS in place of NaCl (see Fig. 5D). The high-K⁺ solution (142.3 mM; Fig. 5E) was prepared by replacing NaCl with KCl in HBS (pH adjusted to 7.4 with KOH). The K⁺-free solution was prepared by replacing KCl with equimolar CsCl in HBS (see Figs. 8–10). In the Na⁺-free solution, NaCl in HBS was totally replaced with equimolar N-methyl-d-glucamine plus HCl (see Fig. 8B).

The pipette solution for the nystatin perforated patch recording contained (in mM) 120 potassium gluconate, 30 KCl, and 10 HEPES, and pH was adjusted to 7.2 with KOH. Nystatin (Sigma Chemical) was dissolved in methanol as a 10 mg/ml stock solution and added to external solutions to obtain a final concentration of ~400 μg/ml. The pipette solution for the conventional whole cell mode contained (in mM) 20 CsCl, 95 cesium aspartate, 5 Na₂ATP, 5 MgCl₂, 5 EGTA, and 5 HEPES titrated to pH 7.2 with CsOH.

Fura 2-AM (Dendo Laboratories, Kumamoto, Japan) and fura PE3-AM (Texas Fluorescence Laboratory) were dissolved in DMSO as 1 μg/μl stock solutions, respectively, and diluted in 1 ml HBS with 2 μl 10% cremophor EL (Sigma Chemical). The final concentration of the Ca²⁺ indicators was 5 μM. Niflumic acid (Sigma Chemical) was dissolved in DMSO as 1 µg/µl stock solutions, respectively, and diluted in 1 ml HBS or simply omitted from HBS. The low-Cl⁻ solutions contained (in mM) NaCl, NaCl in HBS was totally replaced with equimolar KCl, and NaCl in HBS was totally replaced with equimolar KCl, and 10 HEPES, and pH was adjusted to 7.2 with KOH. In some experiments, sodium isethionate was used as a substitute for NaCl. The external solution containing tetrathylenammonium ion (TEA) was prepared by adding 20 mM TEA chloride to HBS in place of NaCl (see Fig. 5D). The high-K⁺ solution (142.3 mM; Fig. 5E) was prepared by replacing NaCl with KCl in HBS (pH adjusted to 7.4 with KOH). The K⁺-free solution was prepared by replacing KCl with equimolar CsCl in HBS (see Figs. 8–10). In the Na⁺-free solution, NaCl in HBS was totally replaced with equimolar N-methyl-d-glucamine plus HCl (see Fig. 8B).

The apparatus for applying mechanical stress. The method for applying the fluid stream was directed parallel to the bottom of the chamber at a distance of 300 µm and the flow rate was constant over the whole cell surface. The flow of the fluid stream was essentially the same as that applied to the reservoir. The outlet of the chamber was connected both to a mercury barometer for monitoring the hydrostatic pressure inside the chamber and to an effluent bottle via a valve for controlling the flow rate of the perfusate. The flow rate was maintained at 2–3 ml/min during the experiments. Using this apparatus, the hydrostatic pressure inside the chamber could be increased up to 200 mmHg.

Fluorescence measurement of [Ca²⁺]. The cells on coverslips were incubated either in HBS containing 5 μM fura 2-AM for 30 min at 37°C or in HBS containing 5 μM fura PE3 for 60 min at 37°C. After treatment, the coverslips were rinsed several times with HBS and incubated in HBS for 30 min at 37°C. The coverslip was mounted in a chamber placed on an inverted microscope (TMD-15J, Nikon, Tokyo, Japan) equipped with a ×40 fluorite objective. The fluorescence measurements of [Ca²⁺] from a single cell were performed using a spectrophotofluorometer (CAM-230, Japan Spectroscopic, Tokyo, Japan). The excitation wavelength alternated at 400 Hz between 340 and 380 nm during recording of emission fluorescence at 500 nm. The emitted light was directed through a rectangular iris that limited the light collected to a single cell under observation. The emission intensities at 340 and 380 nm excitation (F340 and F380) were monitored on a chart recorder (Thermal Array recorder WR7700, Graphtec, Tokyo, Japan). F340 and F380, together with the current and voltage signals, were sampled every 1 s onto the hard disk of a computer running Superscope software (version 6.0, PCLAMP, Axon Instruments). The fluorescence intensities at excitation wavelengths 340 nm in the Ca²⁺-free and ionomycin-containing solutions. The basal [Ca²⁺] in normal HBS under our experimental conditions. However, the absolute values of [Ca²⁺] measured in Ca²⁺-free and ionomycin-containing solutions. The basal [Ca²⁺] measured in either fura 2- or fura PE3-loaded HAEC ranged from 50 to 100 nM in normal HBS under our experimental conditions. However, the absolute values of [Ca²⁺] could not be calculated because the dissolution constant of the indicators and Ca²⁺ in the cytosol might be different from those measured in the absence of protein (13). In the present experiments, F340/F380 values were thus used as a relative measurement of [Ca²⁺].

Electrophysiology. Single endothelial cells on coverslips were voltage clamped using either the conventional whole cell technique (7) or the nystatin perforated patch-clamp technique (9). The electrode resistance ranged from 1 to 5 MΩ when electrodes were filled with the pipette solution. The current-voltage (I-V) relationship was measured by voltage ramp pulses that were applied from a holding potential of −52.8 mV to +60 and −140 mV with change in voltage over time (dv/dt) of either ±0.2 or ±0.1 v/s. The current and voltage signals were digitized online at 500 Hz using pCLAMP software (version 6.0.3, Axon Instruments). The reference electrode was usually HBS agar with an integral Hg-HgCl₂ wire. In the experiments in which the extracellular Cl⁻ concentration ([Cl⁻]) was varied, a flowing 3 M KCl solution
was used instead of HBS agar to minimize any possible changes of the junction potential at the tip of the reference electrode (18). The liquid junction potential between the pipette solution and HBS was directly measured to be −12.8 mV on the basis of the assumption that the junction potential at the tip of a 3 M KCl electrode in these solutions was negligible. The voltage values shown in the text were corrected accordingly. The bath solution was warmed by the use of a water jacket around the perfusing tube, so that the temperature of the chamber was kept at 35.5 ± 1.0°C. The flow rate of the bath perfusion was 2–3 ml/min.

Data analysis. The Gd3⁺-induced suppression of the fluid stream-induced [Ca²⁺], increase was analyzed by the following equation

\[ R_{Gd}/R_{cont} = I_{max}/(1 + ([Ca^{2+}]_o/[Gd^{3+}]_o)^n) \]  

where \( R_{Gd} \) represents the magnitude of the decrease in F₃₄₀/F₃₈₀ at various extracellular concentrations of Gd³⁺ ([Gd³⁺]₀) and \( R_{cont} \) is the amplitude of F₃₄₀/F₃₈₀ induced by the fluid stream alone. \( I_{max} \) indicates the maximum inhibition, and \( n \) is the Hill coefficient.

The results are expressed as means ± SE; \( n \) indicates the number of cells examined. The statistical analysis was performed using Student's paired t-test, and values of \( P < 0.05 \) were considered to be statistically significant.

RESULTS

Fluid stream-induced increase of [Ca²⁺] in HAEC. As shown in Fig. 1, a polyethylene tube with an opening of 300 μm (inside diameter) was placed parallel to the bottom of the recording chamber at a distance of 300 μm from a single endothelial cell, and the cell was exposed to a fluid stream of HBS containing 1 mM Ca²⁺. When the flow rate was increased from 0 to 0.45 ml/min, the peak of the [Ca²⁺] increase was observed at ~1 min. During the continuous presence of the fluid stream, [Ca²⁺] appeared to remain elevated for ~2 min and thereafter declined. A further increase of the flow rate to 1.1 ml/min caused an initial peak of [Ca²⁺], which thereafter decreased to a plateau level. A subsequent cessation of the flow reversibly decreased [Ca²⁺], toward the basal level. From an average of five cells, F₃₄₀/F₃₈₀ at the plateau phase was 0.67 ± 0.04 in the control, 0.77 ± 0.04 at 0.45 ml/min, and 0.93 ± 0.03 at 1.10 ml/min. Regarding the initial peak of [Ca²⁺], its magnitude appeared to depend on the flow rate i.e., the transient elevation was more marked at the flow rate of 1.1 ml/min than at 0.45 ml/min. Furthermore, the initial peak was consistently observed if the flow rate changed instantaneously from 0 to 0.45 or 1.1 ml/min (not illustrated). However, such an instantaneous change of the flow rate often caused a detachment of the cells from the coverslip, and the experiments were thus interrupted. To avoid a loss of cells, we changed the flow rate by manually opening or closing the valve of the perfusing tube while confirming the attachment of the cells. As a result, a few seconds were required to reach a new flow rate level, which may thus have resulted in varying degrees of transient [Ca²⁺] elevation among different endothelial cells examined (Figs. 1, 2, 4–6, and 11). The transient nature of the [Ca²⁺] increase could not be analyzed extensively in the present study. The following experiments focused on the sustained elevation of [Ca²⁺].

The effects of the fluid stream were examined in different [Ca²⁺]₀ (Fig. 2A). In the absence of external Ca²⁺, no significant change in [Ca²⁺] was detected in response to the fluid stream. However, the flow-dependent increase of [Ca²⁺], became evident when [Ca²⁺]₀ increased to 5 and 10 mM. The relationship between [Ca²⁺] and the flow rate was measured at [Ca²⁺]₀ of 0, 1, 5, and 10 mM. At each [Ca²⁺]₀, five cells were subjected to an increasing flow from 0 to 0.45 and 1.1 ml/min in a manner similar to that for Fig. 2A. The results are summarized in Fig. 2B. It is clearly shown that the fluid stream increases [Ca²⁺], in a manner that is both flow rate dependent and [Ca²⁺]₀ dependent. In the absence of external Ca²⁺, F₃₄₀/F₃₈₀ during the fluid stream of either 0.45 or 1.1 ml/min was not significantly different from control (0.59 ± 0.03 in control, 0.63 ± 0.04 at 0.45 ml/min, and 0.63 ± 0.04 at 1.1 ml/min; \( P > 0.1 \)). These findings suggest that Ca²⁺ entry across the plasma membrane is involved in the fluid stream-induced rise in [Ca²⁺].

For subsequent experiments conducted to examine the mechanism underlying the fluid stream-induced increase in [Ca²⁺], it was necessary to obtain a substantial response with little interference from any background noise. In the following experiments, we therefore recorded the response to the fluid stream in HBS containing 5 mM Ca²⁺ at 1.1 ml/min, with which almost a maximal response was expected.

Effect of hydrostatic pressure on HAEC. Mechanical stress acting on the endothelial cells in a fluid-stream method may include not only shear stress but also transmural pressure and membrane stretch. To address whether transmural pressure by itself causes an increase in [Ca²⁺], we intentionally changed the hydro-
static pressure in the recording chamber, using a semi-closed circuit apparatus (Fig. 3A). Cells on a coverslip were mounted in an acrylic chamber filled with HBS, and then the chamber was sealed tightly. After the control \([\text{Ca}^{2+}]_o\) had been measured, the internal pressure of the chamber was raised to 80 mmHg with compressed oxygen that was applied to the reservoir for 3 min. Subsequently, the internal pressure was further increased to 140 mmHg for 3 min, and thereafter the pressure was released. Two representative records with 1 mM (Fig. 3B, top) and 10 mM (Fig. 3B, bottom) \([\text{Ca}^{2+}]_o\) are shown. With \([\text{Ca}^{2+}]_o\) of 1 mM, hydrostatic pressure ranging from 0 to 140 mmHg produced virtually no effect on \([\text{Ca}^{2+}]_i\). \(F_{340}/F_{380}\) was 0.63 ± 0.03 in the control, 0.63 ± 0.03 at 80 mmHg, and 0.62 ± 0.03 at 140 mmHg (n = 4; not significant). When the external solution contained 10 mM \([\text{Ca}^{2+}]_o\), however, \([\text{Ca}^{2+}]_i\) was only slightly affected by increasing the hydrostatic pressure. \(F_{340}/F_{380}\) was 0.64 ± 0.03 in the control, 0.68 ± 0.03 at 80 mmHg, and 0.69 ± 0.02 at 140 mmHg (n = 9). The differences between the value in the control and those obtained at 80 or 140 mmHg were statistically significant (P < 0.014). These findings suggest that the transmural pressure by itself may be able to increase \([\text{Ca}^{2+}]_i\). However, the transmural pressure alone is not sufficient to account for the fluid stream-induced rise in \([\text{Ca}^{2+}]_i\), and other types of mechanical stress must be involved.

Inhibition of the \([\text{Ca}^{2+}]_i\) response to fluid stream by various ion channel blockers. To characterize the \([\text{Ca}^{2+}]_i\) influx pathway, the effects of various channel blockers were examined. Fig. 4A shows the effect of Gd\(^{3+}\), known as a nonselective cation channel blocker (21), on the sustained elevation of \([\text{Ca}^{2+}]_i\). The \([\text{Ca}^{2+}]_i\) level was increased by the fluid stream at a rate of 1.1 ml/min with \([\text{Ca}^{2+}]_o\) of 5 mM. During the maintained elevation of \([\text{Ca}^{2+}]_i\), 10 µM Gd\(^{3+}\) was applied and resulted in a suppression of the fluid stream-induced rise in \([\text{Ca}^{2+}]_i\) in a reversible manner. To quantitatively analyze the inhibitory effect of Gd\(^{3+}\), the magnitude of the decrease in \(F_{340}/F_{380}\) was measured at various \([\text{Gd}^{3+}]_o\) and normalized to that of the control response in the absence of Gd\(^{3+}\). The results in 18 cells are summarized in Fig. 4B. The IC\(_{50}\) value obtained using Eq. 2 was 0.43 µM, with maximum inhibition of ~80%. It should be noted that \(F_{340}/F_{380}\) does not linearly reflect \([\text{Ca}^{2+}]_i\) or the influx that is responsible for the fluid stream-induced \([\text{Ca}^{2+}]_i\) elevation. Therefore, the concentration-response curve cannot indicate the stoichiometry for Gd\(^{3+}\) and its binding sites. Nevertheless, the findings indicate that \([\text{Ca}^{2+}]_i\) entry, which is sensitive to micromo-
lar concentrations of Gd\(^{3+}\), contributes to the fluid stream-induced rise in [Ca\(^{2+}\)].

The [Ca\(^{2+}\)] elevation was also inhibited by niflumic acid, which is known to block not only various Cl\(^{-}\) channels (38) but also cation channels (5) (Fig. 5A). Niflumic acid (100 µM) decreased F\(_{340}\)/F\(_{380}\) by 73.4 ± 10.7% (n = 4). In Fig. 5B, the effect of reducing [Cl\(^{-}\)]\(o\) was examined. The sustained elevation of [Ca\(^{2+}\)] was reversibly decreased by reducing [Cl\(^{-}\)]\(o\) from 154.3 to 17.4 mM and substituting sulfonate ions. The inhibition was 52.1 ± 3.9% (n = 5). When isethionate ions were used for Cl\(^{-}\) substitution, the average inhibition was 37.5 ± 4.8% (n = 5).

In a separate series of experiments, the possible contribution of K\(^{+}\) conductance to regulation of Ca\(^{2+}\) entry was examined. As shown in Fig. 5C, 0.5 mM Ba\(^{2+}\) did not appreciably inhibit the elevation of [Ca\(^{2+}\)] (n = 4). Essentially similar results were obtained in three of four cells when another K\(^{+}\) channel blocker, 20 mM TEA, was applied on top of the response to the fluid stream (Fig. 5D). In one of four cells, however, 20 mM TEA further increased [Ca\(^{2+}\)], by 43%, and the effect was reversible after washout of TEA (not illustrated). On the other hand, [Ca\(^{2+}\)] was reversibly decreased by increasing the extracellular K\(^{+}\) concentration ([K\(^{+}\)]\(o\); Fig. 5E). The inhibition was 38.8 ± 7.1% at [K\(^{+}\)]\(o\) of 142.3 mM (n = 8).

Fluid stream-induced membrane current and increase in [Ca\(^{2+}\)]. It is well known that in endothelial cells [Ca\(^{2+}\)] increases in response to hyperpolarization and decreases upon depolarization (10, 16, 37). It is thus possible that the fluid stream-induced change in [Ca\(^{2+}\)] or its inhibition by the various blockers described so far might be caused by a secondary change in the driving force for Ca\(^{2+}\) entry. To investigate the mechanisms underlying the fluid stream-induced Ca\(^{2+}\) entry, changes in the membrane current associated with the increase of [Ca\(^{2+}\)] were recorded by the nystatin perforated patch-clamp technique. After membrane perforation was established, the membrane potential was held at −52.8 mV and the voltage ramp of ±0.2 V/s was applied every 20 s to obtain the I-V relationship (Fig. 6). In response to a fluid stream (1.1 ml/min) containing 5 mM Ca\(^{2+}\), the membrane current shifted to a downward direction, accompanied by a gradual increase in [Ca\(^{2+}\)] (Fig. 6A). It was a consistent finding that the activation of the current preceded the increase in [Ca\(^{2+}\)], i.e., the current reached a peak within several tens of seconds and [Ca\(^{2+}\)] increased thereafter. It was also noted that the inward current gradually decreased after its activation despite the continuous presence of the fluid stream, thus indicating a desensitization of the response. However, the extent of the decay varied from cell to cell. In some cells, the current decreased as shown in Fig. 6A, whereas the current amplitude was maintained at almost a constant level in others (see Fig. 9). In Fig. 6B, the I-V curves recorded before and during the application of the fluid stream are illustrated. In the control (Fig. 6B, trace a), the I-V curve showed a marked inward rectification at poten-
entials more negative than \(-80\) mV, thus indicating the presence of inwardly rectifying K\(^+\) channels. The resting membrane potential was \(-77.7\) mV, as indicated by the zero-current potential. During the exposure to the fluid stream (Fig. 6B, trace b), the current deflection in response to ramp pulse became larger and the membrane potential was depolarized to \(-36.6\) mV. The fluid stream-induced current (trace b - trace a) was obtained by digitally subtracting the control current from the current in the presence of the fluid stream and is plotted in Fig. 6C. The I-V curve of the fluid stream-induced current showed an outward rectification with a reversal potential (\(E_{\text{rev}}\)) of \(-24.8\) mV.

The same protocol was applied to nine cells, and \(E_{\text{rev}}\) of the fluid stream-induced current averaged \(-28.3 \pm 1.9\) mV. In Fig. 7, the change in membrane potential in these nine cells is shown. Under unstimulated conditions (in the control), the membrane potential varied from \(-7.9\) to \(-87.2\) mV and thus correlated with previous findings (3, 8, 19). During the exposure to the fluid stream, however, the membrane potential averaged \(-28.7 \pm 2.6\) mV and became close to the \(E_{\text{rev}}\) of the fluid stream-induced current.

Fluid stream-induced current, \(E_{\text{rev}}\) of about \(-28\) mV cannot be explained by a change in K\(^+\) selective conductances, but it does suggest that Cl\(^-\) and/or a nonselective cation current is responsible for the fluid stream-induced current. The ion selectivity of the inward current was examined by the conventional whole cell patch-clamp method using Cs\(^+\)-rich internal and K\(^+\)-free external solutions (Fig. 8A). Under the blockade of K\(^+\) currents, the control I-V curve (Fig. 8A, middle,
induced current (Fig. 8). The configuration of the extracellular K\(^+\) that recorded in the presence of intracellular and presence of fluid stream, was qualitatively similar to the control current from the current obtained in the presence of fluid stream. Bar indicates application of fluid stream with a flow rate of 1.1 ml/min. Dashed line, 0 current level. A. Arrows, zero-current potential before and during application of the fluid stream (Fig. 9). All cells were marked by corresponding letters in A. Arrows, zero-current potential before and during application of fluid stream. The fluid stream-induced current could be recorded even when external Na\(^+\) was completely removed (Fig. 8B). The average \(E_{\text{rev}}\) was \(-31.5 \pm 2.5\) mV (\(n = 6\)). The value was slightly more positive than that obtained in the presence of Na\(^+\).

In contrast to the results of the Na\(^+\) replacement study, \(E_{\text{rev}}\) of the fluid stream-induced current was markedly influenced by varying [Cl\(^-\)]. As shown in Fig. 9, the current response to the fluid stream was recorded with 70 (A), 35 (B), and 17.4 (C) mM Cl\(^-\). The flow rate was 1.1 ml/min, and the bathing solution contained 5 mM Ca\(^{2+}\). The I-V curves recorded before and during the application of the fluid stream are plotted in Fig. 9, A–C, middle. The difference currents between the membrane currents was carried out by the nystatin perforated patch-clamp method to examine the voltage-dependent [Ca\(^{2+}\)] change induced by fluid stream. The simultaneous measurement of [Ca\(^{2+}\)] and the membrane currents was carried out by the nystatin perforated patch-clamp method to examine the voltage-
dependent change of \([\text{Ca}^{2+}]_i\). In the absence of the fluid stream, \([\text{Ca}^{2+}]_i\) was affected neither by depolarization nor by hyperpolarization of the membrane potential (Fig. 11A). In contrast, during exposure to the fluid stream, \([\text{Ca}^{2+}]_i\) decreased in response to depolarization from \(-52.8\) to \(-2.8\) mV in a reversible manner, although hyperpolarization to \(-102.8\) mV only slightly increased \([\text{Ca}^{2+}]_i\) (Fig. 11B). To quantitatively analyze the voltage-dependent change in \([\text{Ca}^{2+}]_i\), the magnitude of the fluid stream-induced rise in \(F_{340}/F_{380}\) was measured at various membrane potentials and was normalized to that obtained at \(-52.8\) mV in the same cell. The data obtained from 14 cells are summarized in Fig. 11C. It was demonstrated that \([\text{Ca}^{2+}]_i\) increased in response to hyperpolarization. Furthermore, \([\text{Ca}^{2+}]_i\) appeared to be saturated at potentials more negative than \(-60\) mV.

Fig. 8. Fluid stream-induced current. Whole cell currents were recorded under blockade of K⁺ currents using Cs⁺-rich internal solution and K⁺-free external solution. A: results obtained in Na⁺-containing external solution. Left: original current traces. Vertical deflections indicate response to ramp pulses, and horizontal bar indicates application of fluid stream. Dashed lines, zero-current level. Middle I-V relationships measured at times indicated by corresponding letters in traces at left. Right: I-V curve of difference current obtained by subtracting control current (a) from current obtained in presence of fluid stream (b). B: experiment was carried out with Na⁺- and K⁺-free external solutions. Same explanation as for A. Traces shown in A and B are representative of 5 and 6 different experiments, respectively. In all experiments, external solution contained 5 mM Ca²⁺ and flow rate was 1.1 ml/min.

Fig. 9. Effect of Cl⁻ substitution on fluid stream-induced current. Fluid stream-induced current was recorded with [Cl⁻]₀ of 70 (A), 35 (B), and 17.4 (C) mM. In each case, top shows original current trace on chart recorder, middle shows I-V curves recorded before (a in A, c in B, e in C) and during (b in A, d in B, f in C) application of fluid stream, and bottom shows I-V curve of difference current (b - a in A, d - c in B, f - e in C). In all experiments, external solutions contained 5 mM Ca²⁺ and flow rate was 1.1 ml/min.
DISCUSSION

The major finding reported herein is that the fluid stream induces an increase in $\left[\text{Ca}^{2+}\right]_i$, accompanied by the activation of a $\text{Cl}^-$ current in HAEC. The fluid stream as a method to provide mechanical stimuli, used in the present study, can be a useful method for recording changes in both the ionic currents and $\left[\text{Ca}^{2+}\right]_i$ (33) but does not necessarily provide a well-defined shear stress (4, 34). Even though the tip of the perfusing tube was placed very close to a single endothelial cell, the flow of the solution was observed to radiate from the tip under the microscope. Thus the results must have been influenced by a disturbed laminar flow. However, we consider that the responses to the fluid stream may reflect those in certain physiological hemodynamic environments in which endothelial cells are subjected to complex mechanical forces, including shear stress, membrane stretch, and transmural pressure.

The present experiments also provide some insights into the possible role of the $\text{Cl}^-$ current in regulating $\left[\text{Ca}^{2+}\right]_i$ during the exposure to the fluid stream. First,
the fluid stream-stimulated [Ca\(^{2+}\)] was not affected by K\(^{+}\) channel blockers, TEA and Ba\(^{2+}\). It is thus unlikely that the observed [Ca\(^{2+}\)] increase is driven by the activation of the K\(^{+}\) current and the resulting hyperpolarization. Second, [Ca\(^{2+}\)] was not affected by varying the membrane potential alone before the application of the fluid stream. During exposure to the fluid stream, however, [Ca\(^{2+}\)] decreased upon depolarization under the voltage-clamp conditions, thus indicating that the fluid stream-induced [Ca\(^{2+}\)] increase is caused by the activation of Ca\(^{2+}\) entry through the plasma membrane. Third, the fluid stream caused either depolarization or hyperpolarization depending on the membrane potentials in the control (unstimulated) condition, i.e., depolarization occurred in those cells that had a negative membrane potential in the control, whereas the cells having less negative resting potentials were hyperpolarized in response to the fluid stream (Fig. 7). As a result, the membrane potential became stable near −30 mV. Fourth, the fluid stream-induced increase of [Ca\(^{2+}\)] was suppressed by reducing [Cl\(^{-}\)]. This should be the case if the membrane potential is determined largely by Cl\(^{-}\) during the stimulation of the fluid stream. A reduction of [Cl\(^{-}\)], would depolarize the membrane, thereby decreasing the driving force for Ca\(^{2+}\) entry. Although the inhibition of the fluid stream-induced [Ca\(^{2+}\)] changes due to a high-K\(^{+}\) solution can be explained by depolarization, this finding does not necessarily indicate that the membrane potential is determined by K\(^{+}\) at physiological [K\(^{+}\)]. On the other hand, the observation of little or no effect of K\(^{+}\) channel blockers suggests that, at physiological [K\(^{+}\)], the contribution of K\(^{+}\) conductance is not larger than that of the Cl\(^{-}\) current.

On the basis of the above observations, it appears that the Cl\(^{-}\) current may play a functional role in the fluid stream-induced [Ca\(^{2+}\)] elevation. If the fluid stream facilitated Ca\(^{2+}\) entry with little change in the membrane conductance, then [Ca\(^{2+}\)] would have varied from cell to cell, depending on the membrane potential of the individual cells. In the [Ca\(^{2+}\)] measurements, however, we observed a consistent, quantitatively similar increase in [Ca\(^{2+}\)], in response to the fluid stream in the various cells examined (for example, see Fig. 5). We thus consider that the activation of the Cl\(^{-}\) current stabilizes the membrane potential near the equilibrium potential for Cl\(^{-}\) (E\(_{Cl}\)) and provides a constant driving force for Ca\(^{2+}\) entry. Ono et al. (28) showed the physiological E\(_{Cl}\) to be about −28 mV when measured by the gramicidin perforated patch-clamp method. At this potential, a moderate increase of [Ca\(^{2+}\)] would occur, according to the [Ca\(^{2+}\)]-membrane potential relationship shown in Fig. 11. It should be noted that the Cl\(^{-}\) current decayed more or less despite the continuous presence of the fluid stream. The degree of the decay varied among the individual cells. Some cells showed a clear decay (Fig. 6), whereas no decay was detected in others (Figs. 8 and 9). Nevertheless, because the input impedance of endothelial cells is relatively high at a potential range between −70 and 0 mV (3, 35), the opening of only a small number of the channels might be sufficient to cause a substantial change in the membrane potential.

Mechanical stress-induced Ca\(^{2+}\) transient. A fluid stream induced a transient increase in [Ca\(^{2+}\)], followed by a sustained elevation. It is very unlikely that the fluid stream-induced rise in [Ca\(^{2+}\)] is due to transmural pressure, since the hydrostatic pressure in the fluid stream method might be negligibly small and since an increase in the hydrostatic pressure alone increased [Ca\(^{2+}\)] only slightly. Thus the fluid stream-induced [Ca\(^{2+}\)] appears to be caused by shear stress and/or membrane stretch, according to the usual classification of mechanical forces (see introduction). It remains, however, controversial as to whether or not pure shear stress by itself induces an increase in [Ca\(^{2+}\)]. For example, the well-defined shear stress from 0.08 to 8 dyn/cm\(^{2}\) produced by the use of a parallel-plate flow chamber caused only a transient increase in [Ca\(^{2+}\)] in BAEC, probably due to a release from the intracellular stores (34). On the other hand, Geiger et al. (4) reported both a transient increase and a sustained elevation of [Ca\(^{2+}\)]. Schilling et al. (31) observed no change in [Ca\(^{2+}\)] in response to shear stress in the range between 2.4 and 25 dyn/cm\(^{2}\) in calf pulmonary artery endothelial cells. Because of the inconsistency of the experimental results among the various investigators, we cannot definitively conclude that the increase in [Ca\(^{2+}\)] observed in the present study is caused by shear stress. Alternatively, shear stress of a much greater amplitude than that tried in the previous studies might be required for an increase in [Ca\(^{2+}\)]. Provided that a laminar flow is maintained at the tip of the polyethylene tube in the present study, flow rates of 1.10 ml/min would have elicited fluid shear stress of 53.0 dyn/cm\(^{2}\) on the cell surface, according to the geometry of the perfusing system (27).

On the other hand, Gd\(^{3+}\), a blocker of nonselective cation channels, suppressed the fluid stream-simulated rise in [Ca\(^{2+}\)]. This is in line with the previous finding that the stretch-induced increase of [Ca\(^{2+}\)] was inhibited with micromolar concentrations of Gd\(^{3+}\) in human umbilical vein endothelial cells (HUVEC) cultured on silicon membranes (21). Plausible nonselective cation channels were found to be activated when the cell membrane was directly distended by negative pressure in the cell-attached patch-clamp condition (15, 29). It is thus possible that the fluid stream causes, more or less, a deformation of the endothelial cell membrane, which may lead to the activation of Ca\(^{2+}\)-permeable cation channels by a common intracellular mechanism on the stretch-induced channel (14).

Although Ca\(^{2+}\)-permeable channels were expected to be active in the fluorescence measurements in the present study, no such conductance could be detected by the patch-clamp method. This finding is in sharp contrast to a clear activation of the nonselective cation current observed in cultured HUVEC, despite the use of an essentially similar method (33); the amplitude was ~300 pA at ~60 mV. We consider that the amplitude of the Ca\(^{2+}\)-permeable current might be too small to be resolved by the patch-clamp method and that the
current could have been masked by an apparent activation of the Cl\(^{-}\) current in the present study. This view might be supported by the finding that the \(E_{\text{rev}}\) (Cl\(^{-}\)) relationship deviated from linearity with decreasing [Cl\(^{-}\)]. Nevertheless, such an undetectable current seems sufficient to produce an increase in [Ca\(^{2+}\)]. For example, Oike et al. (25) reported that the Ca\(^{2+}\) influx caused by depletion of the store was only 1 pA at -80 mV in cultured HUVEC.

Fluid stream-induced ionic currents. The present results strongly suggest that the fluid stream-induced current is carried by Cl\(^{-}\), since the \(E_{\text{rev}}\) of about -30 mV was not largely affected by changes in either [K\(^{+}\)]\(_i\) or the external Na\(^{+}\) concentration but was dependent on [Cl\(^{-}\)]. However, the measured \(E_{\text{rev}}\) of the fluid stream-induced current was shown to be more positive than the \(E_{\text{Cl}}\) (about -40 mV). In addition, the value was more positive in the K\(^{-}\)-free solutions (about -28 mV) than in K\(^{-}\)-free solutions (about -36 mV in K\(^{-}\)-free, Na\(^{+}\)-containing solutions and about -32 mV in K\(^{-}\)-free, Na\(^{-}\)-free solutions). The slightly more positive values in the K\(^{-}\)-free, Na\(^{-}\)-free solutions than in the K\(^{-}\)-free, Na\(^{-}\)-containing solutions might be explained by the junction potential at the tip of the reference electrode (-4.5 mV when we measured the junction potential between HBS and the Na\(^{-}\)-free solution). However, the difference between the K\(^{-}\)-containing solutions and K\(^{-}\)-free solutions cannot be explained by any possible changes in K\(^{+}\) currents, since the configuration of the I-V curves of the fluid stream-induced current was quite similar irrespective of whether the solutions contained K\(^{+}\). The possibility of including an unknown junction potential in our measurements is not probable because we obtained an \(E_{\text{rev}}\) of the inwardly rectifying K\(^{+}\) current using K\(^{-}\)-containing solutions of about -85 mV (Nakao, Ono, and Iijima, unpublished data). We have no ready explanation for these results at present.

In BAEC grown in microcapillary tubes, fluid shear stress activated an inwardly rectifying K\(^{+}\) channel (12, 27). The K\(^{+}\) current developed as a function of shear stress, with half-maximal activation of only 0.7 dyn/cm\(^2\) (27). In the present study, we failed to detect either an increase in K\(^{+}\)-conductance or hyperpolarization in response to the fluid stream, probably because a well-defined shear stress was not achieved by the present method. Although Cl\(^{-}\) current is a major component of fluid stream-induced current in HAEC, the precise mechanisms linking the applied mechanical force to the activation of the Cl\(^{-}\) current are still unknown. The fluid stream-induced Cl\(^{-}\) current could be recorded even though the pipette solution contained 5 mM EGTA. Furthermore, the bathing solutions in all experiments were isotonic. These findings may indicate that the Ca\(^{2+}\)-activated Cl\(^{-}\) channels and/or volume-sensitive Cl\(^{-}\) channels, both of which were found to be present in vascular endothelial cells (22, 23), are not responsible for the fluid stream-stimulated Cl\(^{-}\) current. However, we cannot completely rule out the possibility that the Cl\(^{-}\) current was activated by a rise in [Ca\(^{2+}\)]. It might be argued that 5 mM EGTA is not sufficient to completely prevent an increase in [Ca\(^{2+}\)], and that a local Ca\(^{2+}\) increase would have triggered the activation of a Cl\(^{-}\) current. If this were the case, a delay between the onset of the Cl\(^{-}\) current and the Ca\(^{2+}\) rise (Fig. 6) would thus be expected because fura 2 measurements provide only an estimation averaged over the entire cell. On the other hand, it might be possible that deformation of the endothelial cell membrane, caused by the fluid stream, activates the Cl\(^{-}\) channels via a change in either the intracellular cytoskeleton or related structures. Indeed, some interaction between the cytoskeleton and the channel was suggested to be involved in the activation of swelling- or stretch-activated Cl\(^{-}\) channels in epithelial cells (20), although this was not the case for the volume-sensitive Cl\(^{-}\) current of endothelial cells (26).

In conclusion, we have, for the first time, demonstrated the involvement of the Cl\(^{-}\) current in the endothelial response to a fluid stream. We propose that the activation of the Cl\(^{-}\) current plays a role, at least partly, in modulating the Ca\(^{2+}\) influx by altering the membrane potential of endothelial cells.

We are grateful to Dr. B. Quinn for grammatical reading of the manuscript and to Hitomi Meguro for excellent technical support in cell culture. We also thank Mika Shiraiwa for secretarial service.

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture (J iapan) and in part by the J iapan Heart Foundation and by an IBM J iapan research grant for 1996.

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Received 26 J anuary 1998; accepted in final form 6 October 1998.

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