Flow-activated Chloride Channels in Vascular Endothelium

SHEAR STRESS SENSITIVITY, DESENSITIZATION DYNAMICS, AND PHYSIOLOGICAL IMPLICATIONS*

Received for publication, June 19, 2006, and in revised form, September 6, 2006. Published, JBC Papers in Press, September 13, 2006, DOI 10.1074/jbc.M605866200

Mamta Gautam1, Yue Shen2, Twanda L. Thirkill, Gordon C. Douglas2, and Abdul I. Barakat†1

From the 1Department of Mechanical and Aeronautical Engineering and the 2Department of Cell Biology and Human Anatomy, University of California, Davis, California 95616

Although activation of outward rectifying Cl− channels is one of the fastest responses of endothelial cells (ECs) to shear stress, little is known about these channels. In this study, we used whole-cell patch clamp recordings to characterize the flow-activated Cl− current in bovine aortic ECs (BAECs). Application of shear stress induced rapid development of a Cl− current that was effectively blocked by the Cl− channel antagonist 5-nitro-2-(3-phenylpropylamino)benzoic acid (100 μM). The current initiated at a shear stress as low as 0.3 dyne/cm², attained its peak within minutes of flow onset, and saturated above 3.5 dynes/cm² (∼2.5–3.5-fold increase over pre-flow levels). The Cl− current desensitized slowly in response to sustained flow, and step increases in shear stress elicited increased current only if the shear stress levels were below the 3.5 dynes/cm² saturation level. Oscillatory flow with a physiological oscillation frequency of 1 Hz, as occurs in disturbed flow zones prone to atherosclerosis, failed to elicit the Cl− current, whereas lower oscillation frequencies led to partial recovery of the current. Nonreversing pulsatile flow, generally considered protective of atherosclerosis, was as effective in eliciting the current as steady flow. Measurements using fluids of different viscosities indicated that the Cl− current is responsive to shear stress rather than shear rate. We also show that the Cl− channel-mediated hyperpolarization and leads to cell membrane depolarization (11, 12). Although characteristics of flow-sensitive K+ channels, including their unitary conductance, dependence on the magnitude of applied shear stress, and regulation, have been reported in previous studies (9, 13, 14), little is known about flow-activated Cl− channels.

Shear stress rapidly phosphorylates the serine/threonine kinase Akt (also called protein kinase B or Rac kinase) (15, 16). Akt phosphorylation regulates endothelial nitric-oxide synthase activation, integrin-mediated signaling, matrix adhesion, and suppression of cell apoptosis (17–19). The mechanisms governing Akt activation by shear stress are not known.

In this study, we used the whole-cell patch clamp technique in bovine aortic ECs (BAECs) to establish the sensitivity of the flow-activated Cl− current to the amplitude of applied shear stress, probe the desensitization characteristics of the current, investigate the responsiveness of the current to sudden changes in shear stress, and demonstrate that the current is responsive to shear stress rather than shear rate. We also show that the Cl− current regulates shear stress-induced Akt phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Flow Experiments—BAECs procured from Cell Systems Corp. (Kirkland, WA) were cultured in Dulbecco’s modified Eagle’s medium/F-12 nutrient media supplemented with antibiotics (penicillin/streptomycin) and 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) and used in passage 4–7. For patch clamp experiments, cells were plated in 1 × 1-mm square cross-section borosilicate glass capillary tubes (Vitrocom, Mountain Lakes, NJ). As detailed elsewhere (20), this flow system allows determination of the shear stress to which BAECs are exposed during recording. Cells located near one end of the capillary tube were used for patch clamp recordings, whereas the other end was connected to a syringe pump (Cole Parmer) capable of generating either steady or purely

The responsiveness of vascular endothelial cells (ECs) to fluid mechanical shear forces is essential for vasoregulation and arterial wall remodeling (1, 2). Furthermore, the fact that ECs respond differently to different types of shear stress is likely the basis for the localization of early atherosclerotic lesions in arterial regions exposed to low and/or oscillatory shear stress (3, 4).
oscillatory flow (zero net flow rate) at prescribed flow rates (and hence known wall shear stresses) and frequencies. Nonreversing pulsatile flow was generated by combining the outputs of a syringe pump supplying steady flow with another syringe pump supplying oscillatory flow.

Western Blot Analysis for Akt Phosphorylation—For Akt studies, BAECs were plated on tissue culture-treated plastic slides (Permanox, Nalge Nunc International, Naperville, IL) and starved for 12 h prior to experiments in Dulbecco’s modified Eagle’s medium/F-12 containing 0.1% fatty acid-free bovine serum albumin (Gemini Bio-Products, Woodland, CA) and 1% penicillin (10,000 IU/ml) and streptomycin (10,000 µg/ml). Steady and oscillatory flow Akt studies were performed in a parallel plate flow chamber as described elsewhere (21). Flow periods ranged from 5 to 60 min. After removal from the flow chamber, the cells were washed twice in ice-cold phosphate-buffered saline. The cells were then disrupted with lysis buffer containing 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate (MP Biomedicals Inc., Aurora, OH), 1 mM sodium vanadate, 0.1% protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, and 1 µM microcystin (MP Biomedicals Inc.). The cell lysates were then centrifuged at 14,000 x g for 15 min at 4 °C. The protein concentration of each sample supernatant was determined by the modified Lowry’s method (22). Equal amounts of protein were resolved in 8% SDS-PAGE and blot ted onto nitrocellulose membranes (Bio-Rad). After blocking with 5% bovine serum albumin, the membranes were incubated (1 h at room temperature) with phospho-specific polyclonal antibodies that recognize the phosphorylated site Ser(P)473 in Akt (Cell Signaling Technology, Beverly, MA). After incubation with secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit IgG), the blots were incubated with SuperSignal® West Pico chemiluminescence substrate (Pierce) and exposed to x-ray film. The membranes were then stripped with Restore™ Western blot stripping buffer (Pierce) and re-probed with total Akt antibody (Cell Signaling Technology) as an internal control. Densities of the bands were analyzed using Kodak 1D image analysis software (Eastman Kodak Co.). Band densities of phosphorylated Akt were expressed relative to the density of the total Akt level.

Electrophysiological Measurements—Cl⁻ currents from BAECs were recorded using the whole-cell voltage clamp technique. Currents were recorded by applying the following voltage ramp protocol; the voltage was maintained at a holding potential of −70 mV for 30 ms, followed by a step to +100 mV for 40 ms, followed by a ramp to −150 mV over 400 ms, and then a step back to the holding potential. The inter-ramp interval was 5 s. Currents were recorded for 40 s before flow onset to ensure a stable pre-flow baseline. For data analyses, all currents were normalized to the "pre-flow" current value measured immediately prior to flow initiation. After making whole-cell capacitance compensation, the currents were recorded using an Axopatch 200B patch clamp amplifier running pCLAMP9 software (Axon Instruments, Union City, CA). Acquired currents were filtered at 1 kHz and sampled at 2 kHz. Patch pipettes were pulled from borosilicate glass tubing (Sutter Instrument Co., Novato, CA) and heat-polished at the tip to give a resistance of 2–3 megohms when filled with pipette solution.

Reagents and Solutions—Unless otherwise stated, chemicals were purchased from Sigma. For patch clamp experiments, the external solution contained the following (in mM): 140 NaCl, 2 CaCl₂, 10 HEPES, and 10 glucose, pH 7.4, with 1 mM NaOH. The pipette (internal) solution contained the following (in mM): 140 cesium glutamate (to block flow-activated K⁺ channels), 1 CaCl₂, 10 HEPES, and 10 EGTA, pH 7.4, with 1 mM CsOH. Both internal and external solutions had measured osmolalities of 310 mOsm. In some experiments, the viscosity of the external solution was increased using dextran (MW = 100,000–200,000) and was measured using a Cannon-Fenske routine viscometer.

**Activation of whole-cell Cl⁻ current by steady shear stress in BAECs.** A, sample recording showing the time evolution of the Cl⁻ current in response to a shear stress of 3.5 dynes/cm². B, average time evolution of the Cl⁻ current activated by a shear stress of 3.5 dynes/cm² (n = 8). C, sample Cl⁻ current recording in response to a shear stress of 3.5 dynes/cm² in the presence of the Cl⁻ channel blocker NPPB (100 µM). D, effect of NPPB (100 µM) on the flow-activated Cl⁻ current. Results are based on recordings at a shear stress of 3.5 dynes/cm² in the absence (n = 6) or presence (n = 6) of NPPB. Data in B and D are means ± S.E., and * denotes statistically significant difference (p < 0.05) relative to pre-flow values.
Flow-activated Chloride Currents in Endothelial Cells

FIGURE 2. Dependence of Cl\(^{-}\) current amplitude on shear stress level. A, representative current traces indicating the flow-activated current in response to steady shear stresses of 0.3, 3.5, and 14 dynes/cm\(^2\). B, peak Cl\(^{-}\) current (mean ± S.E.) measured 2 min after flow onset as a function of applied shear stress. Data are for shear stress levels of 0.3 (n = 6), 3.5 (n = 9), and 14 (n = 5) dynes/cm\(^2\). * denotes statistically significant difference (p < 0.05) relative to pre-flow values.

Data Analyses and Statistics—Data are presented as mean ± S.E. Patch clamp data analyses were carried out using Clampfit (Axon Instruments) and Origin 7 software (Northampton, MA). For patch clamp data, statistical comparison of means was performed using either the Wilcoxon matched pairs test and one-way analysis of variance followed by a post hoc test (multi-group comparison) or unpaired t test (two-group comparison in Fig. 7). For Western blots, one-way repeated-measure analysis of variance and then began desensitization; however, the extent of desensitization varied among cells (Fig. 3A). We had demonstrated in previous studies that the flow-induced outward current is Cl\(^{-}\)-specific (11, 12). This was confirmed in this study; in the presence of the Cl\(^{-}\) channel blocker NPPB (100 μM), the flow-induced current was completely inhibited (Fig. 1, C and D). The flow-activated inward-rectifying K\(^{+}\) current (10, 14, 15) was not observed because all recordings were performed in the presence of the K\(^{+}\) channel blocker cesium in the pipette solution (see “Experimental Procedures”).

Effect of Shear Stress Magnitude on Cl\(^{-}\) Current—To determine the sensitivity of the flow-activated Cl\(^{-}\) current to shear stress magnitude, the current amplitude induced by a 2-min application of a steady shear stress in the range 0.3–14 dynes/cm\(^2\) was recorded. As shown in Fig. 2, A and B, a shear stress as small as 0.3 dynes/cm\(^2\) elicited a Cl\(^{-}\) current whose peak amplitude was significantly larger than pre-flow levels (1.2 ± 0.06-fold increase; p < 0.05). Shear stresses of 1.1 and 3.5 dynes/cm\(^2\) led to further progressive increases in the Cl\(^{-}\) current to 1.7 ± 0.2 and 2.3 ± 0.3 times pre-flow levels, respectively. The current saturated above 3.5 dynes/cm\(^2\) as shear stresses of 7 and 14 dynes/cm\(^2\) failed to elicit larger currents.

Desensitization Characteristics of Cl\(^{-}\) Current—The experiments described thus far involved exposure of BAECs to short flow episodes. To probe possible desensitization of the flow-activated Cl\(^{-}\) current in response to sustained flow, we performed recordings on BAECs subjected to a steady shear stress of 3.5 dynes/cm\(^2\) for a period of 10 min. This shear stress level was selected because it corresponds to maximal current activation (Fig. 2B). In the presence of sustained flow, the Cl\(^{-}\) current attained a peak 208 ± 27 s (range 105–395 s) after flow initiation and then began desensitizing; however, the extent of desensitization varied among cells (Fig. 3A). To quantify this variability, we define a dimensionless “desensitization index” (DI) as DI = (|I\(_f\) - I\(_m\)|)/(I\(_m\) - I\(_0\)|), where I\(_f\) and I\(_m\) respectively, denote the normalized Cl\(^{-}\) current at the end and beginning of the 10-min recording period, and I\(_m\) denotes the maximum normalized current during the recording period. Thus, DI ranges from 0 to 1 with DI = 0 denoting complete desensitization, and DI = 1 indicating no desensitization. As illustrated in the histogram in Fig. 3B, the desensitization was very pro-

(Industrial Research Glassware Ltd., Roselle, NJ). The viscosities of solutions containing 0, 5, and 10% dextran were 0.95, 2.8, and 6.62 cP, respectively.

RESULTS

Activation of Cl\(^{-}\) Current by Steady Shear Stress—In virtually all BAECs patched, steady shear stress elicited whole-cell outward currents that initiated immediately upon flow onset and developed relatively slowly (Fig. 1, A and B). We had demonstrated in previous studies that the flow-induced outward current is Cl\(^{-}\)-specific (11, 12). This was confirmed in this study; in the presence of the Cl\(^{-}\) channel blocker NPPB (100 μM), the flow-induced current was completely inhibited (Fig. 1, C and D). The flow-activated inward-rectifying K\(^{+}\) current (10, 14, 15) was not observed because all recordings were performed in the presence of the K\(^{+}\) channel blocker cesium in the pipette solution (see “Experimental Procedures”).

Effect of Shear Stress Magnitude on Cl\(^{-}\) Current—To determine the sensitivity of the flow-activated Cl\(^{-}\) current to shear stress magnitude, the current amplitude induced by a 2-min application of a steady shear stress in the range 0.3–14 dynes/cm\(^2\) was recorded. As shown in Fig. 2, A and B, a shear stress as small as 0.3 dynes/cm\(^2\) elicited a Cl\(^{-}\) current whose peak amplitude was significantly larger than pre-flow levels (1.2 ± 0.06-fold increase; p < 0.05). Shear stresses of 1.1 and 3.5 dynes/cm\(^2\) led to further progressive increases in the Cl\(^{-}\) current to 1.7 ± 0.2 and 2.3 ± 0.3 times pre-flow levels, respectively. The current saturated above 3.5 dynes/cm\(^2\) as shear stresses of 7 and 14 dynes/cm\(^2\) failed to elicit larger currents.

Desensitization Characteristics of Cl\(^{-}\) Current—The experiments described thus far involved exposure of BAECs to short flow episodes. To probe possible desensitization of the flow-activated Cl\(^{-}\) current in response to sustained flow, we performed recordings on BAECs subjected to a steady shear stress of 3.5 dynes/cm\(^2\) for a period of 10 min. This shear stress level was selected because it corresponds to maximal current activation (Fig. 2B). In the presence of sustained flow, the Cl\(^{-}\) current attained a peak 208 ± 27 s (range 105–395 s) after flow initiation and then began desensitizing; however, the extent of desensitization varied among cells (Fig. 3A). To quantify this variability, we define a dimensionless “desensitization index” (DI) as DI = (|I\(_f\) - I\(_m\)|)/(I\(_m\) - I\(_0\)|), where I\(_f\) and I\(_m\) respectively, denote the normalized Cl\(^{-}\) current at the end and beginning of the 10-min recording period, and I\(_m\) denotes the maximum normalized current during the recording period. Thus, DI ranges from 0 to 1 with DI = 0 denoting complete desensitization, and DI = 1 indicating no desensitization. As illustrated in the histogram in Fig. 3B, the desensitization was very pro-
nounced (DI < 0.25) in 6 of 13 cells. In 3 cells, the desensitization was moderate (0.25 ≤ DI ≤ 0.75). Finally, in the remaining 4 cells, the desensitization was modest (DI > 0.75).

Response of Cl\textsuperscript{−} Current to Step Changes in Shear Stress—In all experiments thus far, BAECs were subjected to a single shear stress level. Because shear stress levels in vivo can change suddenly in response to changes in stress and/or activity level, we studied the response of the flow-activated Cl\textsuperscript{−} current to step changes in shear stress. The cells were subjected to three consecutive and progressively increasing shear stress levels, each of which was imposed for 120 s. Flow was interrupted briefly (10 s) between shear stress steps to change flow rate settings. Because of the shear stress dependence of the Cl\textsuperscript{−} current (Fig. 2B), two sets of step flow experiments were conducted as follows: the first at shear stresses that elicit submaximal current where the cells were subjected to steps of 0.3, 1.1, and 3.5 dynes/cm\textsuperscript{2}; the second set at shear stresses that induce maximal current where the cells were exposed to steps of 3.5, 7, and 14 dynes/cm\textsuperscript{2}.

The results demonstrate that the Cl\textsuperscript{−} current response to step changes in shear stress is different for the two sets of experiments. For the first set, where the shear stresses were below the level that elicits maximal current, the amplitude of the Cl\textsuperscript{−} current increased with each step in shear stress (Fig. 4A). On the other hand, recordings from the second set, where the shear stresses were above the level that induces maximal current, demonstrated current desensitization following the first flow stimulus and consequent insensitivity to subsequent shear stress steps (Fig. 4B). These results suggest that flow-activated Cl\textsuperscript{−} currents in ECs respond to sudden changes in shear stress only if the shear stress levels are below those eliciting maximal current.

Response of Cl\textsuperscript{−} Current to Repeated Flow Episodes—Under certain conditions in vivo, such as during ischemia-reperfusion, ECs experience an interruption in flow followed by flow resumption. To probe the impact of such a situation on the flow-activated Cl\textsuperscript{−} current, we recorded the current in BAECs subjected to two 2-min flow episodes separated by a 160-s no-flow period. For all experiments, the shear stress associated with the first flow episode was 3.5 dynes/cm\textsuperscript{2}, whereas the shear stress for the second episode was 3.5, 7, or 14 dynes/cm\textsuperscript{2}. The results indicate that in all cases, the first flow episode elicited a significant current (~2–3.5-fold increase above pre-flow levels) consistent with the results presented above (Fig. 2B). The second flow episode, however, stimulated a significant current only when it was at a higher shear stress than the first episode (Fig. 5). Whenever a Cl\textsuperscript{−} current was stimulated by the second
Flow-activated Chloride Currents in Endothelial Cells

Flow-activated Chloride Currents in Endothelial Cells

Desensitized after the first episode and is therefore not responsive to a second flow cycle at the same shear stress level as the first, whereas a second cycle at a higher shear stress does elicit limited current.

Response of Cl⁻ Current to Unsteady Shear Stress—Flow in large arteries is pulsatile; therefore, arterial ECs are exposed to highly unsteady flow. Although most arterial sites experience nonreversing pulsatile flow, flow in local regions near branches and bifurcations may be purely oscillatory. Interestingly, arterial sites subjected to oscillatory flow are especially prone to the development of early atherosclerotic lesions, whereas regions exposed to nonreversing pulsatile flow remain largely spared (3, 4). We compared the effects of a steady shear stress of 3.5 dynes/cm², a 1-Hz nonreversing pulsatile shear stress of 3.5 ± 3.5 dynes/cm², and an oscillatory shear stress of 0 ± 3 dynes/cm² with frequencies in the range 0.04–1 Hz on the flow-activated Cl⁻ current.

The results show that both steady and nonreversing pulsatile flow induced significant and comparable Cl⁻ currents in BAECs (Fig. 6). More specifically, steady flow led to a peak current whose amplitude was 2.3 ± 0.3-fold that of the pre-flow current; whereas the equivalent value for nonreversing pulsatile flow was 2.5 ± 0.3. On the other hand, the effect of oscillatory flow depended on the frequency of oscillation. Although oscillatory flow with a physiological frequency of 1 Hz failed to elicit the Cl⁻ current (normalized current of 1.1 ± 0.1), subphysiological frequencies of 0.04 and 0.2 Hz elicited significant currents (1.4 ± 0.1 and 1.7 ± 0.2, respectively), although this current was smaller than that activated by either steady or 1-Hz nonreversing pulsatile flow.

Cl⁻ Channel Responsiveness to Shear Stress Versus Shear Rate—We asked whether the activation of Cl⁻ channels by flow was a result of the “direct” impact of the fluid mechanical force on the channels or channel-associated structures (a shear stress effect) or an “indirect” effect of flow on the delivery and/or removal of soluble factors to the cell surface (a shear rate effect). To address this question, we measured Cl⁻ currents induced by steady flow at a given flow rate after changing the viscosity of the external solution using different dextran concentrations. This approach has been used previously to demonstrate that the extent of intracellular calcium mobilization in ECs correlates with shear stress rather than shear rate (23). Maintaining the flow rate constant with solutions of different viscosities maintains the same shear rate while providing different shear stress levels. The following three types of solution were used: 0% dextran (normal external solution) whose viscosity was measured as 0.95 cP, 5% dextran solution (2.8 cP), and 10% dextran solution (6.6 cP).

The results demonstrate that the magnitude of flow-induced Cl⁻ current correlates with shear stress rather than shear rate (Fig. 7). For instance, whereas a flow rate of 0.25 ml/min in 0% dextran solution (corresponding to a shear rate of 0.3 s⁻¹ and a shear stress of 0.3 dyne/cm²) elicits minimal current (1.2 ± 0.1-fold increase over pre-flow levels), the same shear rate in 10% dextran solution (shear stress of 2.1 dyne/cm²) induces a significantly larger current (2.4 ± 0.3-fold increase). Similarly, at a flow rate of 1 ml/min (shear rate of 1.2 s⁻¹), a shear stress of 1.1 dynes/cm² elicits a smaller current than either 3.5 or 7 dynes/cm².
Flow-activated Chloride Currents in Endothelial Cells

Physiological Implications of Shear Stress-induced Cl\(^{-}\) Channel Activation—To understand the role of flow-sensitive Cl\(^{-}\) channels in regulating downstream flow responses in ECs, we probed the involvement of these channels in shear stress-induced Akt phosphorylation. BAECs exposed to a steady shear stress of 15 dynes/cm\(^2\) for 5, 15, 30, and 60 min exhibited a time-dependent change in Akt phosphorylation at the site Ser\(^{473}\) (Fig. 8A). After 5 min of flow, Akt phosphorylation increased 3.8 ± 0.9-fold relative to cells in static culture. This value remained essentially constant (3.5 ± 0.8) 15 min after the onset of flow but eventually decreased to base-line levels by 60 min. At all time points except 60 min, Akt phosphorylation levels were significantly higher than those of static control (\(p < 0.05\)). These results are similar to those reported previously (16).

To test the involvement of flow-sensitive Cl\(^{-}\) channels in shear stress-induced Akt phosphorylation, we studied Akt phosphorylation in BAECs subjected to 1-Hz oscillatory flow, which, as already shown (Fig. 6), fails to elicit the Cl\(^{-}\) current. A shear stress of 0 ± 15 dynes/cm\(^2\) applied for 5–60 min resulted in a significantly smaller increase in Akt phosphorylation than that induced by steady flow (Fig. 8B). More specifically, at the 5-min time point, oscillatory flow elicited a 1.7 ± 0.2-fold increase in Akt phosphorylation (versus 3.8 ± 0.9 for steady flow, \(p < 0.05\)). Similar results were obtained at the 15- and 30-min time points (1.5 ± 0.2 versus 3.5 ± 0.8, \(p < 0.05\); 1.3 ± 0.1 versus 3.2 ± 0.5, \(p < 0.05\)). These results support an involvement of flow-sensitive Cl\(^{-}\) channels in the regulation of shear stress-induced Akt phosphorylation.

We also probed the impact of NPPB (100 \(\mu M\)), which blocks flow-activated Cl\(^{-}\) channels (Fig. 1), on shear stress-induced Akt phosphorylation (Fig. 8C). Application of a steady shear stress of 15 dynes/cm\(^2\) for 15 min in the presence of NPPB induced only a 1.6 ± 0.4-fold increase in Akt phosphorylation relative to control cells in static culture versus a 3.1 ± 0.9-fold increase in the absence of NPPB (\(p < 0.05\)). On the other hand, blocking flow-sensitive K\(^{+}\) channels with Ba\(^{2+}\) (100 \(\mu M\)) had no effect on shear stress-induced Akt phosphorylation (4.5 ± 1.4-fold increase relative to control cells in static culture in the presence of Ba\(^{2+}\) versus 3.9 ± 1.2 in the absence of Ba\(^{2+}\), \(p > 0.05\)). Incubating the cells in the ion channel blockers in the absence of flow had no impact on Akt phosphorylation. These observations further support a role for flow-sensitive Cl\(^{-}\) channels (but not K\(^{+}\) channels) in regulating shear stress-induced Akt phosphorylation in BAECs.

DISCUSSION

Flow-sensitive ion channels play an important role in shear stress sensing in vascular endothelium (5, 8). Although previous studies have shown that flow-activated Cl\(^{-}\) channels are stimulated immediately upon the onset of flow and that activation of these channels leads to EC membrane depolarization that antagonizes K\(^{+}\) channel-mediated hyperpolarization (11), little is known about the sensitivity of these channels to shear stress...
and the physiological implications of their activation. In this study, we used whole-cell patch clamp recordings to characterize the dependence of the Cl\(^-\) current in BAECs on the level and type of applied shear stress, the desensitization kinetics of the current, and the responsiveness of the current to steps in shear stress as well as to repeated shear stress episodes. We also studied the role of this current in regulating shear stress-induced Akt phosphorylation.

Our results demonstrate that flow-sensitive Cl\(^-\) channels are activated by a shear stress as small as 0.3 dyne/cm\(^2\), a value largely similar to that previously reported for flow-activated K\(^+\) channels (9). However, unlike the K\(^+\) current that saturates at \(\sim 10–15\) dynes/cm\(^2\), Cl\(^-\) current saturation occurs at \(\sim 3.5\) dynes/cm\(^2\). In medium and large arteries, the mean physiological shear stress in regions of undisturbed flow is \(\sim 10–20\) dynes/cm\(^2\), and the mean shear stress in disturbed flow regions that are prone to atherosclerosis is considerably lower (0–5 dynes/cm\(^2\)) (3, 4). We propose that the difference in saturation shear stress between Cl\(^-\) and K\(^+\) channels may point to a system where the two channels function cooperatively to provide sensitivity to a broader shear stress range than would either channel alone. Within this context, Cl\(^-\) channels would be primarily responsible for sensing relatively low shear stress levels, whereas K\(^+\) channels would sense relatively high shear stress levels.

Our results also demonstrate that although the Cl\(^-\) current initiates immediately upon flow onset, it develops slowly and attains its peak only after \(\sim 200\) s. The slow development of flow-sensitive Cl\(^-\) currents is consistent with the observation made in previous studies (11, 12) that although both K\(^+\) and Cl\(^-\) channels are activated immediately upon the onset of flow, K\(^+\) channel-mediated membrane hyperpolarization precedes Cl\(^-\) channel-mediated depolarization, despite the larger electrochemical gradient for Cl\(^-\) than for K\(^+\).

In response to sustained flow, BAECs exhibit varying degrees of Cl\(^-\) current desensitization. The basis for the variability in desensitization behavior among cells remains to be determined but is not related to cell passage or time in culture. Indeed, it is unclear if the limited Cl\(^-\) current desensitization observed in some cells reflects a fundamental difference in these cells or simply slower desensitization dynamics. In principle, recording Cl\(^-\) currents in cells subjected to even longer periods of sustained flow than those used here would
Flow-activated Chloride Currents in Endothelial Cells

address this question; however, this is logistically impractical because of the difficulty in maintaining a whole-cell seal for longer time periods. In other systems, there is evidence for the existence of cellular subpopulations exhibiting different desensitization characteristics. For instance, nicotinic acetylcholine receptors in rat sympathetic ganglion neurons exhibit rapid desensitization in 90% of the cells, whereas the remaining 10% show slower desensitization kinetics (24). In that case, the two distinct desensitization patterns are because of differences in subunit composition of the channels. We cannot exclude the possibility that somewhat analogous differences might be present in flow-activated Cl\(^{-}\) channels in ECs. Another possibility for the differences in desensitization characteristics among BAECs is that desensitization does occur in all cells but that cell-to-cell differences exist in shear stress threshold required for its occurrence.

Our results show that step increases in shear stress induce increased Cl\(^{-}\) current only if the shear stress levels fall below those leading to maximal current (∼3.5 dynes/cm\(^2\)). Above the saturation shear stress, the current is nonresponsive to step increases in shear stress and exhibits desensitization similar to that observed for sustained flow at a single shear stress level. If this behavior occurs in vivo, then sudden increases in shear stress associated with changes in stress and/or activity level would only be expected to elicit alterations in Cl\(^{-}\) current if the shear stress excursions fall below the saturation levels defined in this study.

Cessation of flow results in slow and progressive decrease of the Cl\(^{-}\) current toward pre-flow base-line levels. Cells stimulated with flow during this recovery period are only minimally responsive to flow and only if the stimulation is at a higher shear stress than the initial stimulus. Flow stoppage and subsequent resumption occur during episodes of ischemia-reperfusion in vivo. The present results suggest that for short periods of flow interruption, reperfusion would be expected to elicit only minimal Cl\(^{-}\) currents. On the other hand, if the flow cessation period were sufficiently long, then the Cl\(^{-}\) channels would resensitize and recover their full responsiveness to flow.

Arterial regions exposed to oscillatory flow are particularly prone to the development of atherosclerosis, whereas regions exposed to nonreversing pulsatile flow are largely protected (3, 4). We have recently reported that although flow-sensitive K\(^{+}\) channels are equally responsive to steady flow as they are to oscillatory flow with a physiological oscillation frequency of 1 Hz, Cl\(^{-}\) channels respond to steady flow but are largely insensitive to 1-Hz oscillatory flow (12). In this study, we have confirmed the nonresponsiveness of the Cl\(^{-}\) current to 1-Hz oscillatory flow. Stimulating BAECs with oscillatory flow at subphysiological frequencies (0.04 or 0.2 Hz) leads to partial recovery of the Cl\(^{-}\) current. This is not surprising because in the limit of very low frequencies, oscillatory flow approaches steady flow. Consistent with previous observations in coronary ECs using membrane potential sensitive fluorescent dyes (25), our results have also shown that 1-Hz nonreversing pulsatile flow has the same effect on the Cl\(^{-}\) current as steady flow.

Phosphorylation of the serine/threonine kinase Akt plays an important role in regulating fundamental aspects of EC function, including endothelial nitric-oxide synthase activation, integrin signaling, and cell apoptosis (17-20). Previous studies have established that Akt phosphorylation is regulated by steady shear stress (15, 16). The present results show that pharmacologically blocking flow-sensitive Cl\(^{-}\) channels significantly attenuates shear stress-induced Akt phosphorylation. However, there is no significant change in Akt activation when flow-sensitive K\(^{+}\) channels are blocked. Furthermore, 1-Hz oscillatory flow, which correlates in vivo with the development of early atherosclerotic lesions and activates flow-sensitive K\(^{+}\) channels but fails to stimulate Cl\(^{-}\) channels, elicits significantly smaller Akt phosphorylation than steady flow. These observations are consistent with the notion that shear stress-induced Akt phosphorylation occurs through mechanisms that are dependent on flow-sensitive Cl\(^{-}\) channels but not K\(^{+}\) channels. If confirmed in vivo, these findings would suggest that activation of flow-sensitive Cl\(^{-}\) channels might play an important protective role against atherosclerosis. This issue naturally merits future investigation.

Our studies also indicate that the extent of Cl\(^{-}\) current activation correlates with the level of applied shear stress and not the applied shear rate. This finding suggests that the impact of flow on Cl\(^{-}\) channels is direct rather than through an effect on the convective and/or diffusive delivery or removal of specific fluid-borne agonists. Interestingly, the extent of mobilization of intracellular calcium in ECs has also been shown to be responsive to shear stress rather than shear rate (23). It remains unclear how shear stress may directly activate Cl\(^{-}\) channels. As has been described elsewhere (8, 26), if the extracellular domain of a Cl\(^{-}\) channel is modeled as a sphere with a diameter of ∼10 nm that is adherent to a planar surface (the EC surface) and is subjected to Stokes flow at a physiological shear stress of 1–10 dynes/cm\(^2\), then the energy imparted to the channel is expected to be 2–3 orders of magnitude smaller than the thermal energy of the channel. Therefore, the fluid mechanical force would not be “felt” by the channel, which renders it unlikely that channel activation occurs as a result of a direct conformational change induced by the applied fluid mechanical force. Rather, a more likely pathway might be channel gating through an effect of flow on cell membrane tension, membrane subdomains to which the channels are directly coupled, or intracellular cytoskeletal elements and/or cytoskeleton-associated structures.

The molecular identity of flow-sensitive Cl\(^{-}\) channels remains to be determined. Previous studies have demonstrated that volume-regulated anion channels in BAECs are also responsive to shear stress and that these channels desensitize following repeated shear stimulation (27); however, volume-regulated anion channels appear to require both hypotonic conditions and ATP for activation. The Cl\(^{-}\) current in this study occurs under iso-osmotic conditions and in the absence of ATP in the patch pipette solution. Efforts aimed at cloning flow-activated Cl\(^{-}\) channels would significantly enhance our understanding of the role of these channels in regulating EC function under both normal and pathological conditions.

Acknowledgment—We thank Prof. Kathryn McCarthy for help in making the viscosity measurements.
REFERENCES
1. Pohl, U., Holtz, J., Busse, R., and Bassenge, E. (1986) *Hypertension* **8**, 37–44
2. Langille, B. L., and O’Donnell, F. (1986) *Science* **231**, 405–407
3. Nerem, R. M. (1992) *J. Biomech. Eng.* **114**, 274–282
4. Ku, D. N., Giddens, D. P., Zarins, C. K., and Glagov, S. (1985) *Arteriosclerosis* **5**, 293–302
5. Davies, P. F. (1995) *Physiol. Rev.* **75**, 519–560
6. Garcia-Cardena, G., Comander, J., Anderson, K. R., Blackman, B. R., and Gimbrone, M. A., Jr. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 4478–4485
7. Li, Y. S., Haga, J. H., and Chien, S. (2005) *J. Biomech.* **38**, 1949–1971
8. Barakat, A. I., Lieu, D. K., and Gojova, A. (2006) *Biomaterials* **27**, 671–678
9. Olesen, S. P., Clapham, D. E., and Davies, P. F. (1988) *Nature* **331**, 168–170
10. Nakache, M., and Gaub, H. E. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1841–1843
11. Barakat, A. I., Leaver, E. V., Pappone, P. A., and Davies, P. F. (1999) *Circ. Res.* **85**, 820–828
12. Lieu, D. K., Pappone, P. A., and Barakat, A. I. (2004) *Am. J. Physiol.* **286**, C1367–C1375
13. Hoger, J. H., Ilyin, V. I., Forsyth, S., and Hoger, A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 7780–7785
14. Jacobs, E. R., Cheliakine, C., Gebremedhin, D., Birks, E. K., Davies, P. F., and Harder, D. R. (1995) *Pfluegers Arch.* **431**, 129–131
15. Dimmeler, S., Assmus, B., Hermann, C., Haendeler, J., and Zeiher, A. M. (1998) *Circ. Res.* **83**, 334–341
16. Go, Y. M., Boo, Y. C., Park, H., Maland, M. C., Patel, R., Pritchard, K. A., Jr., Fujio, Y., Walsh, K., Darley-Usmar, V., and Jo, H. (2001) *J. Appl. Physiol.* **91**, 1574–1581
17. Dimmeler, S., Fleming, L., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) *Nature* **399**, 601–605
18. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) *Cell* **88**, 435–437
19. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997) *EMBO J.* **16**, 2783–2793
20. Wiesner, T. F., Berk, B. C., and Nerem, R. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3726–3731
21. Suvatne, J., Barakat, A. I., and O’Donnell, M. E. (2001) *Am. J. Physiol.* **280**, C216–C227
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
23. Ando, J., Ohtsuka, A., Korenaga, R., Kawamura, T., and Kamiya, A. (1993) *Biochem. Biophys. Res. Commun.* **190**, 716–723
24. Britt, J. C., and Brenner, H. R. (1997) *Pfluegers Arch.* **434**, 38–48
25. Qiu, W. P., Hu, Q., Paolocci, N., Ziegelstein, R. C., and Kass, D. A. (2003) *Am. J. Physiol.* **285**, H341–H346
26. Barakat, A. I. (2001) *J. Theor. Biol.* **210**, 221–236
27. Romanenko, V. G., Davies, P. F., and Levitan, I. (2002) *Am. J. Physiol.* **282**, C708–C718