A Flow-Activated Chloride-Selective Membrane Current in Vascular Endothelial Cells

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Abstract—Shear stress–induced activation of endothelial ion channels, one of the earliest responses to flow, is implicated in mechanosignal transduction that results in the regulation of vascular tone. The effects of laminar flow on endothelial membrane potential were studied in vitro using both fluorescent potentiometric dye measurements and whole-cell patch-clamp recordings. The application of flow stimulated membrane hyperpolarization, which was reversed to depolarization within 35 to 160 seconds. The depolarization was caused by a Cl⁻–selective membrane current activated by flow independently of the K⁺ channel-mediated hyperpolarization. Thus, flow activated both K⁺ and Cl⁻ currents, with the net membrane potential being determined by the balance of the responses. Membrane potential sensitivity to flow was unchanged by flow preconditioning that elongated and aligned the cells. (Circ Res. 1999;85:820-828.)

Key Words: mechanotransduction □ shear stress □ ion channel □ potentiometric dye □ patch clamp

The endothelium is a biological mechanotransducer that senses shear and stretch forces associated with blood flow and converts these mechanical stimuli into biochemical signals.1–3 In vivo, endothelial mechanotransduction is central for both acute vasoregulation and chronic vascular remodeling.4,5 In vitro studies have demonstrated that fluid mechanical shear stress elicits acute endothelial responses that include activation of ion channels6 and G proteins,7–9 mobilization of intracellular calcium,10,11 and induction of protein kinase pathways.12 Subsequent transcription factor activation13 and binding to shear stress response–related sequences of several flow-responsive genes1 results in important functional and structural changes in the cells that include extensive topographic and cytoskeletal reorganization, cellular elongation, and alignment in the direction of shear stress.14–16

The mechanisms that initiate shear stress signaling are poorly understood. They may arise from discrete surface molecules that undergo deformation at the luminal surface or from an integrated change of intracellular tension,1 or both. An important very early response to flow, however, is the activation of a K⁺ current that hyperpolarizes the cell16,17 and is driven by shear stress–responsive inward-rectifying K⁺ channels present on the luminal surface of cultured endothelium.18 In studies using membrane potential–sensitive dyes, we noted a biphasic endothelial response to the initiation of flow in which membrane hyperpolarization was quickly attenuated and reversed to depolarization.19 In this paper, using both whole-cell patch-clamp recordings and measurements from potentiometric fluorescent dyes, we demonstrate the presence of a shear stress–induced chloride-selective current in endothelial cells that antagonizes flow-induced hyperpolarization. The membrane potential responsiveness to flow persists in flow-conditioned cells that have undergone significant topographic and cytoskeletal reorganization.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (BAECs) were isolated and cultured by standard procedures.20 Cells in passages 15 to 25 were used. After trypsinization, the cell suspension was plated at subconfluent density either onto standard square (22×22-mm) glass coverslips (patch-clamp experiments) or into 1×1-mm square cross-section borosilicate glass capillary flow tubes6 (VitroCom, Inc) (potentiometric dye measurements). The cells were bathed in DMEM (high sucrose; Gibco) containing 10 mmol/L HEPES, 2 mmol/mL glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated calf serum (Gibco).

Reagents

The membrane potential–sensitive fluorescent dye bis(1,3-diethyliothiobarbiturate)trimethine oxonol, abbreviated to “bisoxonol,” was purchased from Molecular Probes, Inc. DIDS and diphenylamine carboxylate (DPC) were purchased from Sigma Chemical Co. The patch pipette fill solution consisted of (in mmol/L) potassium aspartate 100, KCl 35, K₂-EGTA 10, CaCl₂ 1, MOPS 10, ATP 1, and MgCl₂ 2, and pH was adjusted to 7.2 with KOH (∼6.5). ATP/MgCl₂ solution (pH 7.2 with KOH) was added to the fill solution from frozen stock at the beginning of each day. Normal Ringer’s solution contained (in mmol/L) NaCl 140, KCl 5, CaCl₂ 2, MgCl₂ 1, and MOPS 10, as well as 5.6 d-glucose, pH 7.4 with NaOH (∼6.5). Low-Cl⁻ Ringer’s solution contained (in mmol/L) sodium aspartate 140, KCl 4, CaCl₂ 2, MgCl₂ 0.5, and MOPS 10, pH 7.4 with NaOH. Low-Na⁺ Ringer’s solution contained (in mmol/L) N-methyl-D-glucamine (NMDG) 140, KCl 5, CaCl₂ 2, MOPS 10,
and 5.6 D-glucose, pH 7.4 with HCl (≈130). Fill solutions had measured osmolalities of ≈275 mosm. External solutions had measured osmolalities of 265 to 310 mosm.

**Patch-Clamp Recordings**

All patch-clamp recordings were performed at room temperature on BAECs cultured on coverslips which were subsequently mounted in a Warner recording/perfusion chamber (Warner Instrument Corp). A schematic of this chamber is illustrated in Figure 1. Steady laminar flow was administered via a syringe pump (Cole Parmer). Preliminary experiments indicated that responsiveness of cells to flow may depend on the waiting period experienced before exposure to shear stress. This possibility was not investigated in detail; instead, a uniform minimum waiting period was imposed. All data presented were from cells exposed to at least 20 minutes of no-flow conditions before and between experimental runs.

Whole-cell voltages were recorded from cells in confluent monolayers using standard patch-electrode recording techniques. Utilizing confluent cells took advantage of the electric coupling via gap junctions known to be present between adjacent BAECs. Currents from cells adjacent to the recording cell reduced the significance of shunts to ground through the pipette seal. This provided more stable and less depolarized membrane voltage recordings in current-clamp mode compared with that obtained in single subconfluent cells. However, this technique has 2 disadvantages, as follows: (1) ion reversal potentials are not precisely known, because only the recording cell is dialyzed whereas the voltage is measured from the reversal potentials are not precisely known, because only the

**Membrane Potential Measurements Using Potentiometric Dyes**

Experiments using the potentiometric fluorescent dye bisoxonol were performed on single (subconfluent) cells cultured in borosilicate glass capillary tubes. BAECs were washed with PBS and then incubated in PBS containing 5 × 10⁻⁷ mol/L of the bisoxonol dye for 15 minutes at 37°C. The dye was added in ethanol stock solution, with the final ethanol concentration in the incubated cells never exceeding 0.05%. In some experiments, a solution made to approximate the ionic composition of the culture medium was used. This solution consisted of (in mmol/L) KCl 4, NaCl 150, CaCl₂ 1, MgCl₂ 2, and HEPES 10, pH 7.4 with HCl.

Fluorimetric measurements of membrane potential were performed using a Spex ARCM-1000 microspectrophotometer (Spex Industries). The fluorimeter was interfaced to a Nikon Diaphot inverted microscope equipped with a Nikon Fluor objective (×40). The photometer contained a pinhole diaphragm to regulate the viewing field. The results represent measurements on single cells at room temperature located at or near the center midline of the tube. Dye-loaded cells were excited at 495 nm; emission at 515 nm was collected at 1.0-second intervals. Background fluorescence was automatically subtracted from all measurements. Similar results were obtained whether the perfusate was PBS (in most experiments) or the solution approximating the ionic composition of the culture medium.

Flow was induced by connecting the capillary tube containing dye-loaded cells to a 60-mL syringe mounted on a syringe pump (Harvard Apparatus) via a length of Silastic tubing (Masterflex, Cole Parmer). Perfusion rates were calibrated by direct measurement of collected volume for a range of pump gear ratios. Shear stress levels were calculated from the flow rates and tube geometry on the basis of the fully developed Poiseuille flow solution in a tube of circular cross section as previously described. Because the capillary tubes used here had square cross sections, the equivalent (or hydraulic) diameter used in the Poiseuille flow expression corresponded to the length of a side of the square tube. The perfusate contained the same concentration of bisoxonol dye as that in which the cells were incubated (5 × 10⁻⁷ mol/L). This was necessary to avoid concentration gradient–driven dye washout from the cells.

**Flow Loop**

In experiments on flow-conditioned cells, BAECs were subjected to flow by insertion of the capillary tubes into a recirculating flow loop. Components of the loop were connected by small diameter Silastic tubing (Cole Parmer). A reservoir of culture medium (125 mL) was maintained at 37°C adjacent to the microscope stage that was enclosed by a temperature-regulated Plexiglas incubator, which was maintained at 37°C. Medium, gently gassed with air (5% CO₂), was drawn through the system by a peristaltic flow pump (Cole Parmer). To dampen pulsatility, the main reservoir was vented, and 2 buffer reservoirs were inserted between the pump and flow tube.

**Filamentous Actin (F-Actin) Fluorescence Staining**

Cells were fixed with 4% formaldehyde in PBS for 20 minutes at 37°C. After 3 washes with PBS, the cells were permeabilized by 0.1% Triton X-100 in PBS for 3 minutes at room temperature and washed twice with PBS and then 3 times with 50 mmol/L ammonium chloride (pH 7.3) for 5 minutes each wash. After rinsing twice with PBS, F-actin was stained by addition of NBD-phallacidin (Molecular Probes) at a dilution in PBS of 1:100 of a 3 mmol/L stock solution, for 20 minutes at room temperature followed by 3 final washes in
Acute Membrane Potential Shear Responses

The dependence of bisoxonol fluorescence intensity on membrane potential has previously been extensively studied. Membrane depolarization leads to an increase in fluorescence intensity as a result of a rise in intracellular concentration of the negatively charged bisoxonol, whereas membrane hyperpolarization has the opposite effect. We have confirmed this behavior in control experiments in which the membranes of BAECs cultured in capillary flow tubes were either depolarized with 100 mmol/L KCl or hyperpolarized with 2 μmol/L ATP (data not shown).

The variation of bisoxonol fluorescence intensity with time in response to flow was measured in single (subconfluent) BAECs previously unstrained by flow (n=17). Results obtained from a representative cell are illustrated in Figure 2A. The baseline intensity was recorded for the first 300 seconds, after which flow at a steady rate of 0.7 mL/min (generating a wall shear stress of 1 dyne/cm² at the midline of each face of the square cross-section tube) was initiated and maintained for 25 minutes (1500 seconds). This shear stress level was selected because it approximates that required for half-maximal activation of the flow-sensitive inward-rectifying K⁺ channel initially reported by Olesen et al but is insufficiently high for the induction of significant topographic and cytoskeletal reorganization. The fluorescence intensity initially sharply decreased in response to flow, indicating membrane hyperpolarization consistent with previous reports using patch-clamp recordings and membrane potential-sensitive fluorescent dyes. With continued flow, however, the fluorescence intensity increased above the original baseline value, reaching a plateau at a depolarized state. Flow, therefore, induced initial cell hyperpolarization followed by depolarization. Membrane depolarization also occurred in a few experiments in which flow was arrested immediately after hyperpolarization (data not shown).

The bisoxonol data provide a qualitative picture of BAEC membrane potential responsiveness to flow. Whole-cell patch-clamp recordings were performed to validate and quantify the bisoxonol shear experiments. Recordings in both current-clamp mode on cells in a confluent monolayer and voltage-clamp mode on single (subconfluent) cells were made. All patch-clamp experiments were performed at a steady flow rate of 3 mL/min. The corresponding shear stress to which the cells in the Warner chamber were exposed in this system is not precisely known; however, assuming a linear velocity variation in the direction orthogonal to flow, the shear stress is estimated to be in the range of 0.5 to 1 dyne/cm².

Figure 2B illustrates current-clamp recordings of the response of BAEC membrane potential to the steady shear stress in normal Ringer’s solution. In these experiments, a stable resting membrane potential (−69±0.5 mV, range −60 to −76 mV; n=23 cells) was recorded for a period of 180 seconds before initiating flow. Flow was then maintained for 240 seconds before being turned off. With onset of flow, the membrane voltage rapidly hyperpolarized, reaching a peak hyperpolarization amplitude of 1.7±0.2 mV (range 0.4 to 6 mV) within 16±1 seconds (range 4 to 43 seconds). Hyperpolarization was then reversed so that the membrane potential crossed the resting voltage 64±6 seconds (range 35 to 158 seconds) after the onset of flow and then continued to increase to a depolarization of 4.4±1.5 mV (range 0 to 36 mV). The dynamics of the depolarization were not the same in all cells; peak depolarization occurred at (Figure 2B), before, or after cessation of flow. As mentioned in Materials and Methods, a no-flow waiting period of at least 20 minutes was imposed before exposure to shear stress. In a limited number of experiments in which a shorter waiting period was used, the initial hyperpolarization event appeared unaffected, but the magnitude of the subsequent depolarization was reduced.

Although all cells in this study responded to flow with a rapid hyperpolarization, the depolarizing response was less consistent, occurring in 22 of 33 cells (15 of 23 current-clamp, 7 of 10 voltage-clamp cells) in experiments in normal Ringer’s solution. Some cells responded to flow with a hyperpolarization that did not resolve to the preflow resting potential (Figure 3A) but instead remained stable at a more hyperpolarized level. In 4 of 5 cells (in either current- or voltage-clamp mode) that did not exhibit flow-induced depolarization in normal Ringer’s solution, it was found that on
replacement of the external solution with low-Cl⁻ Ringer’s solution, the same flow paradigm elicited a depolarizing response (Figure 3B). This suggests a potential involvement of Cl⁻ ions in the flow-induced depolarizing response.

Comparison of Figure 2A with Figure 2B suggests that, whereas the bisoxonol dye system qualitatively captures the behavior of BAEC membrane potential responsiveness to flow, the time constants and relative magnitudes of the flow-induced changes in dye fluorescence intensity differ from the physiological behavior measured in the patch-clamp experiments. The reason for this difference remains unknown but may relate to an interaction between the flow and the dynamics of bisoxonol partitioning between the cytoplasmic and extracellular spaces. However, the minimal invasiveness of the dye to both the cells and the flow field makes this technique attractive for qualitative studies of membrane potential flow responses.

Depolarization Is Due to a Shear Stress–Induced Cl⁻-Selective Membrane Current

The fact that the flow-induced depolarization was maximized in low-external Cl⁻ Ringer’s solution suggested the involvement of a Cl⁻-permeable conductance. Endothelial cells express a variety of Cl⁻ channels. Cells were therefore exposed in dye experiments to the 2 known Cl⁻ channel blockers DIDS (1 mmol/L) and DPC (1 to 2 mmol/L) and subsequently subjected to a steady shear stress of 1 dyne/cm². In the presence of Cl⁻ channel inhibitors, there was a sustained hyperpolarization without depolarization of the cells (Figure 4), suggesting the involvement of Cl⁻ channels in the flow-induced depolarization response. DIDS and DPC did not block the depolarization in every case; DIDS was effective in 3 of 6 cells, whereas DPC blocked flow-induced depolarization in 5 of 6 cells.

More definitive evidence of flow activation of a Cl⁻-selective current was provided by whole-cell voltage-clamp experiments. Cells were subjected to the same flow protocol as used in the current-clamp experiments. In cells treated with 1 mmol/L external Ba²⁺ to block the flow-sensitive K⁺ current, whole-cell conductance was increased by ~50% during flow and did not return to resting levels until several minutes after flow had been stopped (Figure 5A). Voltage ramps from this experiment intersected at -18 mV (Figure 5D), indicating that the reversal potential of the flow-activated current was near this value and consistent with Cl⁻ (E Cl⁻ = -36 mV) carrying a significant portion of the current. The reversal potential for the flow-activated current (intersection point of voltage ramps) shifted with E Cl⁻ on replacement of external Cl⁻ with aspartate (current reversal potential E Cl⁻ = -28 mV; E Cl⁻ = -31 mV), indicating that the current was largely mediated by Cl⁻ ions (Figure 5B). In contrast, the reversal potential of the flow-activated current was, for the most part, insensitive to replacement of external Na⁺ with the impermeant cation NMDG (current reversal potential E Cl⁻ = -11 mV; E Na⁺ = -34 mV); the intersection point would have shifted to a more hyperpolarized level if a Na⁺-selective current were involved. These results are consistent with the activation of an anion-selective current.

Figure 6 illustrates the net (leak-subtracted) currents elicited...
by flow in the data of Figure 5. This figure more clearly demonstrates the reversal potentials, and it illustrates that the flow-induced current is altered considerably in low external Cl\(^{−}\) while remaining virtually unchanged in low external Na\(^{+}\). The fact that the reversal potentials obtained here do not exactly match \(E_{\text{Cl}}\) suggests that the flow-activated Cl\(^{−}\) current is imperfectly selective or that other conductances are activated. For example, a nonselective cation component, as has been demonstrated in other Cl\(^{−}\) currents, would shift the reversal potential of the observed current away from \(E_{\text{Cl}}\) and toward 0 mV, as was seen in these experiments (Figure 5D through 5F). Alternatively, the Cl\(^{−}\) current observed here could have some finite permeability to aspartate. In the conditions presented in Figure 5, \(E_{\text{aspartate}}\) was positive in the cases of normal and low-Na\(^{+}\) Ringer’s solution, whereas \(E_{\text{aspartate}}\) was negative in the case of low-Cl\(^{−}\) Ringer’s solution. Again, this would be consistent with the shift in reversal potential toward 0 mV observed in all conditions. Lastly, the discrepancy between calculated and observed reversal potentials could be due in part to junction potentials in our patch-clamp setup that were not corrected for. However, this voltage error is <7 mV for our solutions.

The fact that the flow-induced Cl\(^{−}\) current was observed in the presence of the K\(^{+}\) channel blocker Ba\(^{2+}\) (Figure 5) suggests that this current is activated independently by flow and is not a consequence of the activation of the flow-sensitive K\(^{+}\) channels. Furthermore, Figures 5B and 6 reveal an additional characteristic of the flow-activated current in low external Cl\(^{−}\) solution, a substantial increase in conductance. The same cell shown in Figure 6A in normal Ringer’s solution showed a >3-fold increase in resting conductance in low-Cl\(^{−}\) Ringer’s solution (Figure 6B). In response to flow, conductance increased by ~100% in both normal and low-Cl\(^{−}\) Ringer’s solution. The increased conductance in low external Cl\(^{−}\) may be due to the recruitment of more channels, an increase in unitary conductance, a direct effect of aspartate (used to replace Cl\(^{−}\) in low-Cl\(^{−}\) Ringer’s solution) on conductance, or a combination of these factors. The increase in resting conductance is likely not due to an increase in nonselective or K\(^{+}\)-based leaks, as the reversal potential in no-flow conditions does not shift more negatively (compare Figure 5D trace 1 with Figure 5E trace 1 from the same cell). More likely, this increase in conductance is Cl\(^{−}\)-based and could be due to activation of the volume-sensitive Cl\(^{−}\) current.
known to be present in macrovascular endothelial cells. Osmolarity of the low-Cl\textsuperscript{−} solution was consistently 10% to 15% less than that of the normal Ringer’s solution, and this may have been sufficient to elicit a volume-activated Cl\textsuperscript{−} current; however, because bath solution composition did not change during shear, the fact that Cl\textsuperscript{−}-based conductance did increase substantially in response to shear in low-Cl\textsuperscript{−} solution indicates that the flow-induced conductance is not due simply to changes in osmolarity.

Flow Preconditioning Does Not Desensitize Flow-Activated Currents

The bisoxonol dye system was used to investigate membrane potential responses to flow in endothelial cells exposed to flow for periods sufficiently long to induce cell alignment. BAECs in capillary tubes were exposed to flow at a shear stress of 13 dyne/cm\textsuperscript{2} in the flow loop for a period of at least 24 hours. In these experiments, cells were confluent on one face of the square cross-section capillary tube and subconfluent on the 2 adjacent faces. At the end of flow preconditioning, the F-actin in cells within the confluent monolayer underwent extensive remodeling (Figure 7), and the cells became elongated and aligned in the general flow direction. Subconfluent cells did not uniformly align but underwent significant cytoskeletal reorganization. The cells were then taken out of the loop and immediately washed and incubated in the bisoxonol dye in a fashion similar to that previously described in the acute flow experiments. Membrane potential measurements on single (subconfluent) cells in response to a shear stress of 1 dyne/cm\textsuperscript{2} were then recorded (n=4).

Figure 8 illustrates the response of flow-preconditioned BAECs to the steady shear stress. All cells hyperpolarized and depolarized in a fashion very similar to that of the response recorded for previously unsheared cells. These results indicate that endothelial cells that have adapted to sustained flow by undergoing extensive flow-induced topographic and cytoskeletal reorganization retain their membrane potential responsiveness to flow.

Discussion

The activation of a K\textsuperscript{+} membrane current is one of the fastest known responses to fluid mechanical shear stress in endothelium and may play a role in the sensing and transduction of fluid shear forces. In this paper, we have used a combination of patch-clamp recordings and measurements from the membrane potential-sensitive fluorescent dye bisoxonol to demonstrate that shear stress also activates a Cl\textsuperscript{−} current in cultured BAECs. As a result of this current, the initial K\textsuperscript{+} channel-mediated membrane hyperpolarization is reversed to a depolarized state within 35 to 160 seconds of the onset of flow, and this depolarization often persists for several minutes after flow cessation. Evidence for the Cl\textsuperscript{−}-selective nature of the depolarizing current was provided by varying external Cl\textsuperscript{−} concentration in the patch-clamp experiments and by using 2 Cl\textsuperscript{−} channel blockers (DIDS and DPC) in the bisoxonol dye studies. Induction of the Cl\textsuperscript{−} current by shear stress persisted in the presence of external Ba\textsuperscript{2+}, a known

Figure 6. Net (leak-subtracted) flow-activated currents from experiments shown in Figure 5. Net currents were obtained by subtracting the lowest conductance ramp (preflow onset) from the highest conductance ramp (postflow onset). Reversal potentials are −18 mV for normal Ringer’s solution, +26 mV for low-Cl\textsuperscript{−} Ringer’s solution, and −11 mV for low-Na\textsuperscript{+} Ringer’s solution.

Figure 7. Distribution of F-actin in BAECs previously not exposed to flow (A) and after 24 hours exposure to unidirectional shear stress at 13 dyne/cm\textsuperscript{2} (B). Note major structural reorganization of the cytoskeleton.
highly homologous (92% homology at the DNA level and ing the selectins, integrins, and immunoglobulins, and it is of the classic families of cell-cell adhesion molecules, includ-

2 the channel.

that this functional duality may bestow mechanosensitivity to

function both as an adhesion molecule and a Cl

homology, it has been suggested that Lu-ECAM-1 may

studies demonstrating that hypotonic volume increase elicits

vated Cl– channels, volume-activated Cl– channels, and

channels that may be regulated by voltage or by cAMP22,23; however, the sensitivity of these

channels to fluid mechanical forces has not been evaluated. More recently, lung-endothelial cell adhesion molecule-1 (Lu-ECAM-1), an endothelial cell surface molecule that mediates adhesion of metastatic melanoma cells to lung endothelium, has been cloned and characterized in cultured BAECS.37 Interestingly, this molecule is not a member of any of the classic families of cell-cell adhesion molecules, including the selectins, integrins, and immunoglobulins, and it is highly homologous (92% homology at the DNA level and 88% at the amino acid level) to a Ca2+-activated Cl– channel present in bovine tracheal epithelium.38 Because of this homology, it has been suggested that Lu-ECAM-1 may function both as an adhesion molecule and a Cl– channel and that this functional duality may bestow mechanosensitivity to the channel.

The only evidence to date of direct activation of endothel-

channels by mechanical stimulation is provided by

studies demonstrating that hypotonic volume increase elicits

currents in cultured human umbilical vein and bovine

artery endothelial cells.27,35 These volume-acti-

vated Cl– channels, which are different from the Ca2+-acti-

vated Cl– channels,36 may also be responsive to fluid me-

chanical shear stress (B. Nilius, personal communication, September 1996). It is unknown whether or not the shear stress–activated Cl– current reported in the present study is mediated by the activation of these volume-regulated Cl– channels; however, it is not expected that the relatively low shear stress levels used in the present study (∼1 dyne/cm²) would lead to significant stretch of the endothelial cell membrane to result in a direct change of cell volume.

The impact of shear stress on endothelial cell membrane ion currents has previously been studied in BAECS by whole-cell patch clamping6,39 and single-channel patch clamping18 and in bovine pulmonary artery endothelial cells by membrane potential–sensitive fluorescent dyes.17 Although all of these studies reported rapid activation by shear stress of a K+-selective membrane current, the precise char-

acter of the resulting membrane potential response to sustained flow varied from persistent hyperpolarization17,18 to

channel desensitization and membrane potential return to the no-flow baseline6 to Ca2+-dependent spontaneous tran-

tsient hyperpolarizing bursts.39 Reasons for these differences remain unclear.

It is also unclear why the Cl– current reported here was not observed in the other studies. One possibility may be related to our observation that in some cells the magnitude of the flow-induced depolarization may be smaller unless there is a relatively long no-flow waiting period (up to 20 minutes) between consecutive flow episodes. This may suggest that the desensitization and resensitization dynamics of the flow-
sensitive K+ current differ from those of the Cl– current. If such differences occur in vivo, then they might be expected to have particular relevance to endothelial cells within flow separation regions in the vicinity of arterial bifurcations where the time-averaged wall shear stress is very low but becomes transiently high during exercise or stress condi-
tions.40 Our results suggest that the length of the time period between stress episodes (relative to the no-flow waiting period) may determine the extent of the flow-induced Cl– current in the endothelial cells within these flow separation zones. It should also be noted that the whole-cell patch-clamp experiments modified cytosolic contents and were performed at room temperature. It is not known whether these factors impact the desensitization and resensitization dynamics of the flow-activated channels. Another possible reason for differences between our results and those of others is that in the previous studies, EC50 was maintained near 0 mV, whereas an EC50 of −36 mV (Figure 5D) was used in the present study; thus, a Cl– current in the other studies could have been interpreted as a nonspecific leak current.

It is unknown why the shear stress–induced Cl– current was not observed in all cells studied. In response to a shear stress of 5 dyne/cm², Hoyer et al39 observed flow-induced K+ bursts in 50% of BAECS studied, whereas 95% of the cells became responsive at 9 and 18 dyne/cm². We tested the idea that the imposed shear stress needs to exceed a critical threshold for Cl– channel activation and that this threshold is not identical in all cells. In a limited number of patch-clamp experiments (data not shown), the flow rate of the perfusate on nonresponsive cells was increased; however, the results were inconclusive, with some cells remaining nonresponsive and others exhibiting only marginal depolarization. Another possibility is that the density of the shear stress–sensitive Cl– channels on the BAECS surface is highly variable from one cell to another. Such topographic heterogeneity has been reported for K+ channels in neurons41 and for volume-acti-

Figure 8. Bisoxonol dye recordings of membrane potential responsiveness to shear stress in BAECS that are chronically

Flow On

0 300 600 900 1200

seconds

Normalized Intensity

0.8 1.0 1.2 1.4

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vated Cl\textsuperscript{-} channels in vascular endothelial cells.\textsuperscript{42} The surface density of Lu-ECAM-1 also appears to vary considerably in freshly isolated BAECs, but the extent of this variability appears to decrease with progressive passages in culture (A.D. Gruber, personal communication, April 1998). To assess the impact of cell passaging, we have performed a limited number of voltage-clamp recordings in BAECs from early passages (passages 4 to 5; data not shown) and have established that the flow-activated Cl\textsuperscript{-} current reported here is consistently observed in these cells. It is interesting that in studies on epithelial cells, anion currents in response to osmotic stress have been reported to occur in only approximately two thirds of the cells studied,\textsuperscript{43,44} a fraction similar to that reported here. Cell-to-cell heterogeneity has also been observed both in vivo and in vitro in several other endothelial flow responses.\textsuperscript{45} Examples include sensitivity to shear stress of VCAM-1, ICAM-1, and [Ca\textsuperscript{2+}].

Use of the fluorescent potentiometric dye bisoxonol for studies of the effect of flow on endothelial cell membrane potential is attractive because of the simplicity of application of the dye as well as its minimal invasiveness to both the cell and the flow field. However, the dye response time is relatively slow (a few seconds),\textsuperscript{46,47} which prevents measurement of precise physiological time constants associated with membrane potential changes. At the relatively low concentrations used in this study, dye-induced cell toxicity appears to be negligible (A.S. Waggoner, personal communication, November 1994) and in no instance during these studies was there any evidence of cytotoxicity. Bisoxonol results were obtained at a shear stress of \(\approx 1\) dyne/cm\textsuperscript{2}; we also attempted similar bisoxonol measurements on cells subjected to larger shear stresses that are more representative of time-average values within large arteries in vivo (5 to 15 dyne/cm\textsuperscript{2}). The results demonstrated an immediate increase in fluorescence intensity on onset of flow, suggesting membrane depolarization in the absence of hyperpolarization (data not shown). J.A. Frangos (personal communication, October 1996) has noted that there exists a nonspecific interaction between the bisoxonol dye system and the flow environment. This interaction becomes very pronounced at the higher flow rates and gives an artificial depolarization signal that overwhelms the hyperpolarization, which is known to occur at physiological shear stresses.\textsuperscript{6,18} The existence of this nonspecific interaction was confirmed by coating the inside surfaces of glass capillary tubes with a lipid layer and applying flow to it. The simple application of flow led to an increase in fluorescence intensity, which resembles membrane depolarization. In combination with the inability of the bisoxonol system to reflect physiological time constants and magnitudes of flow-induced membrane potential changes (Figure 2B), this suggests that care must be exercised in the use of this dye system in studies of flow-induced changes in membrane potential.

Endothelial cells in vivo are exposed to flow chronically and are often aligned in the flow direction.\textsuperscript{48} Our experiments demonstrate that BAEC membrane potential responsiveness to shear stress in flow-preconditioned cells that have undergone extensive topographic and cytoskeletal reorganization is similar to that in previously unsheared cells, ie, initial hyperpolarization that is subsequently reversed to depolarization. Thus, endothelial cells adapt to a chronic flow environment in a manner that maintains their membrane potential sensitivity to shear stress. The mechanisms governing such adaptive processes remain unknown but may involve association of ion channels with cytoskeletal proteins such as actin\textsuperscript{49–51} and annexins.\textsuperscript{36} Volume-activated Cl\textsuperscript{-} channels present in macrovascular endothelial cells have recently been shown to be modulated by annexin II, a member of a family of Ca\textsuperscript{2+}-dependent proteins that aggregate as cytoskeletal structures at cell membranes.\textsuperscript{36}

The functional role of the flow-sensitive Cl\textsuperscript{-} channel reported here remains to be elucidated. It is possible that this channel provides a mechanism for depolarization of the membrane potential after flow-induced K\textsuperscript{+} channel-mediated hyperpolarization. However, both the responsiveness of the Cl\textsuperscript{-} channel to shear stress in the absence of K\textsuperscript{+} channel activation by flow (in presence of external Ba\textsuperscript{2+}) and the fact that the cell ends up at a depolarized level rather than at its preflood membrane voltage suggest that this channel also serves other functions. Hoyer et al\textsuperscript{19} have reported that shear stress–induced endothelial hyperpolarization facilitates calcium influx by increasing the electric driving force; therefore, one possibility is that the activation of the Cl\textsuperscript{-} current by flow acts to limit this influx. A second possibility is that the flow-induced Cl\textsuperscript{-} current may be involved in volume changes that may occur as the cells prepare for the extensive cytoskeletal and morphological changes that occur in response to sustained flow. A third possibility is that the imposition of shear stress may change the metabolic demands on endothelial cells so that regulation of intracellular pH may be required, and the Cl\textsuperscript{-} current may be involved in this regulation. Finally, the Cl\textsuperscript{-} channel may constitute an independent candidate structure involved in mechanosensing. Given the seemingly different dynamics of flow desensitization and resensitization of the K\textsuperscript{+} and Cl\textsuperscript{-} channels, an intriguing notion is that these channels may constitute components of separate systems capable of responding to shear stresses of different temporal scales. Thus, whereas the K\textsuperscript{+} channel system responds to rapid changes in shear stress, the Cl\textsuperscript{-} channel may be primarily involved in responding to temporally slower fluid mechanical stimuli.

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