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# Fluid Flow Modulates Calcium Entry and Activates Membrane Currents in Cultured Human Aortic Endothelial Cells

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**Abstract.** Human aortic endothelial cells (HAEC) respond to flow with $Ca^{2+}$ entry, activation of a nonselective cation channel, activation of a chloride channel, and activation of a calcium-activated potassium channel. Conversely, human capillary endothelial cells were unaffected by similar flow rates. In HAEC the flow induced cytosolic free calcium increase ($[Ca^{2+}]_i$) and the ionic currents associated with it were sustained for up to 15 min after perfusion was stopped. In the absence of extracellular $Ca^{2+}$, fluid flow was unable to evoke the $[Ca^{2+}]_i$ increase or the increase in membrane currents but the response could be restored by addition of extracellular $Ca^{2+}$. Surprisingly, the flow response was inhibited in 50% of the cells by inhibitors of nitric oxide production. The results suggest that the sustained flow response in HAEC may be partially mediated by nitric oxide production and release.

**Key words:** Endothelial cells — Shear stress — Flow — Ion channels

## Introduction

Endothelial cells are ideally situated to respond to shear forces resulting from the flow of blood. Endothelial cells respond to these shear forces with increases in intracellular $Ca^{2+}$ and cGMP and release of vasoactive substances such as nitric oxide, endothelin, prostaglandin and prostacyclin, as well as by induction of endothelial transforming growth factor beta-1 [5, 6, 10, 22, 26, 32, 33]. Large vessels experience higher laminar shear stress from blood flow across their inner surface than capillary vessels that are exposed to low-velocity pulsatile blood flow [6]. Ionic channels responsive to shear stress and stretch have been described from large vessels of bovine, porcine, and human umbilical cord endothelial cells [16, 25, 27, 31]. Those activated by flow include an inward rectifier type channel and activation of a nonselective cation channel [27, 31]. These channels remain activated as long as the shear stress is present but quickly decay upon termination of flow. Increases in intracellular calcium in response to shear stress have also been reported for endothelial cells but again these appear to only last for the time the stress is applied [10]. An endothelial cell's response to blood flow may vary depending upon the vessel type and size, the animal species, and the ion channels present [6, 29] as well as by the dynamics of $Ca^{2+}$ handling. We report here on a flow-activated prolonged increase in intracellular $Ca^{2+}$, mediated in part by $Ca^{2+}$ entry through a nonselective cation channel and on the sustained activation of a chloride channel and a calcium-activated $K^+$ channel by flow in human aortic endothelial cells (HAEC). We find that this flow response is unique to HAEC as it is not found in human capillary endothelial cells and that it is partially mediated via the nitric oxide pathway.

## Materials and Methods

### Cell Culture

Human aortic endothelial cells (HAEC) and human capillary endothelial (HCEC) cells were obtained from Clonetics and grown in endothelial cell growth media (EGM™, Clonetics, Kirkland, WA). The culture medium was changed each day and cells were split at 60–90% confluence. The cells were used up to passage 6.

### Electrophysiological Measurements

Cells were recorded in whole-cell voltage clamp with the patch technique [14]. Patch electrodes were pulled from borosilicate glass (Sutter

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Inst. O.D. 1.0 mm, I.D. 0.5 mm) and had resistance from 3–6 MOhms. The input resistance of cells at −60 mV measured in excess of 1 Gigaohm. Seal resistance was typically 20 Gigaohms or greater. All recordings were done at room temperature. An EPC9 amplifier with the acquisition program Pulse-PulseFit from HEKA (Lambrecht, Germany) was used for recording, data acquisition, and analysis. Series resistance compensation was used and currents were filtered at 3 KHz. The standard pipette solution for whole-cell recordings was (in mmol/l): 120 K-aspartate, 20 KCl, 0.05 EGTA, 5 HEPES (pH 7.2 with KOH), 5 Mg-ATP, 5 phosphocreatine-$Na_2$, 1 $MgCl_2$. In dual recordings 0.05 mM Fura2 acid was added in the standard pipette solution. The standard bath recording solution consisted of (in mmol/l) 140 NaCl, 5.4 KCl, 5 HEPES (pH 7.2 with NaOH), 15 dextrose, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.4 $KH_2PO_4$, 0.3 $Na_2HPO_4$. Cells were activated with flow by perfusing the 35 mm dish at a rate of 1 ml/min with the bath solution. No differences could be seen in the cells' response when they were perfused over a range of 0.5–6 ml/min. No attempt was made to calculate the shear force in dynes/$cm^{-2}$ as this can only be accurately determined for closed perfusion systems with laminar flow. Sodium substitution experiments used a $Na^+$-free bath solution in which NMDG-Cl was substituted for NaCl in the standard bath solution. Chloride substitution experiments used a reduced $Cl^-$ bath solution in which $NaMeSO_3$ was substituted for NaCl. Iberiotoxin (IbTx) was purchased from Peptide Institute, NPPB (5 Nitro-2-(3-phenylpropylamino)benzoic acid was from RBI, Fura2 acid from Molecular Probes (Eugene, OR) and ionomycin and all salts from Sigma (St. Louis, MO).

### Photometry

Intracellular $Ca^{2+}$ was monitored simultaneously with patch clamp recordings in some of the experiments. A T.I.L.L. Photonics (Germany) photometer adapted for the EPC-9 (Applied Scientific Instrumentation) was used to monitor the fura ratio. Calcium concentration was calculated from min/max, and $K_d$ values entered into the Pulse Fura program and verified against a calcium calibration curve generated with Molecular Probes calibration kit F-6774. Calcium concentrations reported with this technique represented the mean of four individual measurements taken during the voltage ramp or pulse.

## Results

Whole cell voltage clamp recordings from human aortic endothelial cells (HAEC) had an average cell capacitance of 56.6 ± 8.4 pF ($n = 80$). The average resting membrane potential of the cells, determined immediately after break in was −50.45 ± 1.77 mV ($n = 23$). Polarizing the cell from −120 to +100 mV from a holding potential of −60 mV elicited both inward and outward currents [17]. Figure 1 shows the response of HAEC to continuous perfusion at 1 ml/min. Figure 1*A* shows the whole cell currents activated before and after flow. The currents were elicited in response to voltage steps from −120 to +100 mV and had fast kinetics of activation with little inactivation. In this cell the outward current increased quickly (within 30 sec) with the onset of perfusion and produced a twofold increase in outward current 7 min after flow. The perfusion was stopped and 7 min later the current slowly reached the steady-state level shown. Figure 1*B* and *C* are recordings from a different HAEC loaded with Fura 2 acid through the pipette and responding to flow. Figure 1*B* shows the change in intracellular $Ca^{2+}$ after initiation of flow. Underneath is the current response during the same time plotted as peak current at +100 mV and −120 mV. The activation of inward and outward currents parallels the increase in intracellular calcium. The current-voltage ramp in Fig. 1*C* illustrates the activation of both inward and outward currents in this cell after perfusion and shows the large shift in the zero current level from −62 mV to +3 mV. The average increase in intracellular $Ca^{2+}$ level in response to flow was 11.5 ± 1.7-fold in 24 cells and the shift in zero current potential was 63.8 ± 2.5 mV ($n = 5$) positive. The flow induced increase in current (and intracellular $Ca^{2+}$) decayed back to preflow levels 15 min after perfusion was stopped in three cells where this was examined.

Human capillary endothelial cells (HCEC) had similar baseline currents [17] but did not respond to flow even at much higher rates (6 ml/min) ($n = 43$). However large outward currents could be activated in them by the $Ca^{2+}$ ionophore A23187 (10 μM) or by perfusion with hypoosmotic external solution (197 mosm *vs.* 300 mosm) suggesting that ion channels were present but not activated by flow.

Figure 2 demonstrates that the flow-induced increase in outward current in HAEC could be prevented when external $Ca^{2+}$ was buffered to low levels by addition of 10 mM BAPTA to the perfusate (Fig. 2*B*). A return to normal 1.8 mM $Ca^{2+}$ containing perfusate restored the cell's ability to respond to flow and a large increase in outward current was immediately observed (Fig. 2*C*). Similar results were seen in five additional cells. After the perfusion was stopped the outward currents induced by flow remained as seen in Fig. 2*D* and in two other cells where they persisted for up to 15 min. Therefore, extracellular $Ca^{2+}$ and flow are both required to trigger the increase in outward currents that is then maintained even in the absence of perfusion. In other cells ($n = 3$) changing from perfusion with normal bath solution to bath solution containing no external $Ca^{2+}$ (2 mM EGTA) produced a rapid decrease in intracellular $Ca^{2+}$ levels and flow activated ionic currents. This supports the role of external $Ca^{2+}$ entry in maintaining the flow response. Four cells recorded with 20 μM BAPTA, zero $Ca^{2+}$ in the pipette solution and nominally free external $Ca^{2+}$ failed to respond to flow. Interestingly, cells recorded with pipette solutions containing 10 or 20 mM BAPTA ($n = 5$) or 10 mM EGTA ($n = 2$) and zero $Ca^{2+}$ in the pipette all responded robustly to flow with bath solution containing normal external $Ca^{2+}$ suggesting an inability to buffer the pertinent intracellular calcium stores through the recording electrode [3].

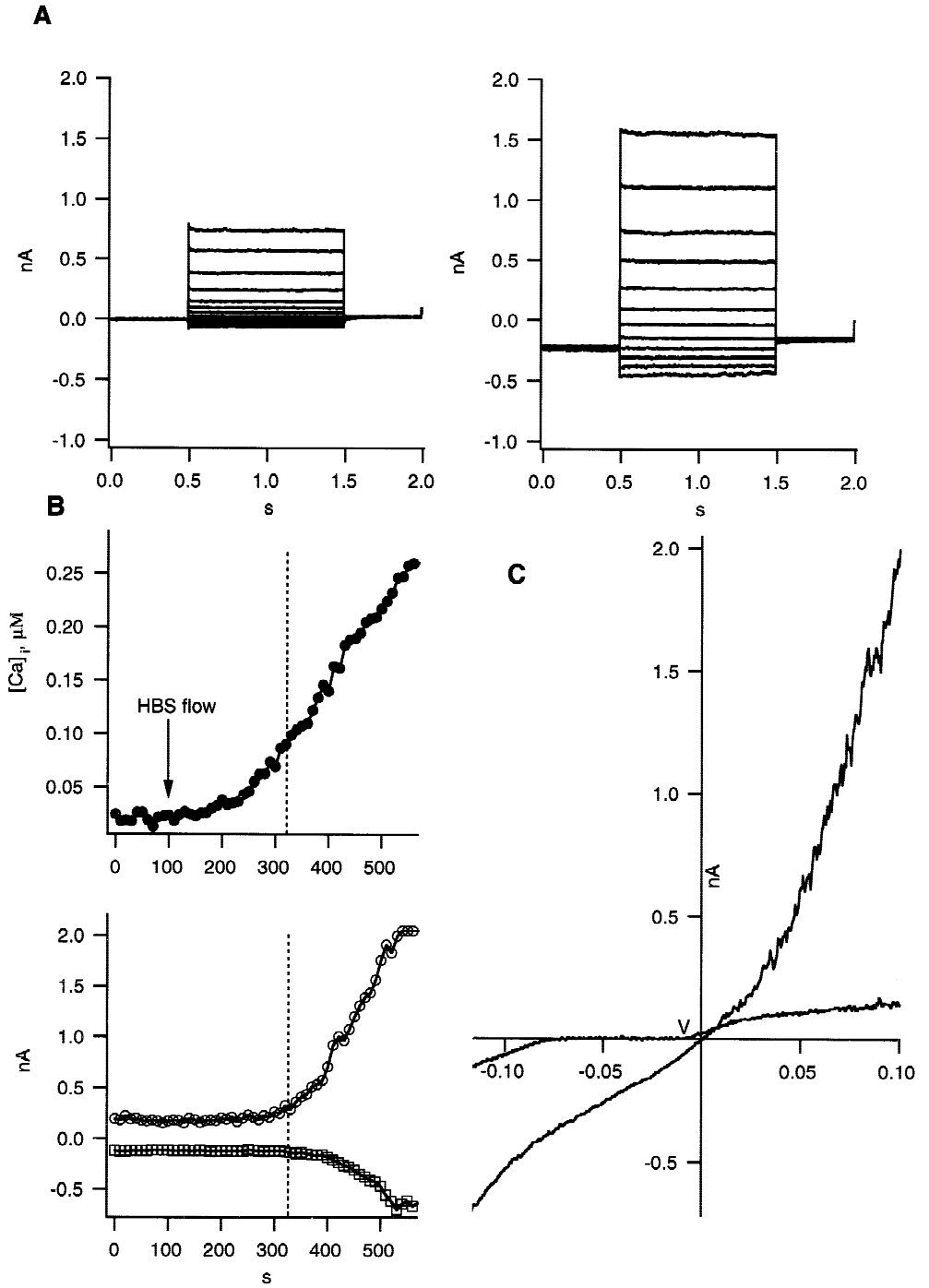


**Fig. 1.** Human aortic endothelial cells (HAEC) response to shear stress induced by perfusion. (*A*) Current traces of a HAEC before and 14 min after perfusion at 1 ml/min. Cell held at −60 mV and pulsed from −120 to +100 mV in 20 mV steps with a duration of 1 sec. (*B*) A different HAEC showing the effect of perfusion on cytosolic $Ca^{2+}$ concentration and currents are shown in the top and bottom panel, respectively. Ramp current traces of 1 sec duration were elicited every 10 sec from −120 to +100 mV with a holding potential of −60 mV. Current data plotted as peak current at −120 (squares) and +100 mV (circles). The dotted line occurs at 325 sec and allows for a comparison of the time for a significant increase in $[Ca^{2+}]_i$ *vs.* the time the ionic currents activate *B.* (*C*) Ramp current traces before and after perfusion for the same cell. Zero current level shifted from −62 to +3 mV following perfusion.

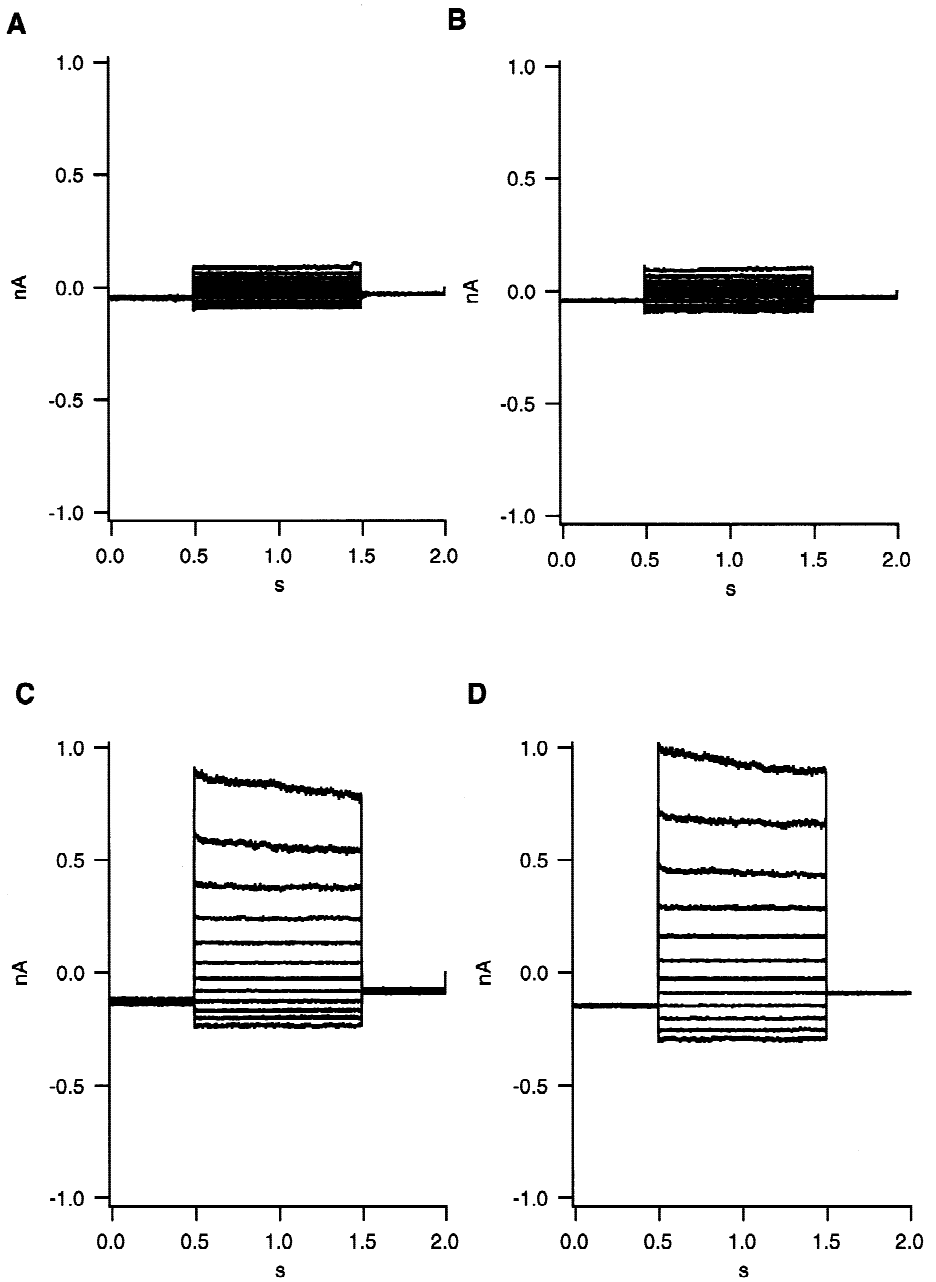


**Fig. 2.** The flow response of HAEC is dependent upon extracellular $Ca^{2+}$. HAEC held at −60 mV and pulsed from −120 to 100 mV in 20 mV steps of 1 sec duration. (*A*) Control in the absence of perfusion. (*B*) After perfusion at 1 ml/min with extracellular solution containing very low $Ca^{2+}$ (10 mM BAPTA, no $Ca^{2+}$ added). (*C*) Same cell now perfused with normal extracellular solution containing 1.8 mM $Ca^{2+}$. (*D*) Same cell after termination of flow in stationary solution.

Figure 3 shows that the ionic currents elicited by flow in HAEC were activated only when extracellular $Ca^{2+}$ levels were relatively high. Figure 3*A* shows changes in intracellular $Ca^{2+}$ from a HAEC loaded with Fura2. In the presence of 0 mM extracellular $Ca^{2+}$, flow produced no increase in intracellular $Ca^{2+}$ and no induction of ionic current (3*B* and *C*). Perfusion with 0.5 mM $Ca^{2+}$ produced a substantial increase in intracellular $Ca^{2+}$ to a steady-state level of 120 nM but this level of intracellular $Ca^{2+}$ was unable to activate significant amounts of ionic current. Perfusion with normal 1.8 mM $Ca^{2+}$ solution increased intracellular $Ca^{2+}$ levels to 230 nM and induced large ionic currents (3*B* and *C*). This experiment shows that extracellular $Ca^{2+}$ entry is involved in

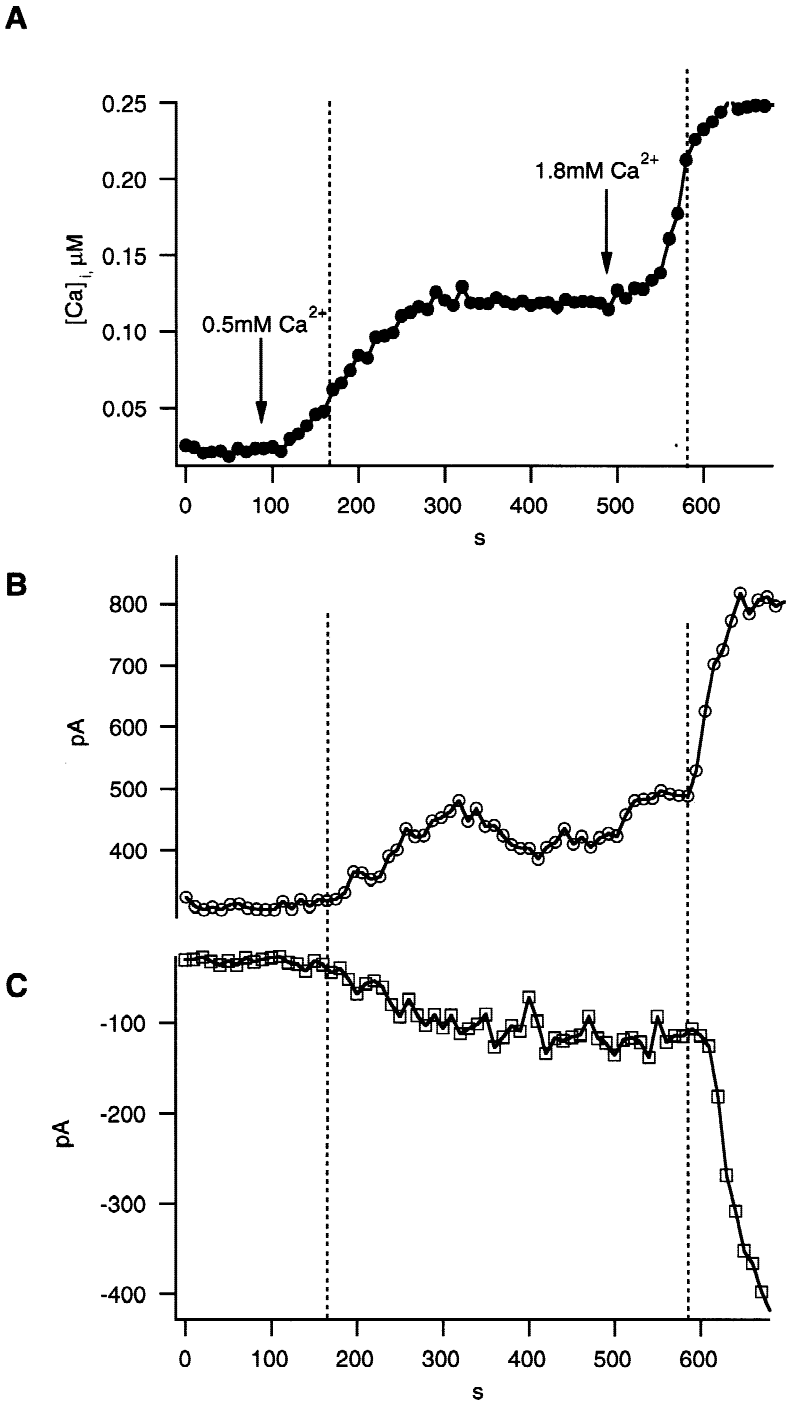


**Fig. 3.** Dependence of intracellular calcium $[Ca^{2+}]_i$ and flow-activated currents on external $Ca^{2+}$. $[Ca^{2+}]_i$ and currents were measured as a function of time from ramp current traces from −120–100 mV for 1 sec. (*A*) $[Ca^{2+}]_i$ (close circles). (*B*) Outward current at 100 mV (open circles). (*C*) Inward current at −120 mV (open squares) before and after perfusion with bath solution containing 0.5 mM $Ca^{2+}$ and 1.8 mM $Ca^{2+}$. Initially the cell was resting in low $Ca^{2+}$ bath solution (1.8 mM $Ca^{2+}$ + 10 mM BAPTA). The dotted lines occur at 170 sec and 580 sec, and allow for a comparison of the time for a significant increase in $[Ca^{2+}]_i$ *vs.* the time the ionic currents activate.

the flow response and that there is a threshold level of intracellular $Ca^{2+}$ required for induction of the flow stimulated ionic currents.

We believe that one component of the ionic current activated by flow is a nonselective current that is further characterized in Fig. 4*A*. The large current-voltage plot shows raw current traces from a HAEC ramped from −120 to +100 mV before and after flow. Flow induced a large increase in both outward and inward current and shifted the reversal potential in the depolarizing direction. Following flow, substitution of external NaCl with NMDG-Cl blocked the inward current and a good portion of the outward current. This suggests that not only is $NMDG^+$ impermeant to the pore but that it also pro-

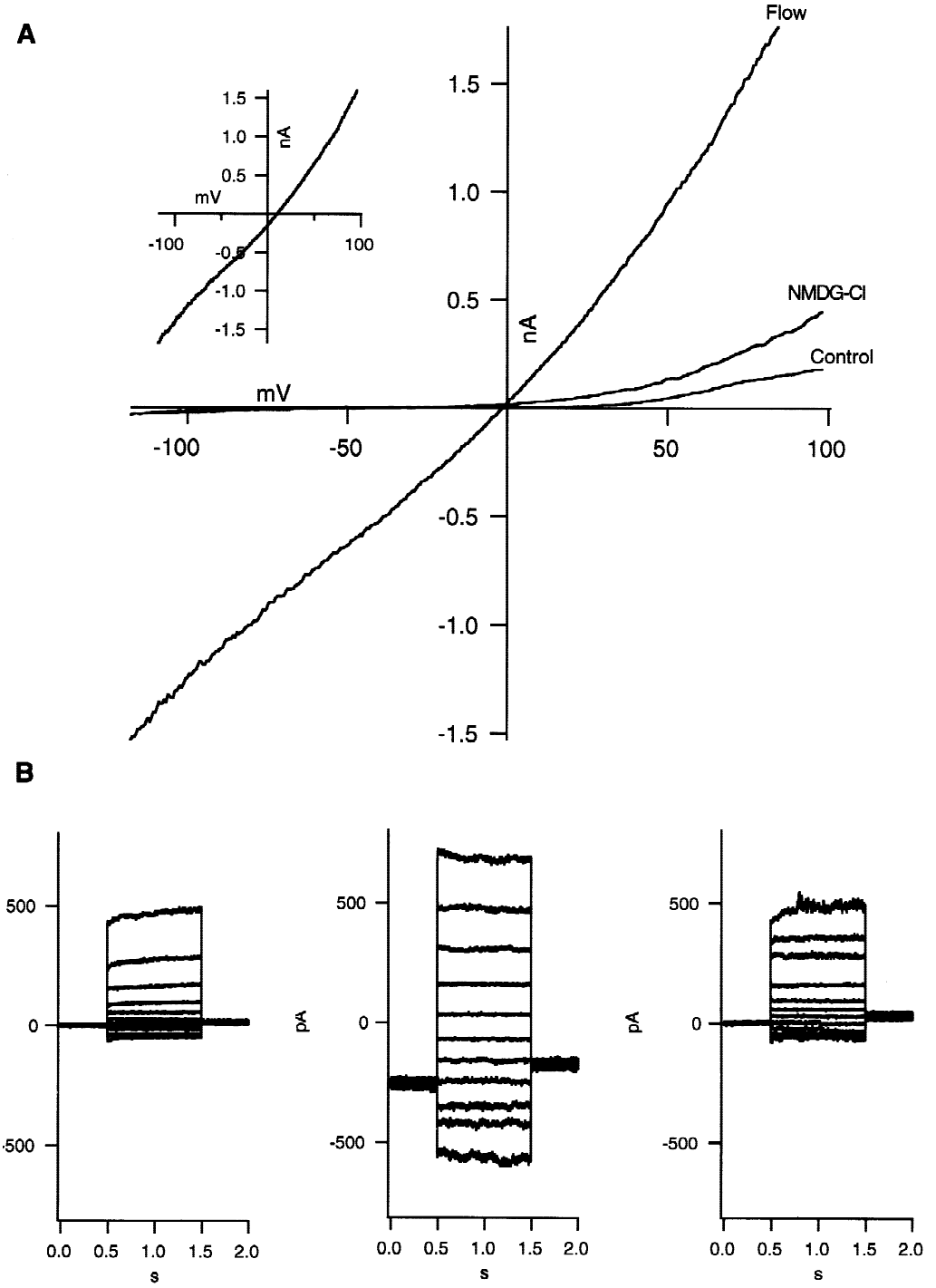


**Fig. 4.** Fluid flow activates a nonselective cation current that reverses at +7 mV and is blocked by substitution of external $Na^+$ with $NMDG^+$. (*A*) Ramp traces from −120 to +100 mV for 1 sec from a holding potential of −60 mV, in stationary bath solution (control), after perfusion (flow), and after substitution of NaCl in the bath solution with NMDG-Cl. The $NMDG^+$ sensitive difference current is shown in the inset and reverses at 7 mV. Figure 4*B* shows raw current traces for another HAEC held at −60 mV and pulsed from −120 to +100 mV in 20 mV voltage steps. The cell started in stationary solution, was perfused with normal external bath solution, and then perfused with bath solution containing 100 μM $Gd^{3+}$.

duces a partial block of outward going $K^+$ ions. The inset plots the $NMDG^+$-sensitive current from the same cell after perfusion with $NMDG^+$ and shows that it has a reversal potential of +7 mV. $NMDG^+$ was observed to block the flow-activated nonselective current in three other HAEC. Figure 4*B* shows raw current traces from another HAEC before and after flow activation of ionic currents. 100 μM $Gd^{3+}$ was added after induction of flow-activated currents and it blocked them back to control levels seen before perfusion. Interestingly, block of the nonselective cation current by substitution of external $Na^+$ with $NMDG^+$ also caused a reduction in intracellular $Ca^{2+}$ levels by 24.2 ± 13% ($n = 5$). This suggests that a portion of the sustained increase in intracellular $Ca^{2+}$ produced by flow is mediated through $Ca^{2+}$ entry, presumably via this nonselective cation channel pathway. This is illustrated in Fig. 5 for a HAEC that has been switched from perfusion with normal bath solution to a perfusate in which $NMDG^+$ has been substituted for $Na^+$. $NMDG^+$ blocked all of the inward and the outward current that was presumably carried by the nonselective cation channel. This produced a fall in intracellular $Ca^{2+}$ levels, supportive of a role for a nonselective channel in a portion of the flow induced $Ca^{2+}$ entry.

Figure 6*A* explores the possible contribution of a chloride current to the flow-activated response. External $Na^+$ was substituted with $NMDG^+$ during perfusion to block the large nonselective cation current. The nonspecific chloride channel blocker NPPB (100 μM) was then added to block the $Cl^-$ current so it could be differentiated from the underlying $K^+$ conductance. The NPPB-sensitive current is shown in the inset and has a reversal potential of −47 mV which is close to the predicted reversal of −48 mV for $Cl^-$. NPPB was effective in blocking the flow-activated $Cl^-$ current in 5 cells that were examined. In a different series of experiments NPPB was shown to be ineffective in blocking the flow induced increase in $Ca^{2+}$ once it was activated, eliminating the possibility that NPPB was blocking the $Cl^-$ current by blocking $Ca^{2+}$ entry. Figure 6*B* shows current step data from another HAEC before and after activation of outward current by flow. Following activation, the cell was switched to an external solution containing the impermeant anion $MeSO_3^-$ that blocked much of the outward current. This suggests that in this particular cell most of the flow activated outward current was carried by a $Cl^-$ influx presumably through a $Cl^-$ channel. $MeSO_3^-$ substitution for external $Cl^-$ reduced flow activated outward currents in five cells that were tested.

The primary $K^+$ current activated following flow is the iberiotoxin sensitive calcium-activated $K^+$ current. Figure 7*A* shows ramp data from −120 to 100 mV in normal solution (HBS) and after substitution of external $Na^+$ by $NMDG^+$. $NMDG^+$ appears to have blocked the inward current through the nonselective cation channel and also reduced the outward $K^+$ efflux through the nonselective channel. Application of 100 nM iberiotoxin blocked most of the remaining outward current activated by flow. The inward rectifier current seen at more negative potentials was not affected. Figure 7*B* shows individual raw current traces from another cell before, after flow, and after addition of 100 nM iberiotoxin. In this particular cell most of the flow-activated current was carried by the $Ca^{2+}$ activated $K^+$ channel which was subsequently blocked by iberiotoxin. Iberiotoxin blocked a portion (70.3 ± 7.7%) of the flow activated outward current in four additional cells.

The increase in intracellular $Ca^{2+}$ and ionic currents during flow could be blocked by inhibitors of nitric oxide synthase in a subset of cells tested (50%). Figure 8*A* shows current traces before flow, after flow with external solution containing 10 μM L-NAME, and after perfusion with normal external solution. The induction of ionic currents by flow was prevented in the continued presence of L-NAME for this particular cell. Figure 8*B* shows Fura2 $Ca^{2+}$ data from another HAEC before flow, after flow with solution containing L-NAME, and for perfusion with normal external solution (HBS). Figure 8*C* shows simultaneous current data from the same cell for peak currents seen at −120 and 100 mV. L-NAME was effective in blocking both the increase in intracellular $Ca^{2+}$ and the subsequent induction of ionic currents in 50% of all cells tested with dual recording ($n = 8$). In five different experiments, another nitric oxide inhibitor, 10 μM L-NIO blocked the increase in intracellular $Ca^{2+}$ in 50.7% of the cells studied ($n = 148$).

## Discussion

Endothelial cells are ideally situated to respond to shear forces resulting from the flow of blood. The walls of large aortic vessels are subjected to different shear and stretch forces than are the walls of small arterioles or capillaries [6]. Endothelial cells can respond to these shearing forces with increases in intracellular $Ca^{2+}$ and release of vasoactive substances such as nitric oxide [6, 18, 26, 34, 36]. Human aortic endothelial cells (HAEC) respond to the shear force produced from moderate (1 ml/min) perfusion of a 35 mm tissue culture dish, with a sustained increase in intracellular $Ca^{2+}$ and ionic currents. Conversely, human capillary endothelial cells (HCEC) were unresponsive to flow (up to 6 ml/min) although they have similar baseline currents [17] and do respond to cell swelling and treatment with the calcium ionophore A23187. It is attractive to postulate that the differences seen between the human aortic and capillary endothelial cells response to flow may reflect true differences seen in vivo but such extrapolation from an in-vitro cell culture system should be discouraged. The studies presented here are from primary endothelial cells

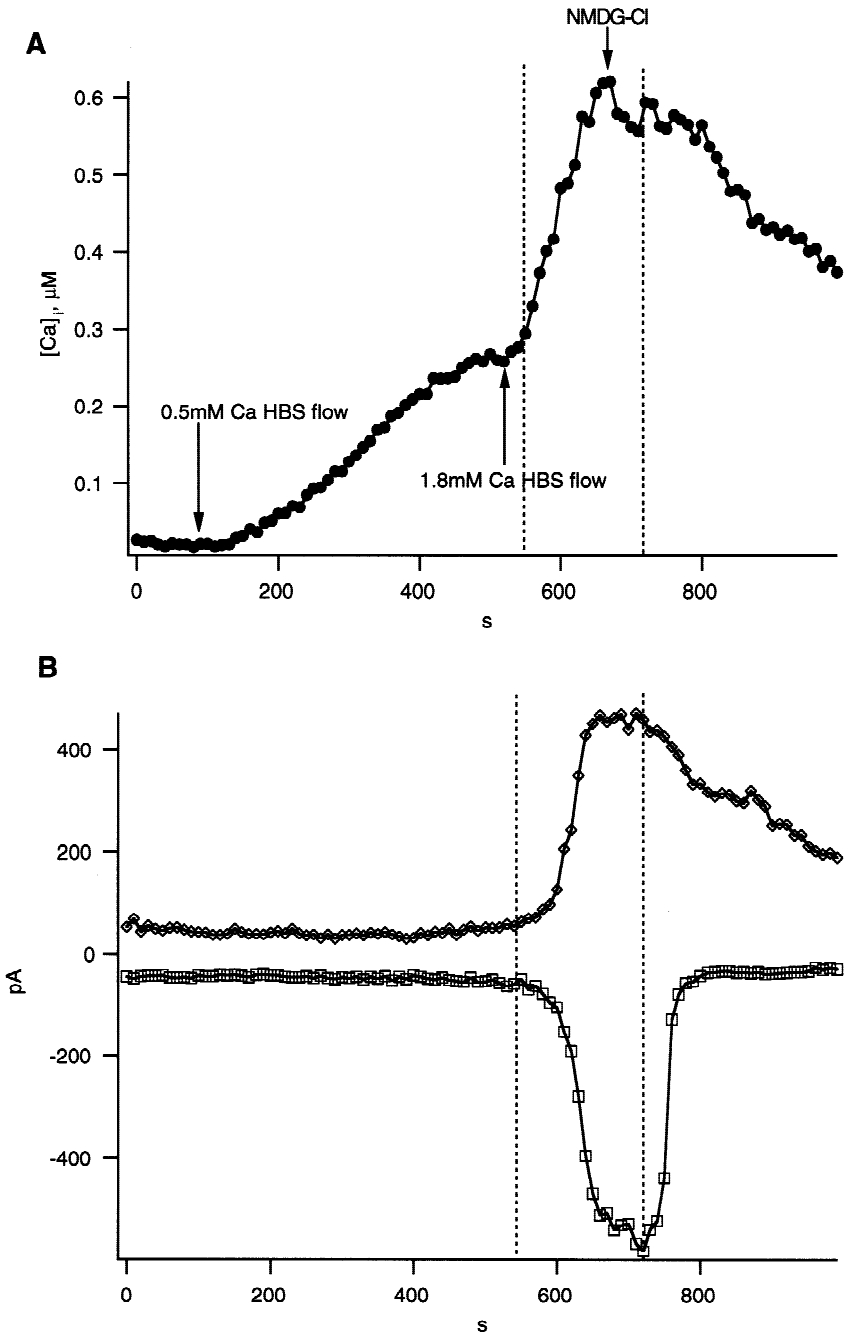


**Fig. 5.** Block of the nonselective cation current by $NMDG^+$ also reduced the increase in intracellular $Ca^{2+}$ produced by flow. (*A*) Shows intracellular $Ca^{2+}$ levels in a HAEC perfused with bath solution containing 0.5 mM $Ca^{2+}$, 1.8 mM $Ca^{2+}$, and after substitution of external $Na^+$ with $NMDG^+$. Perfusion with 1.8 mM $Ca^{2+}$ elicits a large increase in intracellular $Ca^{2+}$ (*A*, solid circles) as well as increases in peak current at +100 mV (*B*, triangles) and −120 mV (*B*, squares). Perfusion with bath solution containing 1.8 mM $Ca^{2+}$ and $NMDG^+$ substituted for $Na^+$ blocks all of the inward current and reduces the outward current and intracellular $Ca^{2+}$ concentration. The dotted lines occur at 545 and 720 sec, and allow for a comparison of the time for a significant increase in $[Ca^{2+}]_i$ *vs.* the time the ionic currents activate.

maintained in cell culture and that their complement of ion channels and associated signaling pathways may be modified from those in vivo.

The increase in ionic currents with flow in HAEC can be divided into three components: (i) A nonselective cation current that is blocked by $NMDG^+$ and $Gd^{3+}$ and reverses at +7 mV. (ii) A voltage-dependent outwardly rectifying $Cl^-$ current that reverses near $E_{Cl^-}$ and is

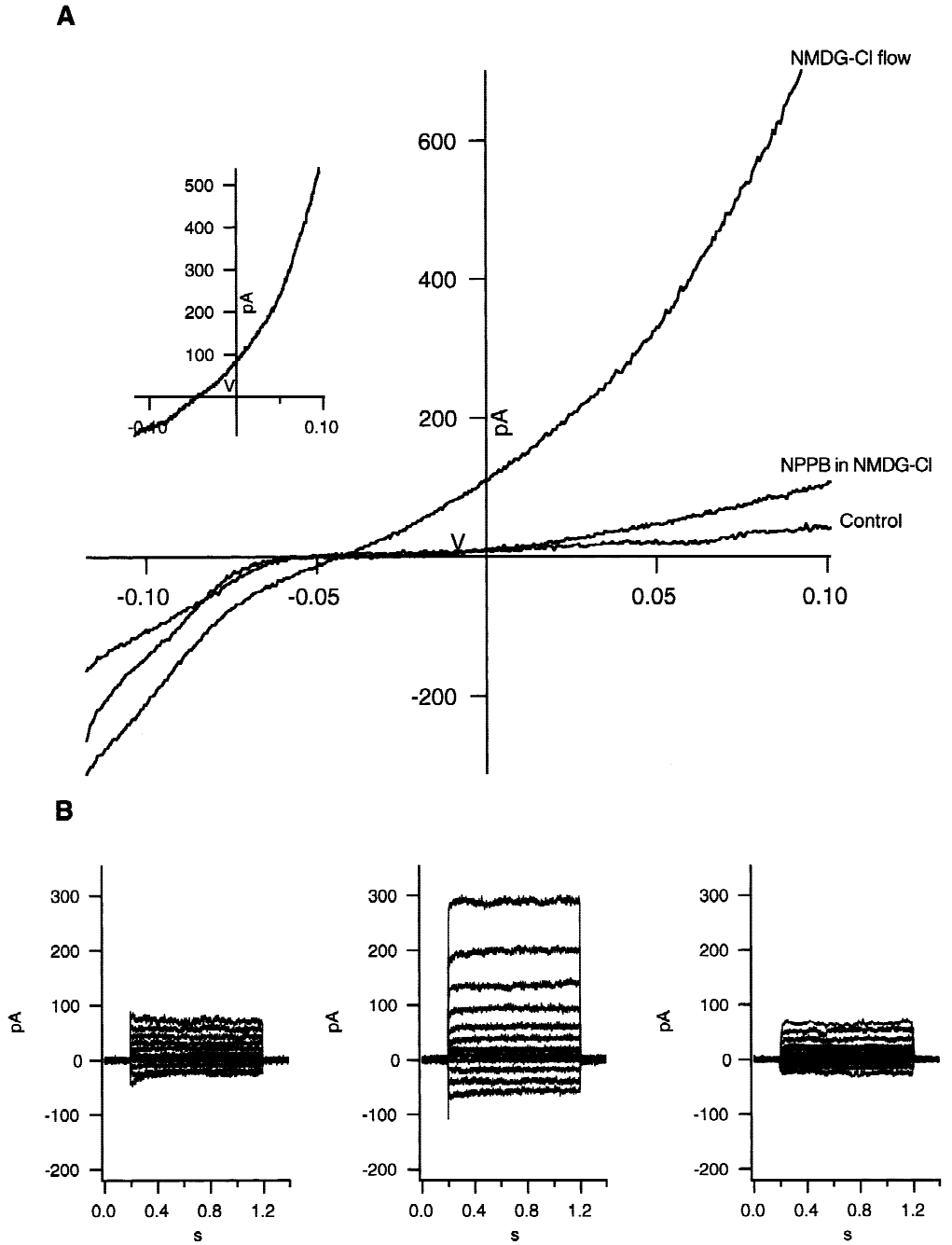


**Fig. 6.** Another component to the flow-activated current in HAECs is blocked by 100 μM of the chloride channel blocker NPPB. (*A*) Ramp current traces from a HAEC in stationary bath solution (control), following perfusion with NMDG-Cl perfusate, and after blocking the chloride current with 100 μM NPPB. $NMDG^+$ was substituted for external $Na^+$ to block the nonselective cation channel that is also activated by flow. The NPPB-sensitive difference current (in the continual presence of $NMDG^+$) is shown in the inset. (*B*) Current traces from another HAEC in response to 20 mV voltage steps from −120–100 mV from a hold potential of −60 mV, in normal stationary bath solution, after perfusion, and following perfusion with bath solution in which $NaMeSO_3$ is substituted for NaCl. $MeSO_3$ is impermeant through many types of $Cl^-$ channels.

blocked by substitution of NaCl with $NaMeSO_3$ and the $Cl^-$ channel blocker NPPB. (iii) A calcium-activated potassium current that is blocked by iberiotoxin. The increase in ionic current is dependent on flow and external $Ca^{2+}$. Increases in intracellular $Ca^{2+}$ levels have been associated with flow in bovine aortic [10, 32] and human umbilical vein endothelial cells [31, 33]. The increase in $[Ca^{2+}]_i$ of HAEC is dependent on external con-

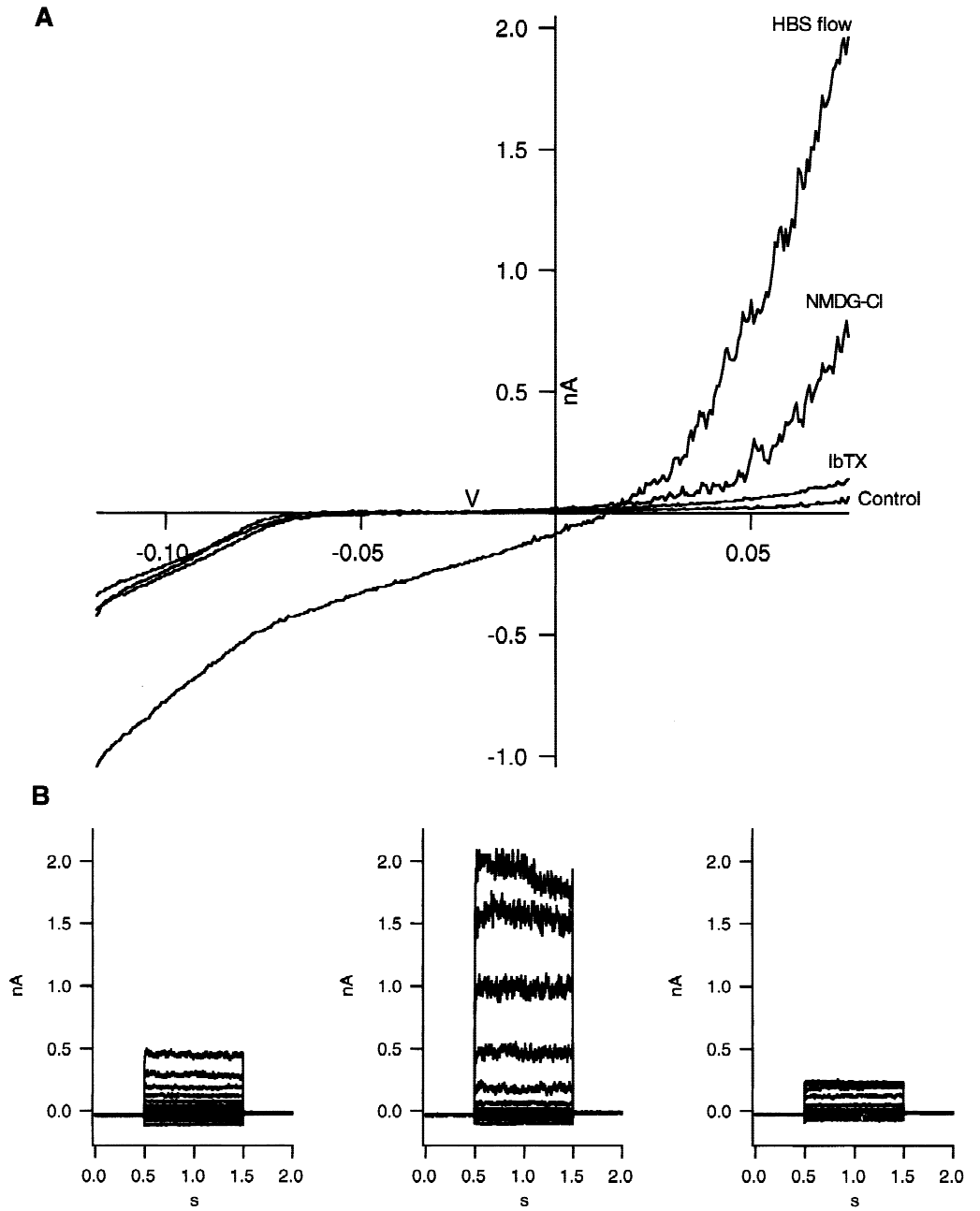


**Fig. 7.** Iberiotoxin block of a flow-activated outward current from a human aortic endothelial cell. (*A*) Current voltage ramps from a HAEC before (control) and after perfusion with normal bath solution (HBS). $NMDG^+$ was substituted for external $Na^+$ to block the nonselective cation channel that is also activated by flow. Iberiotoxin at 100 nM blocked over 80% of the remaining outward current in this cell. Figure 7*B* shows raw current traces from a HAEC held at −60 mV and pulsed from +100 to −120 mV in stationary, perfused solution, and perfused with 100 nM iberiotoxin. In this particular cell most of the outward current after block of the nonselective current was carried by the iberiotoxin-sensitive current.

centrations of $Ca^{2+}$ and may be mediated through a flow activated $Ca^{2+}$ permeable channel [31]. We were unable to detect the presence of such a $Ca^{2+}$ current immediately following perfusion, although the concurrent $Ca^{2+}$ measurement showed a clear increase in $Ca^{2+}$ (Figs. 1, 3, 5 and 8). The inability to detect a $Ca^{2+}$ current even though intracellular $Ca^{2+}$ is increasing has been described in human umbilical vein endothelial cells for the calcium release activated current ($I_{CRAC}$) mediated increase in intracellular $Ca^{2+}$, where it was attributed to the low magnitude and sustained nature of the presumed $Ca^{2+}$ current [11, although *see* 7]. The rise in intracellular $Ca^{2+}$ may directly activate the ionic currents we see or initiate a cascade of intracellular processes that leads

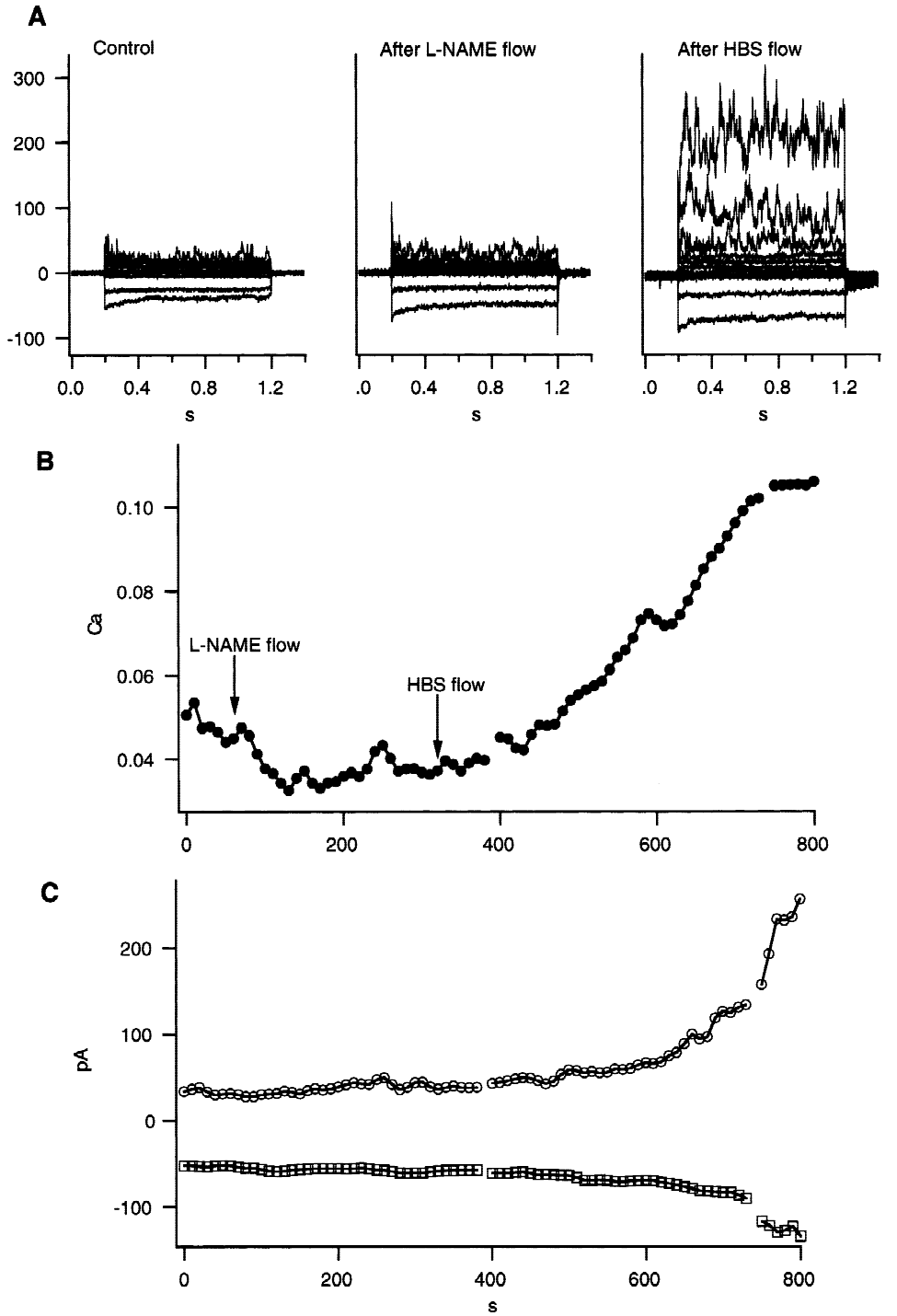


**Fig. 8.** Nitric oxide synthase inhibitor, L-NAME blocks the flow-activated current and $[Ca^{2+}]_i$ increase in subpopulation of HAEC. (*A*) HAEC held at −60 mV and pulsed from −120 to +100 mV in 20 mV voltage steps. Control shows the cell in stationary solution, after perfusion with 100 μM L-NAME and after wash out of L-NAME with normal bath solution. $[Ca^{2+}]_i$ (*B*, solid circles) and peak outward current at +100 mV (*C*, hollow circles) and peak inward current at −120 mV (*C*, hollow squares) *vs.* time from the same cell.

to the increase in currents. The inability of zero $Ca^{2+}$ and 20 mM BAPTA or EGTA inside the pipette to block the flow activation of currents taken together with the strict dependence on external $Ca^{2+}$ suggests that intracellular $Ca^{2+}$ release may not be involved and that the site of increase of intracellular $Ca^{2+}$ is localized to the entry sites and the ion channels that are modulated, and are inaccessible to bulk $Ca^{2+}$ buffering through the pipette [35].

A flow-activated, inwardly rectifying $K^+$ current has been described in cultured bovine aortic endothelial cells [27]. We see no evidence for such a flow-activated inwardly rectifying $K^+$ current from our cells as can be seen by the overlapping inward rectifier current in Fig. 4 for a cell in stationary solution and after perfusion with external $NMDG^+$ to block the nonselective cation current activated by flow. A shear stress-activated cation current has been reported in human umbilical cord endothelial cells. The current reverses at 1.5 mV in normal 1.5 mM external $Ca^{2+}$ bath solution and has a current-voltage relationship similar to the one we observe in HAEC. In contrast to our study the nonselective current activated in that report was only present for as long as the flow was maintained [31].

If such a nonselective cation channel is involved in the HAEC flow response then it may be responsible for a portion of the $Ca^{2+}$ entry resulting in the sustained levels of intracellular $Ca^{2+}$ observed following flow (Fig. 5). Interestingly, the nonselective cation current we see activated in these human endothelial cells following flow is maintained well after the flow has stopped. It may be that the channel is activated by flow and $Ca^{2+}$ entry and that its continued activation is dependent upon high levels of local intracellular $Ca^{2+}$ or other factors activated as a result of $Ca^{2+}$ entry. The current has several properties that link it to the trp-like family of ion channels and there has been a recent report identifying trp in bovine aortic endothelial cells [3].

The flow activated NPPB sensitive chloride current seen in this study has a reversal potential of −47 mV which is close to the predicted Nernst potential of −48 mV based on 149/22 mM $Cl^-$ external and internal, respectively. This chloride current differs from most stretch- or swelling-activated $Cl^-$ currents as once it is activated by flow it remains activated even when the cell is returned to stationary solution.

There are no reports of flow-activated chloride channels from endothelial cells although osmotically sensitive $Cl^-$ channels are found in many cell types [20, 21]. A $Ca^{2+}$ activated $Cl^-$ current has been described in bovine artery endothelial cells that appears very similar to the flow-activated $Cl^-$ current we describe in HAEC. These similarities include: the current kinetics are time and voltage independent and the current voltage relationship shows strong outward rectification [22, 23, 24]. Indeed it seem reasonable to suggest that the flow-activated $Cl^-$ current we see in these HAEC is due to a $Ca^{2+}$-activated $Cl^-$ channel.

The calcium-activated potassium current that is activated following flow is likely to be the large conductance $Ca^{2+}$ activated $K^+$ channel (BKCA) based on its sensitivity to iberiotoxin [2, 12]. The whole cell current recordings of HAEC were “noisy” in appearance and had noninactivating kinetics similar to BKCA channels recorded in other endothelial cell preparations [30].

Endothelial cells are known to contain nitric oxide synthase and to release NO in response to flow [26]. The mechanism by which NO is released is still in dispute with some groups claiming a $Ca^{2+}$-independent mechanism [9, 28] possibly via phosphorylation of NOS [4] while others observe a more conventional mechanism mediated by increases in intracellular $Ca^{2+}$ [19, *see* 8 for review]. We consistently find that 50% of the HAEC have their flow-induced response blocked by NO synthase inhibitors applied either prior to the flow or simultaneous with it. We postulate that by blocking NO synthesis we reduce calcium-independent NO release and that NO feeds back onto the endothelial cells to induce calcium entry and activation of ionic currents. In support of this idea, the NO donor SMOC has been shown to increase intracellular $Ca^{2+}$ levels in a cultured human umbilical vein derived endothelial cell line [13] possibly through $Ca^{2+}$ entry. The 50% response level may occur because NO synthase inhibitors only block NO production and not release and there may be alternate pathways available for induction of flow dependent increases in intracellular calcium and ionic currents.

In summary, flow increased intracellular $Ca^{2+}$ levels in HAEC through an unknown $Ca^{2+}$ entry mechanism and induces the activation of a nonselective cation current that further contributes to $Ca^{2+}$ entry. The overall increase in intracellular $Ca^{2+}$ activates a Ca dependent chloride current, and a $Ca^{2+}$ activated $K^+$ current that is blocked by iberiotoxin. In contrast human capillary endothelial cells were insensitive to flow although their intracellular $Ca^{2+}$ and ionic currents can be increased by cell swelling and $Ca^{2+}$ ionophore. In the HAEC, the flow activated currents, and the $Ca^{2+}$ influx responsible for the increase in intracellular $Ca^{2+}$, are maintained well after the flow is terminated. Inhibitors of NO are successful in blocking both the flow activated current and calcium increase in 50% of HAEC possibly through a calcium independent NO pathway. The observation that calcium entry can occur directly through the flow activated nonselective channel, and the activation of ionic currents that may affect the driving force for calcium, suggests that these flow activated ion channels could play an important role in the increase in intracellular calcium levels seen in the endothelial cell’s response to flow [1, 15, 18].

## References


1. Adams, D.J., Barakeh, J., Laskey, R., van Breemen, C. 1989. Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.* **3:**2389–2400
2. Candia, S., Garcia, M.L. 1992. Mode of action of iberiotoxin, a potent blocker of the large conductance $Ca^{2+}$-activated $K^+$ channel. *Biophys. J.* **63:**583–590
3. Chang, A.S., Chang, S.M., Garcia, R.L., Schilling, W.P. 1997. Concomitant and hormonally regulated expression of trp genes in bovine aortic endothelial cells. *FEBS Lett.* **415:**335–340
4. Corson, M.A., James, N.L., Latta, S.E., Nerem, R.M., Berk, B.C., Harrison, D.G. 1996. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. **79:**984–991
5. Davies, P.F. 1997. Mechanisms involved in endothelial responses to hemodynamic forces. *Atherosclerosis* **131:**S15–7
6. Davies, P.F., Barbee, K.A., Volin, M.V., Robotewskyj, A., Chen, J., Joseph, L., Griem, M.L., Wernick, M.N., Jacobs, E., Polacek, D.C., dePaola, N., Barakat, A.I. 1997. Spatial relationships in early signaling events of flow-mediated endothelial mechanotransduction. *Ann. Rev. Physiol.* **59:**527–549
7. Fasolato, C., Nilius, B. 1998. Store depletion triggers the calcium release-activated calcium current (ICRAC) in macrovascular endothelial cells: a comparison with Jurkat and embryonic kidney cell lines. *Pfluegers Arch.* **436:**69–74
8. Fleming, I., Bauersachs, J., Busse, R. 1997. Calcium-dependent and calcium-independent activation of the endothelial NO synthase. *J. Vasc. Res.* **34:**165–174
9. Fleming, I., Bauersachs, J., Fisslthaler, B., Busse, R. 1998. $Ca^{2+}$ independent activation of endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ. Res.* **82:**686–695
10. Geiger, R.V., Berk, B.C., Alexander, R.W., Nerem, R.M. 1992. Flow-induced calcium transients in single endothelial cells: spatial and temporal analysis. *Am. J. Physiol.* **262:**C1411–C1417
11. Gericke, M., Oike, M., Droogmans, G., Nilius, B. 1994. Inhibition of capacitative $Ca^{2+}$ entry by a $Cl^-$ channel blocker in human endothelial cells. *Eur. J. Pharmacol.* **269:**381–384
12. Giangiacomo, K.M., Sugg, E.E., Garcia-Calvo, M., Leonard, R.J., McManus, O.B., Kaczorowski, G.J., Garcia, M.L. 1993. Synthetic charybdotoxin-iberiotoxin chimeric peptides define toxin binding sites on calcium-activated and voltage-dependent potassium channels. *Biochemistry* **32:**2363–2370
13. Haburcak, M., Wei, L., Viana, F., Prenen, J., Droogmans, G., Nilius, B. 1997. Calcium-activated potassium channels in cultured human endothelial cells are not directly modulated by nitric oxide. *Cell Calcium* **21:**291–300
14. Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100
15. Himmel, H.M., Whorton, A.R., Strauss, H.C. 1993. Intracellular calcium, currents, and stimulus-response coupling in endothelial cells. *Hypertension* **21:**112–127
16. Hoyer, J., Distler, A., Haase, W., Gogelein, H. 1994. $Ca^{2+}$ influx through stretch-activated cation channels activates maxi $K^+$ channels in porcine endocardial endothelium. *Proc. Nat. Acad. Sci. USA* **91:**2367–2371
17. Jow, F., Numann, R., Colatsky, T. 1996. Ion channels in human aorta endothelial cells and human capillary endothelial cells. *Biophys. Soc.* (*Abstr.*) **70(2):**W-pos 130
18. Luckhoff, A., Busse, R. 1990. Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pfluegers Arch.* **416:**305–311
19. Malek, A.M., Jiang, L., Lee, I., Sessa, W.C., Izumo, S., Alper, S.L. 1999. Induction of nitric oxide synthase mRNA by shear stress requires intracellular calcium and G-protein signals and is modulated by PI 3 kinase. *Biochem. Biophys. Res. Comm.* **254:**231–242
20. Nilius, B., Eggermont, J., Voets, T., Droogmans, G. 1996. Volume-activated $Cl^-$ channels. *Gen. Pharmacol.* **27:**1131–1140
21. Nilius, B., Oike, M., Zahradnik, I., Droogmans, G. 1994. Activation of a $Cl^-$ current by hypotonic volume increase in human endothelial cells. *J. Gen. Physiol.* **103:**787–805
22. Nilius, B., Prenen, J., Szucs, G., Wei, L., Tanzi, F., Voets, T., Droogmans, G. 1997. Calcium-activated chloride channels in bovine pulmonary artery endothelial cells. *J. Physiol.* **498:**381–396
23. Nilius, B., Prenen, J., Voets, T., Van den Bremt, K., Eggermont, J., Droogmans, G. 1997. Kinetic and pharmacological properties of the calcium-activated chloride-current in macrovascular endothelial cells. *Cell Calcium* **22:**53–63
24. Nilius, B., Szucs, G., Heinke, S., Voets, T., Droogmans, G. 1997. Multiple types of chloride channels in bovine pulmonary artery endothelial cells. *J. Vasc. Res.* **34:**220–228
25. Nilius, B., Viana, F., Droogmans, G. 1997. Ion channels in vascular endothelium. *Ann. Rev. Physiol.* **59:**145–170
26. Noris, M., Morigi, M., Donadelli, R., Aiello, S., Foppolo, M., Todeschini, M., Orisio, S., Remuzzi, G., Remuzzi, A. 1995. Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ. Res.* **76:**536–543
27. Olesen, S.P., Clapham, D.E., Davies, P.F. 1988. Haemodynamic shear stress activates a $K^+$ current in vascular endothelial cells. *Nature* **331:**168–170
28. O'Neill, W.C. 1995. Flow-mediated NO release from endothelial cells is independent of $K^+$ channel activation or intracellular $Ca^{2+}$. *Am. J. Physiol.* **269:**C863–C869
29. Revest, P.A., Abbott, N.J. 1992. Membrane ion channels of endothelial cells. *Trends in Pharmacol. Sci.* **13:**404–407
30. Rusko, J., Tanzi, F., van Breemen, C., Adams, D.J. 1992. Calcium-activated potassium channels in native endothelial cells from rabbit aorta: conductance, $Ca^{2+}$ sensitivity and block. *J. Physiol.* **455:** 601–621
31. Schwarz, G., Droogmans, G., Nilius, B. 1992. Shear stress-induced membrane currents and calcium transients in human vascular endothelial cells. *Pfluegers Arch.* **421:**394–396
32. Shen, J., Luscinskas, F.W., Connolly, A., Dewey, C.F. Jr., Gimbrone, M.A. Jr. 1992. Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells. *Am. J. Physiol.* **262:**C384–C390
33. Sigurdson, W.J., Sachs, F., Diamond, S.L. 1993. Mechanical perturbation of cultured human endothelial cells causes rapid increases of intracellular calcium. *Am. J. Physiol.* **264:**H1745–H1752
34. Tsao, P.S., Lewis, N.P., Alpert, S., Cooke, J.P. 1995. Exposure to shear stress alters endothelial adhesiveness. Role of nitric oxide. *Circulation* **92:**3513–3519
35. Vaca, L., Kunze, D.L. 1993. Depletion and refilling of intracellular $Ca^{2+}$ stores induce oscillations of $Ca^{2+}$ current. *Am. J. Physiol.* **264:**H1319–H1322
36. Wellman, G.C., Bevan, J.A. 1995. Barium inhibits the endothelium-dependent component of flow but not acetylcholine-induced relaxation in isolated rabbit cerebral arteries. *J. Pharmacol. Exp. Ther.* **274:**47–53