

Hydrodynamic hyperpolarization of endothelial cells

(membrane polarization/hemodynamic/arterial pathophysiology)

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ABSTRACT The orientation and morphology of the endothelium lining the cardiovascular system may result from hemodynamic forces acting on the endothelial cells. To investigate the flow effects at the membrane level, we have examined the variations of the fluorescence intensity of two membrane-sensitive dyes, merocyanine 540 and bis(1,3-diethylthiobarbiturate)trimethineoxonol, (*i*) as a function of flow shear stress and (*ii*) with the onset or cessation of the flow. We found a time-dependent decrease in fluorescence intensity with the onset of the flow with an exponential approach to steady state of the order of 1 min. The process is reversible; when the flow is stopped the fluorescence intensity returns to its original value. The polarization of the endothelial cell membranes or, more precisely, the amplitude of the fluorescence intensity responses is an increasing function of the shear stress (up to 120 dynes/cm²). Assuming the equilibrium potential for K⁺ is more hyperpolarized than the resting potential and using valinomycin, we have deduced from the sign of the ionophore effects that the flow hyperpolarizes the endothelial cell membrane.

Numerous studies have been carried out to elucidate the relationship between blood flow and the genesis and development of arterial wall pathophysiology (1). The influence of hemodynamic related events on the arterial wall itself has been emphasized and more recently this focus has been turned to the endothelial cells. Endothelial cells lining the cardiovascular system are aligned in the direction of blood flow, and the orientation and morphology of these cells may result from hemodynamic forces acting on the cells (2). The first macroscopic response to fluid-imposed shear stress is the remodeling of the cytoskeletal system (3). But the relationship between stress fibers, one of the three types of intracellular filaments, and shearing forces appears complex. Other biochemical responses have already been associated with fluid-related parameters. A high correlation exists between shear stress and histamine-forming capacity, suggesting that the histidine decarboxylase system of the aorta may have the potential for serving as one coupling agent between applied hemodynamic stress and resultant alterations in aortic wall resistance to macromolecules (4). Endothelial cells produce a burst of prostacyclin in response to suddenly imposed arterial-like shear stress (5). It has also been reported that physiological shear stress enhances the Ca²⁺ permeability of human erythrocytes (6). On the other hand, electrochemical parameters account for some pathophysiological processes occurring at the blood-vascular interface. And there have been indications that the electrochemical parameters of the blood cells and vascular wall should be taken into account in all vascular thrombosis phenomena (7). In another biological system, a galvanic response consisting of the orientation of stress fibers perpendicular to the field has been described in cultured *Xenopus* epithelial cells (8).

Moreover, theoretical work on electrophoresis along cell membranes (9) and related experiments have shown that an electric field grossly redistributes concanavalin along embryonic muscle cell membrane (10). Thus, to correlate flow-related parameters and endothelial cell membranes, we addressed the question of how the membrane may sense the flow. Here we report on the hydrodynamic hyperpolarization of the endothelial cell membrane.

MATERIALS AND METHODS

Flow Ring. The flow chamber used to expose cultured endothelial cells to a known hydrodynamic wall shear is a channel of rectangular cross section that has been designed to provide steady uniform laminar flow (Fig. 1). A microscope slide of cultured cells constitutes the bottom of this channel. Flowing solution over the microscope slide approximates flow between infinite parallel plates. The fluid mechanics in such channels have been studied both theoretically and experimentally (11). Knowing the pressure drop through the channel, one may calculate the wall shear stress and the Reynolds number. In our conditions, up to 120 dynes/cm², parabolic velocity profiles were verified by injecting ink into the fluid during control experiments. Some jetting was observed near the entrance; therefore, all observations have been made in the fully developed area.

Cells. Bovine pulmonary artery endothelial cells were established from primary culture and grown in Dulbecco's minimal essential medium (GIBCO) containing 10% fetal calf serum (HyClone, Logan, UT). We used cells between passages 3 and 10. Their endothelial origin was confirmed by the presence of cytoplasmic factor VIII antigen and membrane angiotensin-converting enzyme. The spontaneously derived BALB/c tumor (A20) established in culture was used as control. In all cases, the cells were used 1 or 2 days after growth to confluency or in a subconfluent state.

Fluorescence Membrane-Potential-Sensitive Dyes. In both endothelial and A20 cells, we performed experiments with merocyanine 540 and the bis(1,3-diethylthiobarbiturate)trimethineoxonol (12, 13) (Molecular Probes, Junction City, OR). Merocyanine was particularly suited to the measurement of membrane potential changes because of the presence of oligomers or microcrystals on the plasma membrane, which can compensate for the internalization of these probes by providing a reservoir for membrane-bound monomers.

We have tried a simultaneous electrode measurement, but it has not been possible to correlate flow and electrode signal measurements simultaneously without disturbing either the flow profile or the membrane potential. Moreover, it has been reported that when microelectrodes are allowed to remain inside the endothelial cell membrane the recorded potential displays slow spontaneous changes of several millivolts (14).

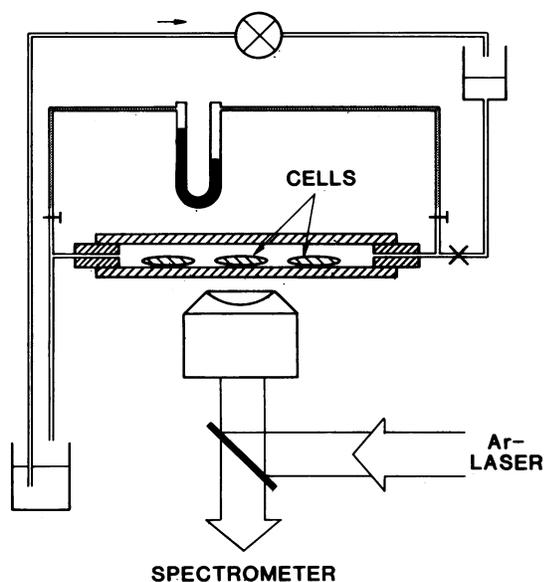


FIG. 1. Set-up used to investigate the flow effects on the endothelial cell membrane. Cells were incubated at room temperature with the membrane-potential-sensitive dyes described in text. Dyes were added from ethanol stock solution. Final concentration was always $\approx 5 \times 10^{-7}$ mol and the final ethanol concentration in the incubated cells never exceeded 0.1%. The standard flowing solution was made to approximate the ionic composition of the culture medium and contained 140 mM NaCl, 6 mM KCl, 1.25 mM CaCl_2 , 0.8 mM MgSO_4 , 1 mM Na_2PO_4 , 10 mM NaHepes, 5.6 mM dextrose (pH 7.2) at room temperature. The flow chamber was milled from a Plexiglas block. The test coverslip was fixed to the frame. The cross-section of the flow path in the chamber was 0.2×20 mm with a length of 60 mm in the center. Flow was achieved with a constant hydrostatic pressure and was regulated by both reservoir level and valve. Under these conditions the flow profile was laminar, even at the highest velocity, since the Reynolds numbers are well below the critical number for turbulence.

In all experiments, the standard flowing solution was made to approximate the ionic composition of the culture medium and valinomycin (Sigma), a cyclic antibiotic known to transport potassium ions selectively across the membrane, was added when required.

RESULTS

Flow-Membrane Fluorescence Intensity Dependence. Fluorescence emission spectra from a single endothelial cell in the presence and absence of flow are shown in Fig. 2 (Upper). The wavelength peak is independent of the flow but the amplitude of the fluorescence responses shows a clear flow dependence. We have been able to verify this result for shear stresses up to 120 dynes/cm² (Fig. 2 Lower). As controls, spectra of membrane-bound dyes and dyes in solution have been scanned. There are no significant changes in either the adsorption wavelength or the emission spectra (data not shown).

Kinetics and Sign of the Cell Membrane Polarization. To further study the flow-dependent endothelial cell membrane phenomenon, we have investigated the time course of the fluorescence intensity with onset and cessation of flow (Fig. 3). The changes in fluorescence intensity are reversible, but after extended periods >1 hr in flowing conditions and under our experimental conditions, the time constant for the decrease of fluorescence intensity with the onset of flow increases substantially. Likewise, the time constant for fluorescence recovery on cessation of flow increases significantly. Thus, the rates shown in Fig. 3 are dependent on the physiological state of the cell. We also note that glutaralde-

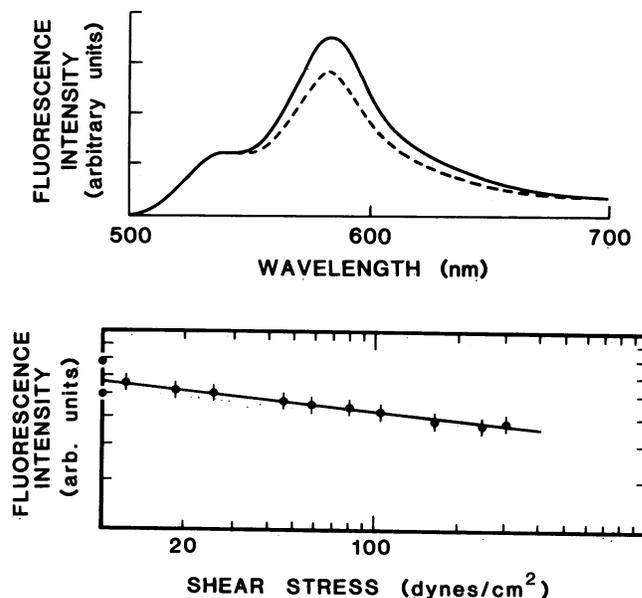


FIG. 2. Fluorescence emission of cell membrane-bound dyes with and without flow. Fluorescence spectroscopy was carried out on a Nikon Diaphot with a $\times 40$ Nikon correction objective. For epi-illumination, the standard filter set B was used with a Spectra Physics Argon ion laser (SP2000). The fluorescence image was switched to the monochromator Spex. The image plane was focused with a lens ($f = 60$ mm) on the entrance slit and filled without losing the area of the grating. The output slit was enlarged 3-fold and projected on the cathode of the RCA 31034 photomultiplier (cooled housing, -30°C). Photon counting was done by a Lecroy 3500 with an EGG 436 discriminator and EGG 535 preamp modules. The monochromator was driven by a Spex CompuDrive. (Upper) Fluorescence emission spectra of merocyanine bound to a single cell in absence of flow (---) and with a flow shear (—) of 10 dynes/cm². The excitation wavelength is 488 nm. The amplitude of the fluorescence response indicates a clear flow dependence. (Lower) Variations of the fluorescence intensity membrane-bound merocyanine as a function of the shear stress. Each single point is an average of 10 experimental results.

hyde fixation renders the membrane insensitive to flow. Moreover, no consistent flow-related changes in either merocyanine or bis(1,3-diethylthiobarbiturate)trimethineoxonol fluorescence intensity were observed with the A20 cell line.

The inclusion of valinomycin ionophore in a no-flow condition results in a decrease in membrane fluorescence intensity for both dyes (Fig. 3). It is known that (i) addition of valinomycin leads to a membrane hyperpolarization (outflux of K^+), and (ii) decreased fluorescence corresponds to hyperpolarization in most cells. Moreover, in the particular case of the bis(1,3-diethylthiobarbiturate)trimethineoxonol, it has been reported (13) that membrane hyperpolarization transfers dye anions from binding sites inside the cell to the external solution and thus leads to a decrease of fluorescence. Consequently, and from the sign of our actual experimental results, we conclude that the changes in fluorescence intensity are the result of an endothelial cell membrane hyperpolarization.

DISCUSSION

The interest in hemodynamics as a factor in arterial wall pathophysiology has raised questions about effects of mechanical events at the wall interface. The orientation and elongation after exposure to flow shear stress is certainly not a passive cell response of the endothelium considered as a viscous elastic continuum. But flow is expected to affect

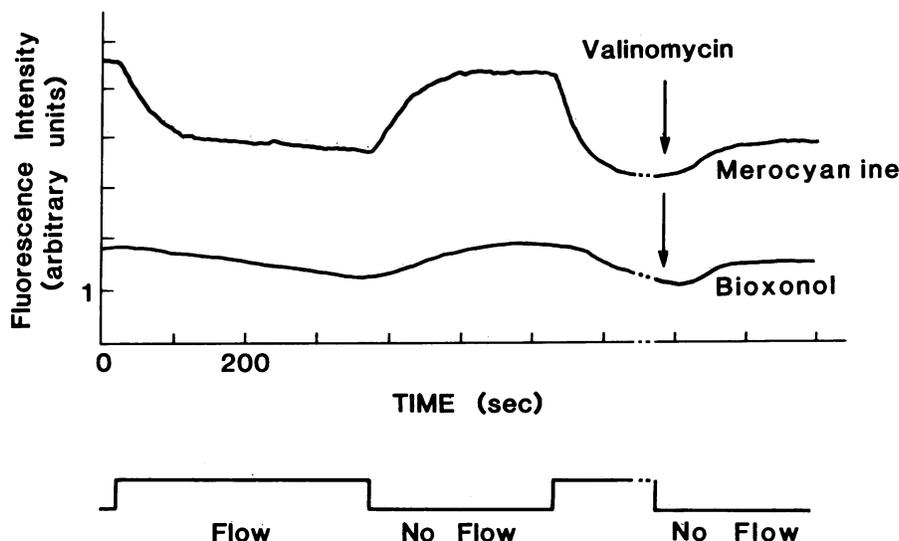


FIG. 3. Fluorescence dependence as a function of time with the onset and cessation of flow. In the reported experiment, the shear stress is 50 dynes/cm². Valinomycin was added at 1 μ M final concentration. The time dependence decrease in merocyanine and bis(1,3-diethylthiobarbiturate)trimethineoxonol (bioxonol) fluorescence intensity with the onset of flow follows an exponential approach to steady state ($t_{1/2}$ = 60 sec). After the flow stop, the recovery time constant is also \approx 1 min.

cells in various ways: (i) a direct mechanical effect at the cell membrane interface (15), (ii) alterations of mass transfer processes (16), (iii) a streaming potential that is due to the movement of charged liquid against an electrostatically charged surface (7). Here, we address the question of how these parameters may affect the cell at the membrane level. We report evidence that flow-related parameters affect the endothelial cell membrane potential. A membrane hyperpolarization whose amplitude is a function of the flow is generated and obeys certain kinetics.

We have already reported on the highly structured endothelial membrane lattice and the specific mobility and distribution of its components related to the molecular nature of the extracellular matrix (17, 18). Based on our actual results, we propose that on the other side of the cell—namely, at the blood-apical membrane interface—the hydrodynamic hyperpolarization plays the role of transducer between the flow and the endothelial cell membrane. Related to what we report here, it is interesting to mention that the permeability of the endothelium increases in regions of acute inflammation and that anti-inflammatory drugs accelerate repolarization of the endothelial cell membranes (19).

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