Shear Stress-mediated Extracellular Signal-regulated Kinase Activation Is Regulated by Sodium in Endothelial Cells

POTENTIAL ROLE FOR A VOLTAGE-DEPENDENT SODIUM CHANNEL*

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Fluid shear stress is an important regulator of endothelial cell (EC) function. To determine whether mechanosensitive ion channels participate in the EC response to shear stress, we characterized the role of ion transport in shear stress-mediated extracellular signal-regulated kinase (ERK1/2) stimulation. Replacement of all extracellular Na⁺ with either N-methyl-D-glucamine or choline chloride increased the ERK1/2 stimulation in response to shear stress by 1.89 ± 0.1-fold. The Na⁺ effect was concentration-dependent (maximal effect, ≤12.5 mM) and was specific for shear stress-mediated ERK1/2 activation as epidermal growth factor-stimulated ERK1/2 activation was unaffected by removal of extracellular Na⁺. Shear stress-mediated ERK1/2 activation was potentiated by the voltage-gated sodium channel antagonist, tetrodotoxin (100 nM), to a magnitude similar to that achieved with extracellular Na⁺ withdrawal. Transfection of Chinese hamster ovary cells with a rat brain type IIa voltage-gated sodium channel completely inhibited shear stress-mediated ERK1/2 activation in these cells. Inhibition was reversed by performing the experiment in sodium-free buffer or by including tetrodotoxin in the buffer. Western blotting of bovine and human EC lysates with SP19 antibody detected a 250-kDa protein consistent with the voltage-gated sodium channel. Degenerate polymerase chain reaction of cDNA from primary human EC yielded transcripts whose sequences were identical to the sodium channel SCN4a and SCN8a α subunit genes. These results indicate that shear stress-mediated ERK1/2 activation is regulated by extracellular sodium and demonstrate that ion transport via Na⁺ channels modulates EC responses to shear stress.

Mechanical stimuli are important modulators of cellular function in tissues, particularly in the cardiovascular system. A key physical force experienced by EC² by virtue of their unique location in the vascular wall is fluid shear stress created by the frictional force of blood flow (1). Changes in fluid shear stress have been shown to regulate EC function, including permeability of plasma lipoproteins, adhesion of leukocytes, and release of pro- and antithrombotic factors, growth factors, and vasoactive substances (reviewed in Refs. 1 and 2). These hemodynamically regulated events may contribute to the pathogenesis of vascular disease as atherosclerotic plaques are preferentially localized to areas of the vascular system that experience turbulent flow and low time-averaged shear stress (3, 4).

Our laboratory has previously reported that ERK1/2 are activated by shear stress in EC (5). Whereas the mechanisms responsible for growth factor-mediated stimulation of ERK1/2 have been well characterized (6), the upstream signaling pathway that leads to activation of ERK1/2 by shear stress remains undefined. Of particular interest are the primary plasma membrane mechanisms by which the physical force of shear stress can be transduced into biochemical signals. Several candidate mechanotransducers have been proposed including G proteins, caveolae, integrins, and mechanosensitive ion channels (2).

A common mechanism that has evolved to sense changes in mechanical stimuli is the mechanosensitive ion channels (1, 7). These channels are widely distributed in tissues and participate in processes such as hearing, balance, touch, and vasoregulation. EC exhibit ion channel responses to mechanical forces that are likely to participate in the signaling response to shear stress. Several different mechanosensitive ion channels are present in EC (8, 9). Shear stress-responsive channels include a cation-selective channel (high calcium conductance) (10), a potassium channel (11–13), and a stretch-activated calcium channel (1, 14). Studies have shown that blockage of mechanosensitive K⁺ channels with barium chloride or tetraethylammonium inhibited shear stress-mediated increases in NO production (15) and transforming growth factor-β release (16), suggesting that transmembrane ion flux and intracellular ion homeostasis are important mediators of the EC response to shear stress. To determine the contribution of mechanosensitive ion channels to the shear stress-mediated regulation of ERK1/2 activity in EC we characterized the effects of varying extracellular cation concentrations and the effects of specific ion transport agonists and antagonists. In the present study we report that Na⁺ entry through voltage-gated sodium channels inhibits shear stress-mediated ERK1/2 activation and demonstrate expression of two voltage-gated sodium channels (identified as SCN4a and SCN8a) in human umbilical vein EC.

¶ The abbreviations used are: EC, endothelial cell; ERK, extracellular signal-regulated kinase; BAEC, bovine aortic EC; HUVEC, human umbilical vein EC; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.
Materials and Methods

Cell Culture—Bovine aortic EC (BAEC) were isolated from fetal calf aortas and maintained in M199 (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Cells used in experiments were at passages <6, as ERK1/2 kinase activation decreased in later passages. Human umbilical vein EC (HUVEC) were obtained as previously described (17). Cells at passages between 1 and 3 were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 20% fetal bovine serum (HyClone Laboratories, Inc.), heparin (Sigma), and EC growth factor. CHO-K1 and CNaIIA-1 cells were obtained from the laboratory of Dr. W. Catterall (University of Washington, Seattle, WA) and maintained in RPMI 1640 supplemented with 5% fetal bovine serum. G418 (200 μg/ml) was added to media used for CNaIIA-1 cells. CHO cells were placed in medium supplemented with 0.1% serum for 24 h prior to experiments to reduce base-line ERK1/2 phosphorylation. All cells were grown on tissue culture dishes coated with 2.5% gelatin (Sigma).

Shear Stress Experiments—Cells were grown in 74 × 36-mm slides of tissue culture plastic cut from the bottom of tissue culture dishes. Two days after reaching confluence, cells were rinsed free of culture media with HBSS (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1.0 mM MgCl2, 20 mM HEPES, pH 7.4) with 10 mM glucose added and either maintained in a static condition or exposed to flow (shear stress = 12 dynes/cm2) in a parallel plate chamber (5) and cone and plate viscometer (18) at 37 °C. After varying times of exposure to fluid shear stress, cells were washed gently with ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4, 1.4 mM KH2PO4, pH 7.3), and ERK1/2 activation was determined.

Preparation of Membrane Fractions—Cells or tissue were homogenized in lysis buffer (50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM Na3VO4, 10 mM HEPES, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, pH 7.4). Cell lysates were prepared by scraping, sonication, and centrifugation (5 min, 4 °C, 14,000 rpm in a microcentrifuge). Sample protein concentrations were determined by DC protein (Bio-Rad) analysis. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions, and proteins were transferred to nitrocellulose filters (Hybond, Amersham Pharmacia Biotech). To ensure quantitative transfer of proteins, the filters were stained with Ponceau S. The membrane was blocked for 2 h at room temperature with a commercial blocking buffer (Life Technologies, Inc.). The blots were incubated overnight at 4 °C with the primary antibody (phospho-specific ERK1/2 antibody was obtained from New England Biolabs, ERK1 and ERK2 antibodies were from Upstate Biotechnology, voltage-gated sodium channel antibody SP19 was provided by Drs. W. Catterall and B. Murphy at the University of Washington (19, 20), and amiloride-sensitive sodium channel antibody was provided by Dr. D. Beno at the University of Alabama) followed by incubation for 1–2 h with secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech) within the linear range of the x-ray film as described previously (5).

Preparation of Membrane Fractions—Cells or tissue were homogenized with fractionation lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 5 mM EGTA, 5 mM 2-mercaptoethanol, 10 mM benzamidene, 1 mg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluorode, 0.1 mg/ml ovalbumin, and 0.1 μg/ml aprotinin, pH 7.4) on ice. After incubation for 5 min, cells were disrupted with a Dounce homogenizer (50 strokes), and centrifugation was performed (100,000 × g for 1 h). The supernatant was saved as the cytosolic fraction. The pellet (membrane fraction) was washed once with lysis buffer, resuspended in 150 μl of lysis buffer that contained 1% Triton X-100, and solubilized for 1 h at 4 °C before sonication. Proteins then underwent Western blot analysis as described above.

Phosphorylation of Na+ Channels by Protein Kinase A—To immunoprecipitate Na+ channels, cell lysates containing 500 μg of protein were incubated with SP19 (2 μg) antibody overnight at 4 °C and then incubated with 20 μl of protein G-agarose beads (Life Technologies, Inc.) for 2 h on a roller system at 4 °C. The beads were washed twice with 1 ml of lysis buffer, twice with LiCl, and once with Tris-EDTA (50 mM LiCl, 100 mM monomer Tris-Cl, pH 7.6, 0.1% Triton X-100, and 1 mmol/liter dithiothreitol), and two times with 1 ml of washing buffer (20 mM HEPES, pH 7.2, 2 mM monomer EGTA, 10 mM monomer MgCl2, 1 mM monomer dithiothreitol, and 0.1% Triton X-100). SP19-immunoprecipitated Na+ channels were phosphorylated by incubation at 37 °C in 50 mM Tris-Cl, pH 7.5, 0.1% Triton X-100, 10 mM MgCl2, 1 mM EGTA, 0.15 μM γ-[32P]ATP (3,000 Ci/mmol), or 100 μM unlabeled ATP in the presence of 0.25 μM purified catalytic subunit of protein kinase A for 1 h. The kinase reaction was terminated by heating at 65 °C for 3 min in 80 mM Tris-HCl, pH 6.8, 10% glycerol, 10 mM dithiothreitol, and 2% SDS.

PCR Primers, cDNA Synthesis, PCR, and Sequencing—Three sets of nested degenerate primers were designed using conserved sequences present in the brain, skeletal muscle, and heart voltage-gated sodium channels or using conserved sequences present in the epithelial sodium channels: Sense 1, 5′-ATIGAYAYTTYAA-3′ and Antisense 1, 5′-GGRITTCCRCATCT-3′; Sense 2, 5′-AAAYHTTGYAYGT-3′ and Antisense 2, 5′-AAIGYTCTCRAATTT-3′; Sense 3, 5′-AAAYHTTGYAYGT-3′ and Antisense 3, 5′-GGRTTCCRAATTT-3′. Degenerate primers were designed for minimal degeneracy, particularly on the 3′ end, and were at least 14-mer in length. mRNA was isolated from primary HUVEC utilizing oligo(dT)-cellulose from the Poly(A)Pure kit (Ambion Inc., Austin, TX) according to the manufacturer’s instructions. cDNA was synthesized using a Superscript cDNA synthesis kit (Life Technologies, Inc.). Polymerase chain reaction was performed using a GeneAmp PCR system 2000 thermal cycle (Perkin-Elmer), Taq polymerase, and the materials provided in the AdvanTage PCR cloning kit (CLONTECH, Palo Alto, CA). Final concentrations in the PCR mixture were as follows: primers (1 μM), dNTP (200 μM), MgCl2 (1.5 mM), cDNA (1–2 ng/reaction), Taq polymerase (1–2 units/reaction), and reaction buffer as supplied in the kit. A single round of amplification (30 cycles) was performed. Amplification was confirmed by agarose gel electrophoresis and visualized by ethidium bromide/UV light. PCR products were cloned into the pT-Adv vector using T4 DNA ligase (4 units of T4 DNA ligase; 50 ng/ml pT-Adv vector, 1 ng of PCR product, and ligation buffer provided in kit) for 16 h at 14 °C and then transformed into competent Escherichia coli and grown on LB agar plates containing 5- bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl-1-thio-β-D-galactopyranoside. Colonies were chosen by white/ blue color selection and then inoculated into fresh media and grown overnight. Plasmids were isolated by mini prep (MiniPrep Spin, QIAGEN), and inserts were sequenced (ABI PRISM dye terminator cycle sequencing kit, Perkin-Elmer) at the Department of Pharmacology sequencing core (University of Washington, Seattle, WA). Insert sequences were compared against those in the GenBank™ data base using the BLAST and Swissprot search algorithms.

Statistical Analysis—Data are presented as mean ± S.E. All experiments were performed at least three times. Significant differences were determined by Student’s t tests (p < 0.05). Desensitometry was measured using NIH Image 1.60. To permit comparison of fold change among different experiments the densitometric value in the static sample, and the ratio was assigned a value of 1.0. The normalized value for each experiment was then determined, and statistical analysis was performed on the normalized ratios.

Results

Shear Stress-mediated ERK1/2 Activation Is Independent of Stretch-activated Calcium Channels—Mechanical stimuli increase intracellular calcium concentration by activating gadolinium-sensitive stretch-activated calcium channels (21). Several laboratories have previously reported that shear stress increases intracellular calcium (22–24). Stretch-activated calcium channels have been reported to be expressed in EC and smooth muscle cells and to transport calcium in response to increased pressure (25). To determine whether shear stress-mediated ERK1/2 activation is dependent on stretch-activated calcium channels, experiments were performed in the presence of gadolinium chloride, which inhibits calcium entry via stretch-activated calcium channels. ERK1/2 was activated ~8-fold by shear stress (12 dynes/cm2 for 10 min) compared with cells under static conditions (Fig. 1). Increasing concentrations of gadolinium chloride had no effect on shear stress-mediated ERK1/2 activation in HUVEC (Fig. 1), suggesting that stretch-activated calcium channels are not necessary for this signal transduction pathway. EGF (100 nm for 10 min) also activated ERK1/2, and activation was unaffected by gadolinium chloride.

Shear Stress-mediated ERK1/2 Activation Is Altered by Extracellular Na+ but Not by Extracellular K+—Previous investigators have demonstrated that shear stress-activated K+...
channels mediate the release of vasoactive mediators from EC (15, 16). Iso-osmotic substitution (replacement of a cation with another cation of equal osmolality) of KCl with N-methyl-D-glucamine chloride had no effect on shear stress-mediated ERK1/2 stimulation (Fig. 2A). Addition of the potassium channel blocker, tetraethylammonium (1 μM-1 mM), also had no effect on shear stress-mediated ERK1/2 activation (not shown, n = 3, p > 0.05). These results indicate that extracellular K+ did not influence shear stress-mediated ERK1/2 activation.

Recent studies indicate that a sodium channel mediates mechanosensing in Caenorhabditis elegans (26). Iso-osmotic substitution of 130 mM NaCl by N-methyl-D-glucamine chloride for 130 mM NaCl increased ERK1/2 stimulation by shear stress (1.89 ± 0.10-fold maximum) in HUVEC (Fig. 2A). Similar results were obtained when choline chloride was used as the iso-osmotic substitute or when BAEC were studied (not shown, n = 3). Characterization of the sodium concentration dependence demonstrated that at Na+ concentrations of ≤12.5 mM, a 2-fold increase in shear stress-mediated ERK1/2 activation relative to the 130 mM Na+ shear stress condition was observed (Fig. 2B). Neither basal levels of ERK1/2 activation nor EGF-mediated ERK1/2 activation was affected by changes in sodium concentration (Fig. 2B), suggesting that the sodium-mediated inhibition of shear stress-mediated ERK1/2 activation was not because of a nonspecific effect.

**Effect of Inhibiting Sodium Transporters on Shear Stress-mediated ERK1/2 Activation**—Because lowering extracellular sodium caused increased ERK1/2 activation and sodium normally moves down a concentration gradient into the cell, the above results suggest that sodium transport across the plasma membrane is required for inhibition of ERK1/2 activation. A sodium gradient is established across the cell membrane by means of the Na+/K+-ATPase, which actively transports sodium out of the cell, resulting in a negative resting potential within the cell. As a result, Na+ flows down both a concentration and an electrochemical gradient into the cell when sodium transporters are activated. Mediators of Na+ transport present in many cells, including EC, are voltage-dependent sodium channels, Na+/Ca2+ exchangers, Na+/H+ exchangers, and the Na+/K+2Cl- cotransporter.

To determine which sodium transporters are responsible for the sodium-dependent effects on shear stress-mediated signaling, we characterized the effect of antagonists of sodium transport mechanisms on shear stress-mediated ERK1/2 activation in HUVEC (Fig. 3). Blocking the Na+/K+-ATPase with ouabain, which raises intracellular levels of sodium, decreased shear stress-mediated ERK1/2 activation to 62 ± 12% of control (n = 4, p < 0.05). These results suggest that a rise in intracellular sodium concentration (whether by increased sodium influx or by decreased sodium efflux) inhibits shear stress-mediated ERK1/2 activation. Ouabain treatment had no effect on ERK1/2 stimulation (Fig. 2A). Addition of the potassium channel blocker, tetraethylammonium (1 μM-1 mM), also had no effect on shear stress-mediated ERK1/2 activation (not shown, n = 3, p > 0.05). These results indicate that extracellular K+ did not influence shear stress-mediated ERK1/2 activation.

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Cell lysates and partially purified membranes were prepared from cultured BAEC and HUVEC. ~50 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis with SP19 antibody. A, Western blot analysis with rat brain and rat heart as positive controls. IB, immunoblot. B, phosphorylation of immunoprecipitated proteins by protein kinase A. IP, immunoprecipitate. Results are representative of three experiments.

K[32]Cl cotransporter with bumetanide had no effect on the shear-stress-mediated ERK1/2 activation, suggesting that sodium entry through these ion transporters does not mediate inhibition of ERK1/2.

Voltage-gated Sodium Channel a Subunit Expression in EC—To determine whether HUVEC express voltage-gated sodium channels, Western blot analysis was performed using an antibody (SP19) directed against the highly conserved inactivation region of the family of voltage-gated sodium channel α subunits (27). Western blotting of cell lysates from BAEC and HUVEC with the SP19 antibody detected an ~250-kDa protein with a molecular mass similar to that of purified voltage-gated sodium channel α subunits (27) (Fig. 4A). Similar results were observed with lysates from rat brain and heart but not kidney. The immunoreactive protein was relatively enriched in membrane fractions from HUVEC and BAEC as measured with the SP19 antibody, indicating that this protein is localized to membranes. Analysis of several lysates from different cell preparations demonstrated two immunoreactive bands between 200 and 250 kDa, suggesting that more than one type of voltage-gated sodium channels is expressed and/or that significant posttranslational modifications occur in EC. To provide further evidence that the 200–250-kDa proteins represent sodium channels, proteins from HUVEC and BAEC lysates were immunoprecipitated with SP19 and then subjected to phosphorylation by recombinant protein kinase A. As reported by other laboratories (19), protein kinase A phosphorylated predominantly 200–250-kDa proteins in SP19 immunoprecipitates, typical of voltage-gated sodium channels (Fig. 4B). No amiloride-sensitive sodium channels were detected using several specific antibodies for Western blot analysis of partially purified HUVEC and BAEC membranes (not shown). These results suggest that the HUVEC and BAEC used for the present experiments express voltage-dependent sodium channels as reported by other investigators (28–30).

Shear Stress-mediated ERK1/2 Activation Is Modulated by Changing Voltage-gated Sodium Channel Activity—To determine the effect of modulating voltage-gated sodium channel function on shear-stress-mediated ERK1/2 activation in EC, we characterized the effects of specific sodium channel agonists and antagonists. Tetrodotoxin, a voltage-gated sodium channel antagonist, increased shear-stress-mediated ERK1/2 activation in a concentration-dependent manner to a magnitude similar to extracellular Na[32] withdrawal (2.3 ± 0.5-fold versus static, Fig. 5, left). Veratridine, a voltage-gated sodium channel agonist, inhibited shear-stress-mediated ERK1/2 activation in a concentration-dependent manner (0.5 ± 0.07-fold versus static, Fig. 5, right). Neither tetrodotoxin nor veratridine had any effect on static or EGF-mediated phospho-ERK1/2 levels, indicating that the effects of these agents were specific. Amiloride (1 μM), an epithelial sodium channel antagonist, had no effect on shear stress-mediated ERK1/2 activation (not shown). These data are consistent with the hypothesis that sodium entry inhibits shear stress-mediated ERK1/2 activation via voltage-sensitive Na[32] channels.

Expression of Rat Brain Voltage-gated Sodium Channels in CHO Cells Inhibits Shear Stress-mediated ERK1/2 Activation—To provide further evidence that sodium transport via voltage-gated sodium channels inhibits shear stress-mediated ERK1/2 activation, CHO cells were stably transfected with cDNA encoding the rat brain type IIA Na[32] channel α subunit (also referred to as SCN2a) to generate CNaIIA-1 cells (31). Exposure of nontransfected CHO cells (CHO-K1) to shear stress of 12 dynes/cm[2] for 10 min increased ERK1/2 activity 5-fold, indicating that CHO-K1 cells were shear stress-responsive (Fig. 6). In contrast, CNaIIA-1 cells failed to show significant ERK1/2 stimulation in response to shear stress. These results indicate that expression of voltage-gated sodium channels inhibits shear stress-mediated ERK1/2 activation (Fig. 6A). ERK1/2 expression was similar in the two cell lines. The transfected sodium channels were functionally coupled to sodium transport, as shown by the findings that removal of extracellular Na[32] or addition of 100 nM tetrodotoxin prevented shear stress-mediated ERK1/2 inhibition in CNaIIA-1 cells (Fig. 6B). These findings support further that Na[32] influx through voltage-gated sodium channels is responsible for shear stress-mediated effects on ERK1/2.

Identification of Voltage-gated Sodium Channels SCN8a and SCN4a in HUVEC—To identify the voltage-gated sodium channel(s) expressed in EC, degenerate PCR primers were designed based on conserved sequences in the voltage-gated sodium channel (see “Materials and Methods”). These primers were used for sequential PCR reactions using cDNA synthesized from primary HUVEC cultures as a template. After two rounds of PCR, four bands (including a band of the predicted length for the voltage gated sodium channels) were obtained. PCR of these four bands yielded two bands (both at predicted sizes for voltage-gated sodium channels) (Fig. 7). After subcloning and transformation, 20 positive colonies were selected at random for sequencing. Sequence analysis indicated the presence of two different clones. The first clone was homologous to rat and mouse SCN8a voltage-gated sodium channel (89% nucleotide homology, 97% amino acid homology). Com-
Channel that involves Na\(^+\) inhibition of ERK1/2 activation by the voltage-gated sodium channel (29). Based on our results, we propose a model (Fig. 8) for the cardiac microvascular EC (28), and human saphenous vein EC, whose relative abundance may be highly regulated in the vascular tree.

DISCUSSION

The major findings of this study are that 1) shear stress-mediated ERK1/2 activation is modulated by extracellular Na\(^+\) but not by extracellular K\(^+\); 2) HUVEC express voltage-gated sodium channels; and 3) sodium entry through voltage-gated sodium channels inhibits shear stress-mediated ERK1/2 phosphorylation but has no effect on basal or EGF-induced ERK1/2 activation. In an alternative model would require that shear stress decrease sodium influx via the voltage-dependent sodium channels, thereby relieving a negative stimulus. In this model, sodium would regulate upstream mediators of ERK1/2 such as Ras and MEK. Proving which model is correct will require patch clamping the membrane patch is dislodged from the pipette with the application of shear stress.

Numerous ion channels have been characterized in EC. These include voltage-gated, stretch-activated, and hormone-regulated conductances (8, 9). Both flow and mechanical stretch have been shown to stimulate ion transport via channels in EC. For example, cell swelling activates an outward-rectifying chloride channel (35). Mechanical stretch stimulates nonselective gadolinium-sensitive cation channels (10, 25, 36), as well as a charybdotoxin-sensitive voltage-gated K\(^+\) channel (37). Shear stress activates several different K\(^+\) channels including an inward rectifier (11, 13) and calcium-activated K\(^+\) channels (38). Thus it is clear that there are several channels present in EC that may mediate mechanotransduction. Three groups have described voltage-gated sodium channels in EC (28–30). The first report demonstrated a tetrodotoxin-resistant (IC\(_{50} = 1 \mu M\)) sodium channel in both HUVEC and EC derived from rat interlobar arteries of the kidney (30). Gosling and colleagues (29) observed a tetrodotoxin-resistant (IC\(_{50} = 4.7 \mu M\)) channel in saphenous vein EC. The channel was tentatively identified as hH1, commonly referred to as the cardiac sodium channel, based on electrophysiologic properties and immunoreactivity with an antibody to the conserved region between domains III and IV of known sodium channel \(\alpha\) subunits. In contrast, Walsh and colleagues (28) characterized a channel from cardiac microvascular EC that was tetrodotoxin-sensitive (IC\(_{50} = 5 \mu M\)) and clearly different from hH1. The tetrodotoxin sensitivity of the channel described here (IC\(_{50} < 100 \mu M\)) differs from both of the previously described channels. However, this IC\(_{50}\) is not inconsistent with tetrodotoxin-resistant channels such as SCN4a and SCN8a, which we cloned from HUVEC. Based on the present findings and previous reports it appears that EC express several sodium channels whose relative abundance may be highly regulated in the vascular tree.

Voltage-gated sodium channels are unlikely to contribute to action potential generation in EC as discussed by other investigators (28, 29). However, I\(_{Na}\) could regulate intracellular calcium via the Na\(^+\)/Ca\(^{2+}\) exchanger. Of particular importance for shear stress-mediated signal transduction, these sodium channels could participate in conducted depolarization between EC or even between EC and smooth muscle cells (39). Such conducted depolarizations may participate in flow-mediated relaxation that is well described in the microcirculation (40).

Previous studies have suggested a role for Na\(^+\) transport and sodium channels in mechanotransduction. The degenerin class of sodium channels in \textit{C. elegans} is homologous to channels present in epithelial tissue (41). Several members of the degenerin family (e.g., MEC-4, MEC-10, and unc-105) have been characterized as Na\(^+\) channels that mediate mechanosensing in \textit{C. elegans}, potentially via interactions with the matrix (26).
The pacinian corpuscle is a mechanically responsive sensor in the nervous system that requires sodium influx for activation. In the cardiovascular system, Bevan’s laboratory (42–44) documented alterations in vascular tone mediated by changes in extracellular sodium and proposed that the primary mechanoreceptor(s) were sensitive to extracellular sodium. The only study that links mechanotransduction by shear stress to voltage-gated sodium channel activation was published by Salter et al. (45). These authors demonstrated membrane depolarization of bone cells exposed to shear stress that was mediated by tetrodotoxin-sensitive sodium channels. Membrane depolarization was inhibited by antibodies against α, β1, and β2 integrins. We previously demonstrated that shear stress-mediated ERK1/2 activation in HUVEC required integrin-matrix interactions (46) and suggested that β1 integrins were especially important (47). Taken together these observations suggest that integrin-mediated changes in signal transduction that regulate sodium channel activity may be a common mechanism for shear stress-dependent regulation of ERK1/2. Alternatively, the voltage-gated sodium channel may possess extracellular matrix-binding domains that interact with the matrix (26). Mechanotransduction may then occur by changes in channel conformation mediated by physical forces transmitted through the cytoskeleton to the matrix.

Several mechanisms by which sodium channels and/or changes in intracellular sodium concentration participate in shear stress-activated signal transduction may be proposed. 1) For Na\(^+\)-responsive signaling molecules, a novel mechanism would require the existence of signaling molecules that are responsive to changes in Na\(^+\) concentration. Of interest, it was recently reported that palytoxin stimulated a sustained activation of c-Jun NH\(_2\)terminal kinase that required extracellular sodium (48). In this study, the authors proposed a Na\(^+\)-dependent signal transduction pathway of unknown composition. In addition, several studies have shown that protein kinase C activity and protein kinase C translocation are inhibited by increases in intracellular Na\(^+\) concentration (49, 50). Because protein kinase C can regulate Na\(^+\) influx by phosphorylation of voltage-gated sodium channels (51) and we have shown that protein kinase C is activated by shear stress (52), protein kinase C isozymes may participate in signal events regulated by shear stress and Na\(^+\). 2) For changes in cell-cell and cell-matrix interaction, shear stress activates ERK1/2 in a β\(_1\) integrin-dependent manner (47) and stimulates phosphorylation of PECAM-1 (53), which is involved in cell-cell interactions. Changes in sodium may influence these interactions and/or there may be direct interactions between the sodium channel and matrix components (26). 3) For regulation of cell volume, water balance is regulated primarily through Na\(^+\) content. Increased sodium influx will obligate water movement and cause cell swelling. However, the effect of cell swelling is to stimulate ERK1/2 activation, whereas the effect of lowering extracellular sodium, which would prevent swelling, enhanced shear stress-mediated ERK1/2 activation. There was also no effect of changing extracellular sodium or adding ouabain on cells maintained in static culture (Fig. 3), suggesting that changes in cell volume alone are not responsible for the observed effect. 4) For altered Na\(^+\)/Ca\(^{2+}\) exchange, increased intracellular sodium may stimulate the activity of the Na\(^+\)/Ca\(^{2+}\) exchanger and, secondarily, stimulate Ca\(^{2+}\) entry. Changes in intracellular Ca\(^{2+}\) appear to be less important for regulation of ERK1/2 activation because a rise in intracellular Ca\(^{2+}\) is not necessary for shear stress-mediated ERK1/2 activation (5, 52), and the increase in intracellular Ca\(^{2+}\) observed in EC exposed to flow does not require extracellular Ca\(^{2+}\) (22).

In summary, the present data show that sodium entry through voltage-gated sodium channels in HUVEC inhibits shear stress-mediated ERK1/2 activation. These results may...
have significance for diseases such as hypertension, where differences in mechanosensitive ion channel expression have been reported (38). Future experiments will be required to identify the functionally relevant voltage-gated sodium channels in EC, to characterize the regulation of the mechanosensitive sodium channel (e.g., protein kinase C isozyme and integrin involvement), and to elucidate the mechanisms by which sodium entry regulates signal transduction.

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