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Regulation of Endothelial Cell Myosin Light Chain Phosphorylation and Permeability by Vanadate

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Abstract The involvement of tyrosine protein phosphorylation in the regulation of endothelial cell (EC) contraction and barrier function is poorly understood. We have previously shown that myosin light chain (MLC) phosphorylation catalyzed by a novel 214 kDa EC myosin light chain kinase (MLCK) isoform is a key event in EC contraction and barrier dysfunction [Garcia et al. (1995): J Cell Physiol 163:510–522; Garcia et al. (1997): Am J Respir Cell Mol Biol 16:487–491]. In this study, we tested the hypothesis that tyrosine phosphatases participate in the regulation of EC contraction and barrier function via modulation of MLCK activity. The tyrosine phosphatase inhibitor, sodium orthovanadate (vanadate), significantly decreased electrical resistance across bovine EC monolayers and increased albumin permeability consistent with EC barrier impairment. Vanadate significantly increased EC MLC phosphorylation in a time-dependent manner (maximal increase observed at 10 min) and augmented both the MLC phosphorylation and permeability responses produced by thrombin, an agonist which rapidly increases tyrosine kinase activities. The vanadate-mediated increase in MLC phosphorylation was not associated with alterations in either phosphorylase A Ser/Thr phosphatase activities or in cytosolic [Ca^{2+}] but was strongly associated with significant increases in EC MLCK phosphotyrosine content. These data suggest that tyrosine phosphatase activities may participate in EC contractile and barrier responses via the regulation of the tyrosine phosphorylation status of EC MLCK. J. Cell. Biochem. 70:141–155, 1998.

Key words: endothelial cell; tyrosine phosphatase; vanadate; permeability; MLCK

Abbreviations: BPAEC, bovine pulmonary artery endothelial cells; BSA, bovine serum albumin; EB-BSA, Evans Blue dye complexed to bovine serum albumin; EC, endothelial cell; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N(,N(-tetraacetic acid; HBSS, Hanks balanced salt solution; HEPES, N-[2 hydroxyethyl] piperasine-N(-[2-ethanesulfonic acid]; MLC, myosin light chain; MLCK, myosin light chain kinase; NP-40, nonylphenoxy polyethoxy ethanol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PTP, protein tyrosine phosphatase; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; Ppase, serine/threonine phosphatase; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; Tris-HCL, tris,[hydroximethyl] aminomethane hydrochloride.

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Endothelial cell (EC) contraction is an essential step in specific models of agonist-induced vascular permeability and tissue edema. Increased contractile force development results in intercellular gap formation and EC barrier dysfunction, processes intrinsic to the pathogenesis of acute lung injury and the adult respiratory distress syndrome [Garcia and Natarajan, 1992; Garcia and Schaphorst, 1995]. Similar to smooth muscle cells, EC contraction is directly associated with the obligatory phosphorylation of the regulatory myosin light chains (MLC) by the Ca^{2+}/CaM-dependent myosin light chain kinase (MLCK) [Garcia et al., 1995; Wysolmerski and Lagunoff, 1990; Patterson et al., 1994], and MLCK activation precedes increases in EC permeability evoked by the coagulant and inflammatory mediator, thrombin [Garcia et al., 1995; Patterson et al., 1994]. To examine mechanisms of edema formation, we have begun to carefully analyze the structure and function of EC MLCK and recently cloned a 214 kDa MLCK

isoform expressed in human and bovine endothelium [Garcia et al., 1997a; Verin et al., 1998]. Amino acid analysis revealed five sites for MLCK phosphorylation by the cAMP-dependent protein kinase A (PKA) [Verin et al., 1998], whose protective effects on EC barrier function are well recognized [Patterson et al., 1994]. We recently demonstrated that increases in both cAMP and PKA activity result in enhanced phosphorylation of endothelial MLCK in situ in association with a marked decrease in MLCK activity [Garcia et al., 1997a]. Similarly, Ser/Thr phosphatase inhibition by calyculin, which produces accumulation of phosphorylated MLC, gap formation, and increased albumin permeability [Verin et al., 1995], also resulted in increased MLCK phosphorylation which significantly attenuated the activity of the kinase [Verin et al., 1998]. These data appear to link EC contractile mechanisms and barrier function with the Ser/Thr phosphorylation status of EC MLCK.

Tyrosine kinases are major participants in multiple cellular processes, and considerable evidence now supports a role for tyrosine phosphorylation in smooth muscle contractility [DiSalvo et al., 1994]. Inhibition of tyrosine phosphatase activity by sodium orthovandate (vanadate) and pervanadate was associated with increased phosphotyrosine protein content and increased contraction of rat gastric muscle strips and aortic rings [Fox et al., 1993]. Smooth muscle contractile responses were reduced by genistein, a tyrosine kinase inhibitor [Filipeanu et al., 1995], and evidence for crosstalk between the tyrosine kinase pathway and the MLCK pathway in smooth muscle has been recently provided [Jin et al., 1996; Stephanie et al., 1996; Sauro et al., 1996]. In preliminary studies to evaluate the role of tyrosine phosphorylation in EC barrier regulation, we noted a protective effect of tyrosine kinase inhibition on thrombin-induced endothelial cell barrier dysfunction [Shi et al., 1998]. Although the target for these tyrosine kinase activities was not demonstrated, we have recently reported that the novel EC MLCK isoform contains a consensus site (Tyr 485) for potential $p60^{src}$-catalyzed tyrosine phosphorylation, as well as several SH2 binding domains, and sites for phosphorylation by tyrosine kinase–regulated Ser/Thr kinases such as MAP kinase and cdc2 kinase [Verin et al., 1998]. In the present study, we have examined the direct effect of protein tyrosine phosphatase activities on EC MLC phosphorylation, MLCK activity, and barrier responses. These data support the hypothesis that tyrosine kinase/phosphatase activities are important participants in the regulation of the EC contractile apparatus and that MLCK is a naturally occurring target for these activities.

MATERIALS AND METHODS

Reagents

Endothelial cell cultures were maintained in DME media (Gibco, Chagrin Falls, OH) supplemented with 20% (v/v) colostrum-free bovine serum (CFBS) (Irvine Scientific, Santa Ana, CA), 15 µg/ml EC growth supplement (Collaborative Research, Bedford, MA), 1% antibiotic and antimycotic solution (penicillin, 10,000 units/ml; streptomycin, 10 µg/ml; amphotericin B, 25 µg/ml) (K.C. Biologicals, Lenexa, KA), and 0.1 mM nonessential amino acids (Gibco). Unless specified, reagents were obtained from Sigma Chemical Company (St. Louis, MO). Phosphate buffer saline (PBS) and Hanks Balanced Salt Solution (HBSS) without phenol red were purchased from Gibco (Grand Island, NY). Sodium orthovanadate Na_3VO_4 (vanadate) was obtained from Fisher Chemical/Fisher Scientific (Fair Lawn, NJ). Bovine thrombin was obtained from Sigma. Polyacrylamide gradient 4–15% ready-to-use gels were purchased from BioRad (Hercules, CA). Anti-MLCK D119 antibody and anti-MLC antibody were generous gifts from Dr. P. Gallagher (Indianapolis, IN) and Dr. J. Stull (Dallas, TX), respectively. Fura-2 acetoxymethyl ester and fura-2, pentapotassium salt, was commercially obtained from Molecular Probes (Eugene, OR) and ionomycin purchased from Calbiochem (La Jolla, CA).

Bovine Pulmonary Artery Endothelial Cell (BPAEC) Cultures

Bovine EC were obtained frozen at 16 passages from American Type Tissue Culture Collection (Rockville, MD; CCL 209), utilized at passage 19–24, and cultured in complete media [Patterson et al., 1994]. The EC cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air and grew to contact-inhibited monolayers with typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin and resuspended in fresh culture medium and passaged to polycarbonate filters for permeability studies into

100 mm^2 dishes for immunoprecipitation studies, into 60 mm^2 dishes for tyrosine kinase activity determination, or into 75 cm^2 flasks for MLC phosphorylation studies.

Myosin Light Chain Phosphorylation in Intact Endothelium

Endothelial cell monolayers grown in 75 cm^2 tissue flasks were analyzed for MLC phosphorylation by urea PAGE as we have previously described [Patterson et al., 1994; Garcia et al., 1995] followed by Western immunoblotting with specific anti-MLC antibodies. The blot was scannned on a BioRad densitometer and the percent MLC phosphorylation determined by dividing the total of phosphorylated and nonphosphorylated areas. This method takes advantage of the fact that the mono- and diphosphorylated forms of MLC migrate more rapidly than nonphosphorylated MLC and are independent of sample loading. Stoichiometry (mol/mol) was calculated per the formula $M/M = P_1 + 2(P_2)/U + P_1 + P_2$ where U = % unphosphorylated endothelial cell MLC and P_1 = % monophosphorylated and P_2 = % diphosphorylated myosin light chains. The diphosphorylated myosin light chain (P_2) is multiplied by a factor of 2 to reflect the presence of two phosphate groups per light chain. The percentage of light chain phosphorylation was calculated by adding the densitometric values of each phosphorylation state for each isoform (i.e., unphosphorylated (U), monophosphorylated (P_1), and diphosphorylated (P_2).

Albumin Clearance Measurement of Endothelial Cell Permeability

Macromolecule permeability of cultured endothelial cell monolayers was performed as we have previously described [Garcia et al., 1986], with some modifications [Patterson et al., 1992]. Gelatinized polycarbonate micropore membranes (Nucleopore, Pleasanton, CA) were mounted on the base of plastic cylinders and BPAEC then seeded on the membranes and grown to confluency. The system consists of two compartments, the upper compartment (luminal) and the lower compartment (abluminal), which are separated by a polycarbonate filter on which the EC monolayer is grown. The lower compartment was stirred continuously and kept at a constant temperature of 37°C by use of a thermally regulated water bath. Medium M199 with 25 mM HEPES was used in both compartments. Bovine serum albumin (4% final concentration) complexed to Evans Blue dye (EB-BSA) was added to the luminal compartment, and samples were taken from the abluminal compartment for 60 min to establish the basal albumin clearance rate (baseline) and then for an additional 60 min period after each specific intervention. Transendothelial cell albumin transport was determined by measuring the absorbance of Evans Blue dye in abluminal chamber samples at 620 nm in a spectrophotometer (Vmax Multiplate Reader; Molecular Devices, Menlo Park, CA). Albumin clearance rates were calculated by linear regression analysis for control and experimental groups.

Measurement of Endothelial Cell Electrical Resistance

Focal adhesion was measured using the technique initially reported by Tiruppathi et al. [1992] with minor modifications [Garcia et al., 1997b]. In this electrical cell–substrate impedance sensing system (Applied Biophysics, Inc., Troy, NY), EC were cultured on a small gold electrode (10^{-4} cm^2), and culture media was used as the electrolyte. The total electrical resistance was measured dynamically across the monolayer and is determined by the combined resistance between the basal surface of the cell and the electrode, reflective of focal adhesion, and the resistance between cells reflecting junctional integrity. Thus, a change in electrical resistance represents a change in cell-cell adhesion and/or cell-matrix adhesion. The small gold electrode and the larger counter electrode (1 cm^2) are connected to a phase-sensitive lock-in amplifier (5301A; EG&G Instruments Corp, Princeton, NJ) with a built-in differential preamplifier (5316A; EG&G Instruments Corp.). A 1 V, 4,000 Hz AC signal was supplied through a 1-M3 resistor to approximate a constant-current source. Voltage and phase data were stored and processed with Pentium 100 MHz computer which controlled the output of the amplifier and relay switches to different electrodes. Experiments were conducted only on wells which achieved >5,000 ohms of steady-state resistance. Resistance was expressed by the in-phase voltage (proportional to the resistance) which was normalized to the initial voltage and expressed as a fraction of the normalized resistance value, as previously described [Garcia et al., 1997b; Tiruppathi et al., 1992].

Measurement of $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ was carried out as previously described using microspectrofluorimetry and the Ca^{2+}-sensitive indicator fura-2 [Irwin et al., 1994]. In brief, EC cultures were grown on gelatin-coated glass-bottom 35 mm culture dishes (MatTek, Ashland, MA), washed three times with M-199 supplement with 5 mM HEPES buffer without phenol red, and then incubated with fura-2/AM (2.5 μM) for 30 min in the dark at 37°C. The cells were then washed three times with the same buffer and incubated for another 15 min to complete hydrolysis of the ester. Albumin (2.5 mg/ml) was added to the buffer to approximate the albumin in the culture medium. The cells were continously perfused with buffer, vanadate, or vanadate with thrombin at 37°C. Measurements of $[Ca^{2+}]_i$ were made in an atmosphere of 95% air, 5% CO_2 at 37°C by illumination on an inverted microscope. Excitation of fura-2 was at 340 and 380 nm with emitted light monitored at 510 nm. Cell-derived fluorescent images were visualized using a ×40, 1.3 NA oil-immersion objective and intensified with a Videoscope KS1381 intensifier (Videoscope International, LTD, Washington, DC) before entering the camera (Dage-MTI, Michigan City, IN). Images were digitized and stored for subsequent analysis (Universal Imaging Co., West Chester, PA). The perfusion device consisted of a heated (37°C), water-jacketed, 12 barrel array of glass tubes emptying into a common glass tip positioned ≤1 mm from the cells being imaged. At the end of the experiment, ionomycin was added to the dish via a separate tube. Air containing 6% CO_2 was bubbled in all bicarbonate-based solutions and blown over the cells during experiments to prevent CO_2 loss from the buffer and maintain pH at approximately 7.4.

In vitro Ca^{2+} calibration was determined using fura-2, pentapotassium salt in a buffer containing (in mM) KCl 130, NaCl 17, HEPES 10, glucose 10 (pH 7.2, 37°C). For maximum and minimum calcium response, 5 mM $CaCl_2$ or 10 mM EGTA was added to this buffer, respectively [Groden et al., 1991]. Calculations of $[Ca^{2+}]_i$ were carried out as previously described [Grynkiowicz et al., 1985] using an apparent dissociation constant of 224 nM.

Phosphotyrosine Immunoprecipitation

For immunoprecipitation under denaturing conditions, confluent EC monolayers from 100 mm^2 dishes were scraped into SDS/denaturing stop solution (1 mM EDTA, 2 mM NaF, 10 mM NaPP, 0.2 mM orthovanadate, 1% SDS 14 mM mercaptoethanol in PBS), and homogenates were boiled for 5 min, diluted 1:10 with PBS to reduce SDS concentration, and incubated with 50 μl of 10% Pansorbin suspension (formalin-hardened and heat-killed Cowan 1 strain Staphylococcus aureus cells, (Calbiochem, La Jolla, CA)) for 30 min at room temperature. Samples were clarified by microcentrifugation, and supernatants were incubated with 2 μl of phosphotyrosine antibody (UBI) for 1 h at room temperature or overnight at 4°C and then with 50 μl of 10% Pansorbin suspension for 60 min at room temperature. Immunocomplexes were pelleted by microcentrifugation, washed, and subjected to SDS-PAGE on 4–15% gradient gels (BioRad) and Western immunoblotting with specific antibodies.

Ser/Thr Phosphatase (PPase) Activities

Serine/threonine Ppase activities were assessed in EC homogenates as we have previously detailed [Verin et al., 1995]. To prepare homogenates, we twice washed confluent EC monolayers in 100 mm^2 dishes incubated for 30 min with vanadate (10 μM to 1 mM) or vehicle with PBS (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4; Sigma) and twice washed them with ice-cold homogenization buffer (50 mM Tris, 0.1 mM EDTA, 28 mM β-mercaptoethanol, pH 7.5) containing protease inhibitors (0.5 mMPMSF, 0.1 mM TLCK, 0.1 mM leupeptin, 2 mM benzamidine). Homogenization buffer (200 μl) was added to the EC monolayers and plates, were quickly frozen at -70°C, scraped, and homogenized by passing the cell suspension several times through a 1 cc tuberculin syringe; then homogenates were aliquoted and kept at -70°C. Aliquots of EC homogenates were diluted as indicated in assay buffer (50 mM Tris, 0.1 mM EDTA, 28 mM β-mercaptoethanol, pH 7.0) containing 1 mg/ml BSA (final concentration). The diluted homogenates containing PPase activity were added (10 μl) to a reaction mixture consisting of 40 μl of [^{32}P] phosphorylase A 5 μM, 1–3 × 10^3 counts/min (cpm)/pmol prepared as we have previously described [Verin et al., 1995] in assay buffer supplemented with 6.26 mM caffeine. In companion experiments, assay buffer was supplemented with 3 nM of okadaic acid. After 15 min (30°C), the reaction was terminated by addition

of 10 µl of 100% (w/v) ice-cold TCA. After 15 min on ice, the suspension was centrifuged for 5 min (IEC Centra-M centrifuge; International Equipment Co., Needham Heights, MA) and aliquots (2 × 20 µl for each sample) of supernatant were loaded on Whatman (Clifton, NJ) 3 MM paper, dried for 15 min, and counted in Scintiverse scintillation solution (Fisher Scientific) by beta scintigraphy (model LS 6000 IC; Beckman, Fullerton, CA). Reactions were carried out in duplicate, and controls consisted of incubations in which PPase-containing cell preparation was replaced by assay buffer with 1 mg/ml BSA. To ensure linear rates of dephosphorylation, we restricted the extent of dephosphorylation to <25%. One unit of phosphatase activity was defined as the amount of enzyme which released 1 nmole of $^{32}P_i$/min.

Western Immunoblotting

Total protein extracts or immunoprecipitates solubilized in sample buffer [Laemmli, 1970] were separated by 4–15% gradient SDS-PAGE (BioRad) gel electrophoresis, transferred to nitrocellulose (18 h at 30 V), and reacted with either antibodies to MLCK (D119), to antiplatelet myosin antibodies (BTI), or to antiphosphotyrosine antibodies (UBI) as previously described [Garcia et al., 1997a]. Immunoreactive proteins were detected using an enhanced chemiluminescence detection system according to the manufacturer's directions (Amersham, Arlington Heights, IL). The relative intensities of the protein in the bands were quantified by scanning densitometry.

Protein Tyrosine Kinase Activity Determinations

Protein tyrosine kinase activity was measured as we have previously described [Garcia et al., 1997b]. Confluent BPAEC monolayers plated on petri dishes (60 × 15 mm) were rinsed twice with 50 mM Tris-HCl buffer solution to remove the culture medium and scraped into a medium containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM EGTA, 10 mM benzamidine-HCl, 10 µM leupeptin, and 1 mM DTT. Cells were collected by low-speed centrifugation (500*g* for 5 min) and the pellet resuspended in 0.3 ml of the medium described above. The cell suspension was sonicated for 10 s at 40°C and then centrifuged at 1,000*g* for 10 min to remove unbroken cells and nuclei. Finally the supernatant (homogenate) was collected, and the volume of the homogenate was adjusted to 1 ml. Protein tyrosine kinase activity was determined in a final reaction volume of 60 µl containing 50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij 35, 15 mM $MgCl_2$, 1 mM sodium orthovanadate, 100 µM ATP, 33 µCi of [γ-^{32}P] ATP, 80 µM of the exogenous tyrosine kinase–specific substrate Raytide™ (Oncogene Science, Inc., Manhasset, NY), and 20 µl homogenate. The reaction mixture was incubated at 30°C for 30 min and the reaction terminated by adding 60 µl of 10% phosphoric acid. The total volume of the reaction mixture was 120 µl, and 60 µl was applied onto 3 cm × 3 cm Whatman P81 paper, dried, and analyzed by liquid scintillation counting. Protein tyrosine kinase activity was expressed as the amount of ^{32}P incorporated (pmol) into the exogenous Raytide™ substrate per minute per 10^6 cells. One unit (U) of the enzyme activity is defined as the amount of enzyme which incorportates 1 pmol of phosphate from ATP into exogenous substrate per minute at 30°C.

MLCK Immunoprecipitation

For immunoprecipitation of EC MLCK under nondenaturing conditions, confluent EC from 100 mm dishes were rinsed one time with M199 media and two times with PBS and then lysed for 5 min on ice with 300 µl NP-40 lysis buffer (1% NP-40, 20 mM MOPS, pH 7.0, 50 mM $MgCl_2$, 10% glycerol, 0.5 mM EGTA) including protease inhibitors (40 µg/ml aprotinin, 18 µg/ml TPCK, 6 µg/ml TLCK, 0.5 mM PMSF) and 28 mM β- mercaptoethanol. The lysate was scraped and microcentrifuged for 5 min at 4°C, and the supernatant was used for immunoprecipitation. Each sample was diluted with 700 µl washing buffer (0.1% NP-40, 50 mM MOPS, pH 7.0, 50 mM $MgCl_2$, 1 mM EDTA) and incubated for 1 h with 1 µl anti-MLCK D119 antibodies at 4°C followed by incubation for 1 h at 4°C with 50 ml 10% Pansorbin suspension consisting of formalin-hardened and heat-killed Cowan 1 strain Staphylococcus aureus cells (Calbiochem). The immunoprecipitated complex was harvested by microcentrifugation, washed three times with washing buffer, and used for MLCK activity assay (see below) or resuspended in 200 µl of the Laemmli sample buffer [Laemmli, 1970] and heat-treated at 100°C for 5 min. Immunoprecipitated proteins were separated from the Pansorbin beads by microcentrifugation for 1 min and subjected to Western immunoblotting

analysis [Garcia et al., 1997b] using specific antibodies to contractile proteins.

Statistics

Linear regression analysis was performed for determination of clearance rates in individual wells with Epistat 2.0 public domain software, and then these slopes were averaged from a number of at least six. Paired *t*-tests were used to compare pretreatment and posttreatment slopes within the same control membrane of BPAEC chamber. ANOVA with Student-Newman-Keul's test was used to compare means of clearance rates of two or more different treatment groups. The significance level was taken to be $P < 0.05$ unless otherwise stated. Data are expressed as mean standard error of the mean.

RESULTS

Role of Protein Tyrosine Phosphatases in Endothelial Cell Barrier Responses

Our initial studies were designed to examine the role of tyrosine phosphorylation in the regulation of the endothelial cell barrier. We first determined that a 100 µM concentration of the protein tyrosine phosphatase inhibitor vanadate (15 min) is sufficient to markedly increase the total phosphotyrosine protein content in endothelial cell homogenates (Fig. 1A). We next determined that vanadate, in addition to its ability to inhibit tyrosine phosphatases, also alters protein tyrosine kinase activity by measuring tyrosine kinase activity using the synthetic substrate Raytide™. Curiously, these results revealed that, in the presence of 100 µM

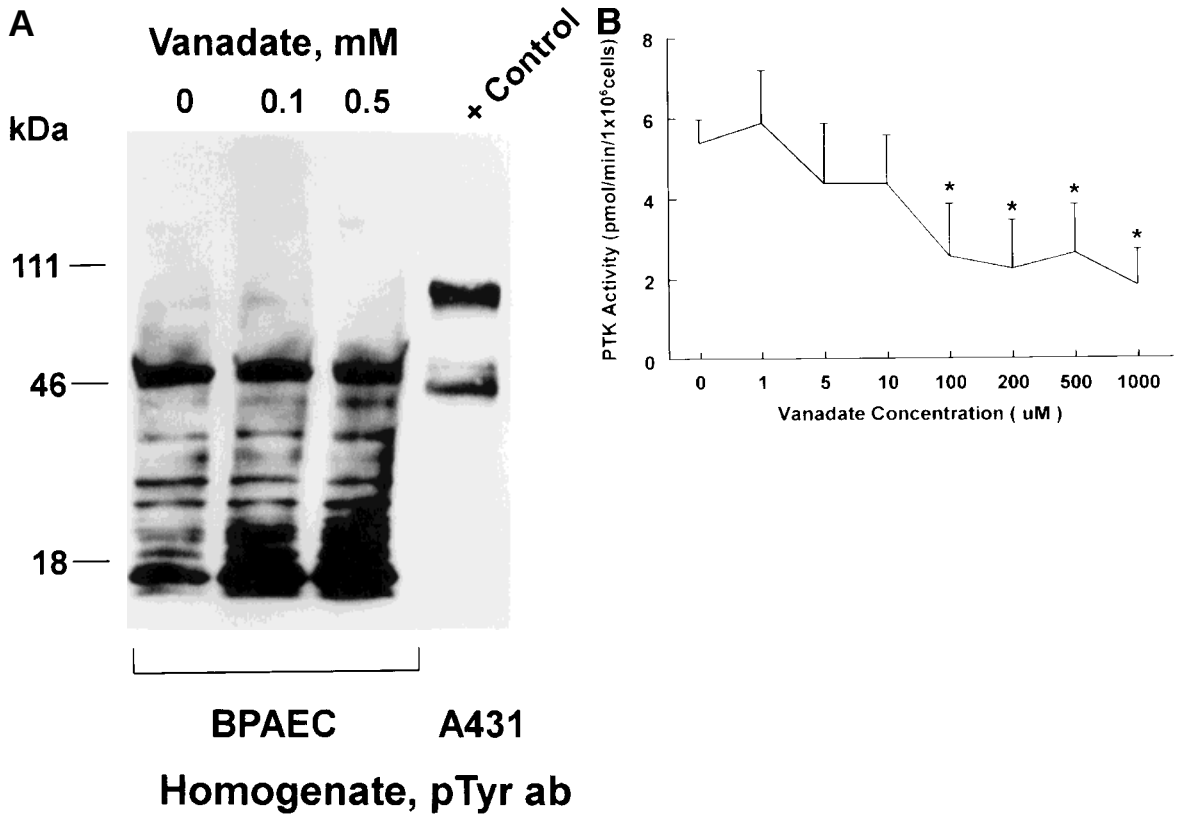

Fig. 1. Effect of vanadate on protein tyrosine phosphorylation in endothelial cell monolayers. **A:** Shown is an antiphosphotyrosine immunoblot of BPAEC homogenates pretreated with vanadate (100 µM and 500 µM for 15 min). Cell lysates from A431 cells previously stimulated with firbroblast growth factor (FGF) were used as a control. Phosphotyrosine antibody staining reveals the predicted rapid and substantial increase in phosphotyrosine protein content in vanadate-treated cells compared with controls. **B:** Confluent BPAEC monolayers (n = 4) were treated with either vehicle or increasing concentrations of sodium orthovanadate for 1 h. Tyrosine kinase (PTK) activity was determined in EC homogenates as described above with Raytide™ substrate and is expressed as the pmol of ^{32}P incorporated into Raytide™/min/10^6 cells. Vanadate decreases tyrosine kinase activity in a dose-dependent manner. *Significant difference from control.

vanadate, protein tyrosine kinase activity decreases as early as 5 min, with maximal inhibition noted at 30 min. Concentrations beyond 500 μM failed to further decrease tyrosine kinase activity (Fig. 1B). Thus, both tyrosine kinase and tyrosine phosphatase activities are inhibited by vanadate. The cumulative effect, however, is a steady rise in the levels of phosphotyrosine protein up to 1 h of vanadate treatment.

Having defined the cumulative effect of vanadate on tyrosine protein phosphorylation, we next determined that vanadate significantly increases EC permeability by measuring the clearance of Evans Blue–albumin (nl/minute) across EC monolayers (Fig. 2A). Increases in the concentration of vanadate beyond 100 μM failed to produce further increases in EC permeability. Utilizing a sensitive index of EC barrier integrity, we next assessed the electrical resistance across EC monolayers grown on gold microelectrodes after treatment with vanadate (Fig. 2B). Although a brief increase in EC electrical resistance was noted immediately (less than 30 min) after vanadate addition, vanadate consistently decreased EC electrical resistance beginning at ~30 min, with the decline in electrical resistance persisting for several hours. These results, indicating compromise of the EC barrier, were observed with vanadate concentrations as low as 10 μM, and there was no further decrease in electrical resistance with concentrations greater than 100 μM. Thus, these physiologic studies indicate a prominent role for tyrosine phosphatases in the regulation of the EC barrier.

Role of Protein Tyrosine Phosphatases in Endothelial Cell Contractile Responses

Having established that tyrosine phosphatase inhibition with vanadate increases EC tyrosine protein phosphorylation and barrier dysfunction, we next investigated the effect of vanadate on MLCK-mediated MLC phosphorylation. EC monolayers grown in 75 cm^2 tissue flasks were challenged with either vehicle or vanadate (100 μM) and at specified time periods the reactions were quickly terminated with ice-cold TCA, and the level of MLC phosphorylation was analyzed by urea gel electrophoresis with immunoblotting (Fig. 2C). Vanadate significantly increased MLC phosphorylation from a stoichiometry of 0.4 mol PO_4/mol MLC to a maximum of ~0.7 mol/mol at 10 min, with MLC phosphorylation declining to near basal levels by 60 min. These results provide a biochemical basis for the physiologic alterations produced by vanadate.

Effect of Vanadate on Thrombin-Induced Permeability and MLC Phosphorylation

Having established that vanadate directly and significantly increases EC permeability and MLC phosphorylation while decreasing EC electrical resistance, we next evaluated the integrated effects of vanadate on the barrier responses elicited by thrombin, an established agonist for EC barrier dysfunction [Garcia et al., 1986; Garcia et al., 1995]. In cells pretreated with vanadate (100 μM for 60 min), there was a significant increase in EC permeability in both the vehicle-treated and thrombin-stimulated monolayers consistent with an additive effect in the presence of both agents (Fig 3A). We have previously shown that thrombin-mediated MLC phosphorylation is maximal at 2 min, subsequently declining to near basal levels by 60 min [Garcia et al., 1995]. To evaluate whether tyrosine phosphatase inhibition influences the thrombin-induced MLC phosphorylation/dephosphorylation response, we pretreated EC monolayers with vanadate (100 μM) for 10 min followed by thrombin (100 nM) challenge for 2 min or 60 min. The thrombin-mediated increase in MLC phosphorylation at 2 min was not altered by vanadate pretreatment. However, in contrast to the predicted decline in MLC phosphorylation after 60 min of thrombin, there was a significant and sustained increase in MLC phosphorylation after thrombin stimulation at 60 min ($P < 0.05$) which occurred only in the presence of tyrosine phosphatase inhibition (Fig. 3B).

Effect of Vanadate on Ser/Thr Phosphatase Activities

We have previously noted that the activity of a myosin-associated Type 1 Ser/Thr phosphatase is a key modulator of the phosphorylation status of EC MLCs [Verin et al., 1995]. Inhibition of this activity with calyculin dramatically increases the level of MLC phosphorylation and reduces EC barrier function [Verin et al., 1995]. To investigate whether the increase in MLC phosphorylation observed with vanadate resulted from vanadate-mediated Ser/Thr phos-

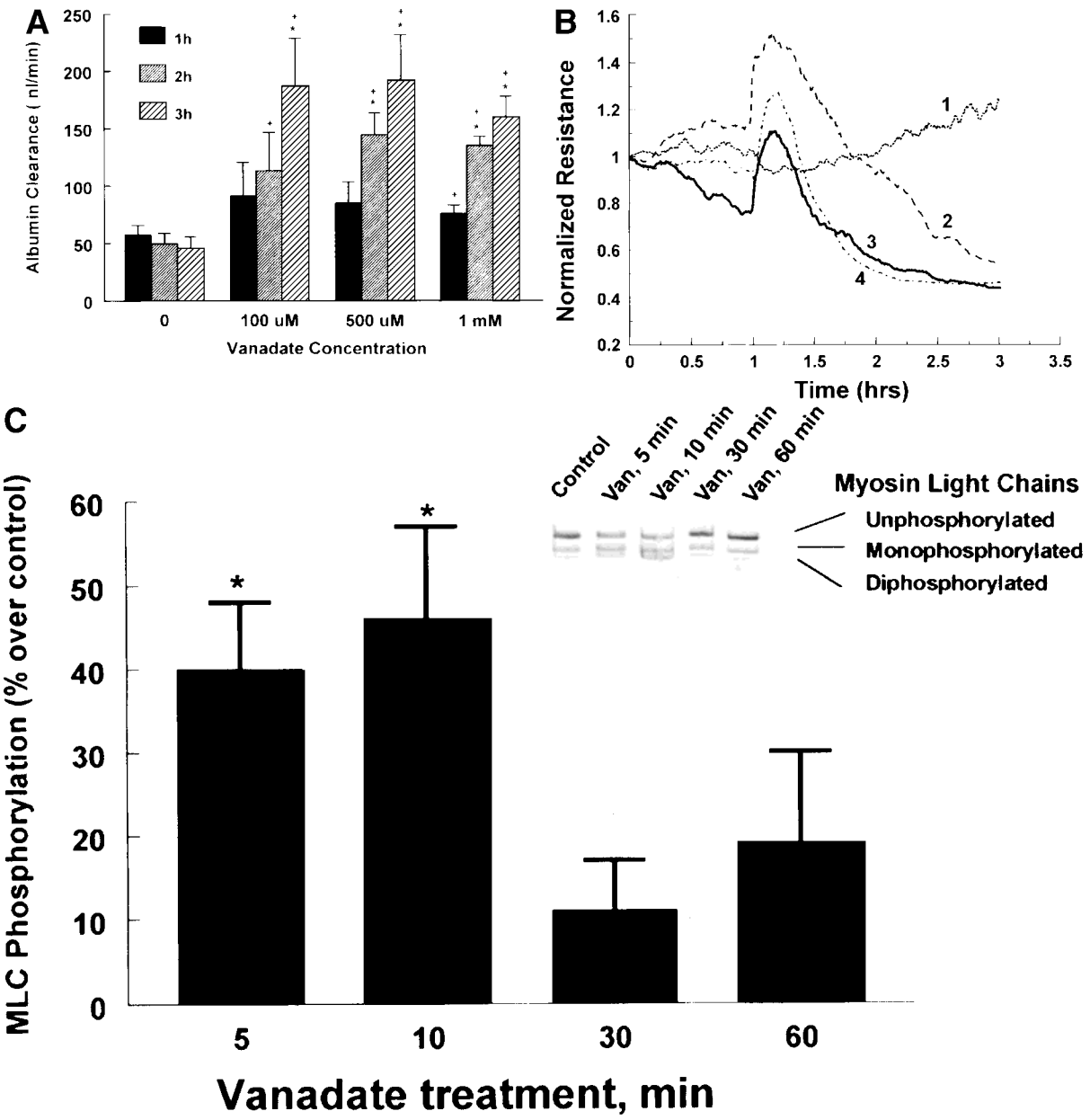

Fig. 2. Effect of vanadate on endothelial cell barrier responses and MLC phosphorylation. **A:** Shown is the clearance rate of Evans Blue–albumin across BPAEC monolayers calculated over 3 h. At time 0, BPAEC were treated with either vehicle or the specified concentration of vanadate. Vanadate increases EC permeability in a time-dependent manner with a maximal effect observed at 100 μM. *Significant difference from control. **B:** BPAEC grown on gold microelectrodes were treated with either vehicle or vanadate (10–1,000 μM) and transendothelial electrical resistance measured as described in Materials and Methods (n = 5). Depicted is a representative experiment where the x axis represents normalized electrical resistance. Lines 1–4 represent 0, 10, 100, and 1,000 μM vanadate, respectively. At each concentration, vanadate appeared to elicit a rapid but brief increase in EC electrical resistance, followed by a decrease beginning at ~30 min findings, consistent with its effects on albumin clearance. **C:** Confluent BPAEC monolayers grown in 75 cm^2 tissue flasks were challenged with either vehicle or vanadate (100 μM) at specified time periods depicted on the x axis, and the level of MLC phosphorylation depicted on the y axis as % of control was analyzed by urea gel electrophoresis. **Inset:** Representative immunoblot indicating the increase in mono- and diphosphorylated MLC after vanadate challenge.

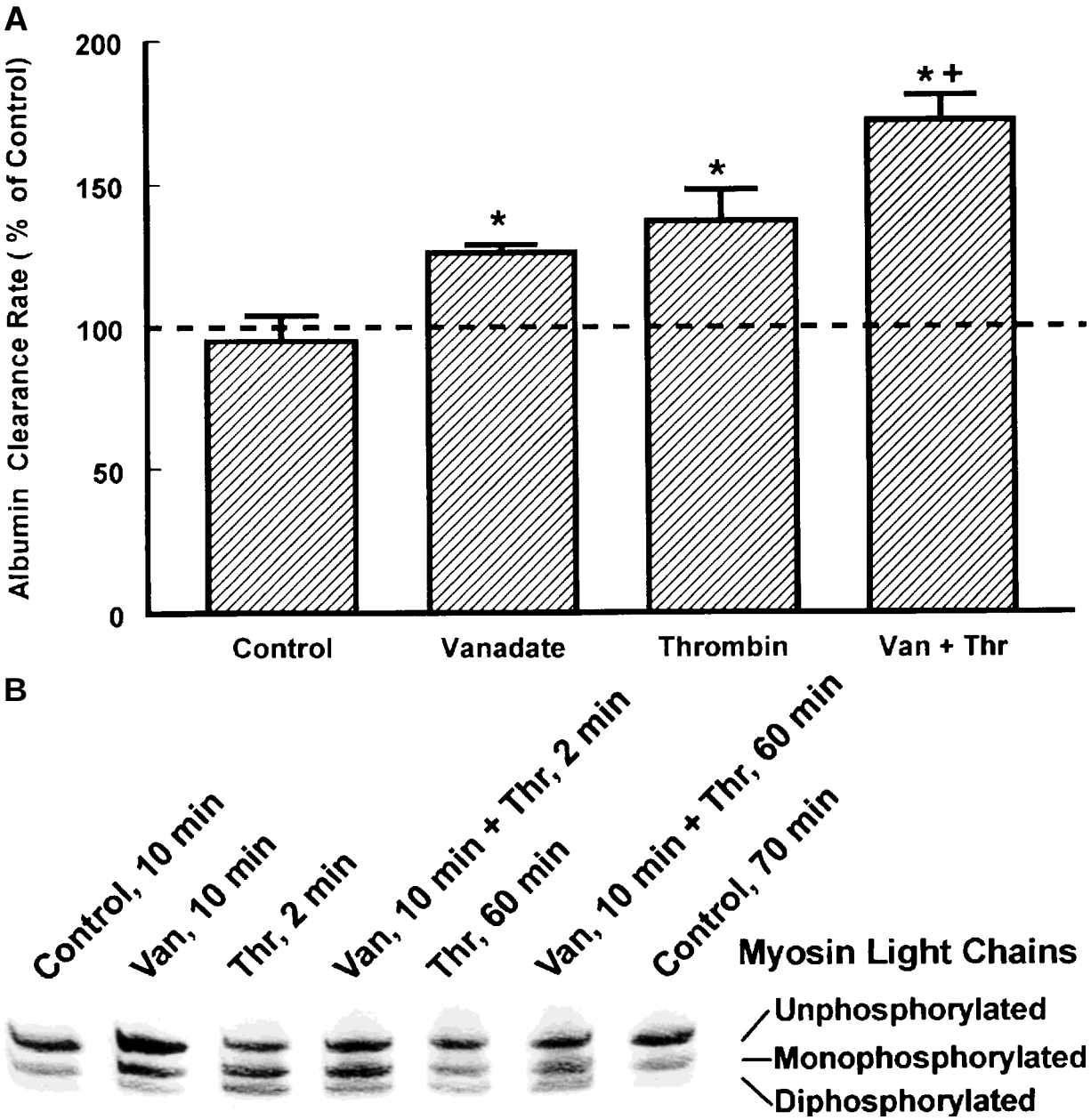

Fig. 3. Effect of vanadate on thrombin-induced BPAEC permeability and MLC phosphorylation. **A:** The clearance (nl/minute) of Evans Blue–albumin across BPAEC monolayers was determined from 120–180 min. At time 0, BPAEC monolayers (n = 12) were treated with either vehicle or vanadate (100 μM). At 60 min, either vehicle or thrombin was added to the luminal compartment. Depicted on the y axis is albumin clearance expressed as % of control. These data show that in the presence of vanadate there is an increase in basal and thrombin-induced albumin clearance. $^{*/+}$Significant difference ($P < 0.05$) from control and thrombin, respectively. **B:** BPAEC were challenged with vanadate (100 μM) and then rechallenged with thrombin (100 nM) for 2 min and 60 min. Shown is an immunoblot depicting the level of MLC phosphorylation analyzed by urea PAGE. Pretreatment with vanadate resulted in increases in thrombin-induced MLC phosphorylation at 60 min.

phatase inhibition, we measured phosphorylase A phosphatase activity (comprised of Type 1 and 2A phosphatases) in EC homogenates after treatment with vanadate. Vanadate in concentrations up to 1 mM failed to significantly affect total phosphorylase A phosphatase activity (Table I). We specifically assessed the effect of vanadate on Type 1 phosphorylase A phosphatase activity by pretreating monolayers with okadaic acid (3 nM), an agent that selectively inhibits Type 2A activity [Verin et al., 1995] and again found that vanadate had no

TABLE I. Effect of Vanadate on Ser/Thr Phosphorylase A Phosphatase (PPase) Activities in EC Monolayers[a]

	Phosphorylase A PPase activity (% of control)	
Vanadate	Total activity	Type 1 PPase activity
10 μM	94 ± 17	96 ± 7
100 μM	114 ± 7	99 ± 9
1 mM	79 ± 25	93 ± 18

[a]Bovine endothelial cell monolayers (n = 4) were treated with varying concentrations of vanadate for 60 min in the absence or presence of 3 nM okadaic acid to inhibit Type 2A PPase [Verin et al., 1995]. Results are expressed as the % of control activity ± SEM. Basal PPase activity was ~5 nmol/min/mg. Vanadate failed to significantly alter either total PPase activity or the okadaic acid–insensitive PPase 1 activity in EC homogenates.

effect on PPase Type 1 activity (Table I). Although myosin-associated PPase activity was not directly measured, these data suggest that the increase in MLC phosphorylation observed after vanadate or after the combination of vanadate and thrombin is unlikely to be due to alterations in Ser/Thr PPase activities.

Effect of Vanadate on $[Ca^{2+}_i]$ and MLCK Tyrosine Phosphorylation

The above studies suggested that the mechanism by which vanadate increases basal and thrombin-mediated MLC phosphorylation, and hence induces barrier impairment, may involve a direct increase in the activity of EC MLCK. As cytosolic Ca^{2+}_i availability is necessary but not sufficient to increase enzymatic activity of the EC MLCK isoform [Garcia et al., 1995], we next determined whether vanadate directly produces a rise in cytosolic Ca^{2+} as a potential mechanism for the activation of the Ca^{2+}/CaM-dependent EC MLCK. These studies failed, however, to demonstrate any alteration in the intracellular Ca^{2+} concentration after vanadate (Fig. 4A,B), suggesting that vanadate-induced MLCK activation is not due to an increase in Ca^{2+} availability. As we have previously shown that alterations in the Ser/Thr phosphorylation status of MLCK regulates enzymatic activity [Garcia et al., 1997a; Verin et al., 1997], we next examined whether the capacity for vanadate to increase MLC phosphorylation was related to alterations in the tyrosine phosphorylation status of MLCK. EC MLCK was immunoprecipitated from EC monolayers with antiphosphotyrosine antibodies under denaturing conditions followed by immunodetection with MLCK antisera. This demonstrated an increase in EC MLCK phosphotyrosine content by virtue of the observed increase in the amount of MLCK recovered in the phosphotyrosine immunoprecipitates from EC pretreaed with vanadate (Fig. 5). Similarly, immunoprecipitation of MLCK with MLCK antisera followed by immunodetection with phosphotyrosine antisera confirmed MLCK as a phosphotyrosine protein (data not shown). Finally, Table II depicts the actual increase in kinase activity in MLCK immunoprecipitates after vanadate treatment. Vanadate produced a modest increase in kinase activity, whereas the tyrosine kinase inhibitor genistein significantly reduced MLCK kinase activity. These data suggest that alterations in MLCK phosphotyrosine status provide a potentially relevant mechanism by which vanadate modulates EC contractile forces. Furthermore, these studies are consistent with our hypothesis that tyrosine phosphorylation directly regulates EC MLCK activity, EC contraction, and barrier properties.

DISCUSSION

Endothelial cells, like other eukaryotic nonmuscle cells, contain contractile components involved in cross-bridge binding of actin and myosin filaments resulting in shortening and tension development [Schnittler et al., 1990; Kolodney and Wysolmerski, 1992; Wysolmerski and Lagunoff, 1990]. Contraction is initiated by the actin-mediated activation of myosin ATPase, an event which involves the obligatory Ser/Thr phosphorylation of the regulatory subunit of the myosin light chain, MLC_{20}. Although this reaction is mediated in smooth muscle and nonmuscle cells by the Ca^{2+}/CaM-dependent MLCK [Adelstein et al., 1981; Kamm et al., 1985], understanding of nonmuscle contractile responses remains limited. Our data indicate that tyrosine phosphorylation, a process clearly involved in cell growth, differentiation, and cell-matrix adhesion [Tonks, 1993; Walton et al., 1993; Wang et al., 1994], is also involved in EC contractility. The observation that genistein, a known receptor- and cytosolic-tyrosine kinase inhibitor [Akiyama et al., 1987, 1991], produced significant decreases in both basal and thrombin-induced EC contractile and barrier-disrupting responses (Table II) [Shi et al., 1998] is consistent with the recent description of the participation of tyrosine kinases in smooth

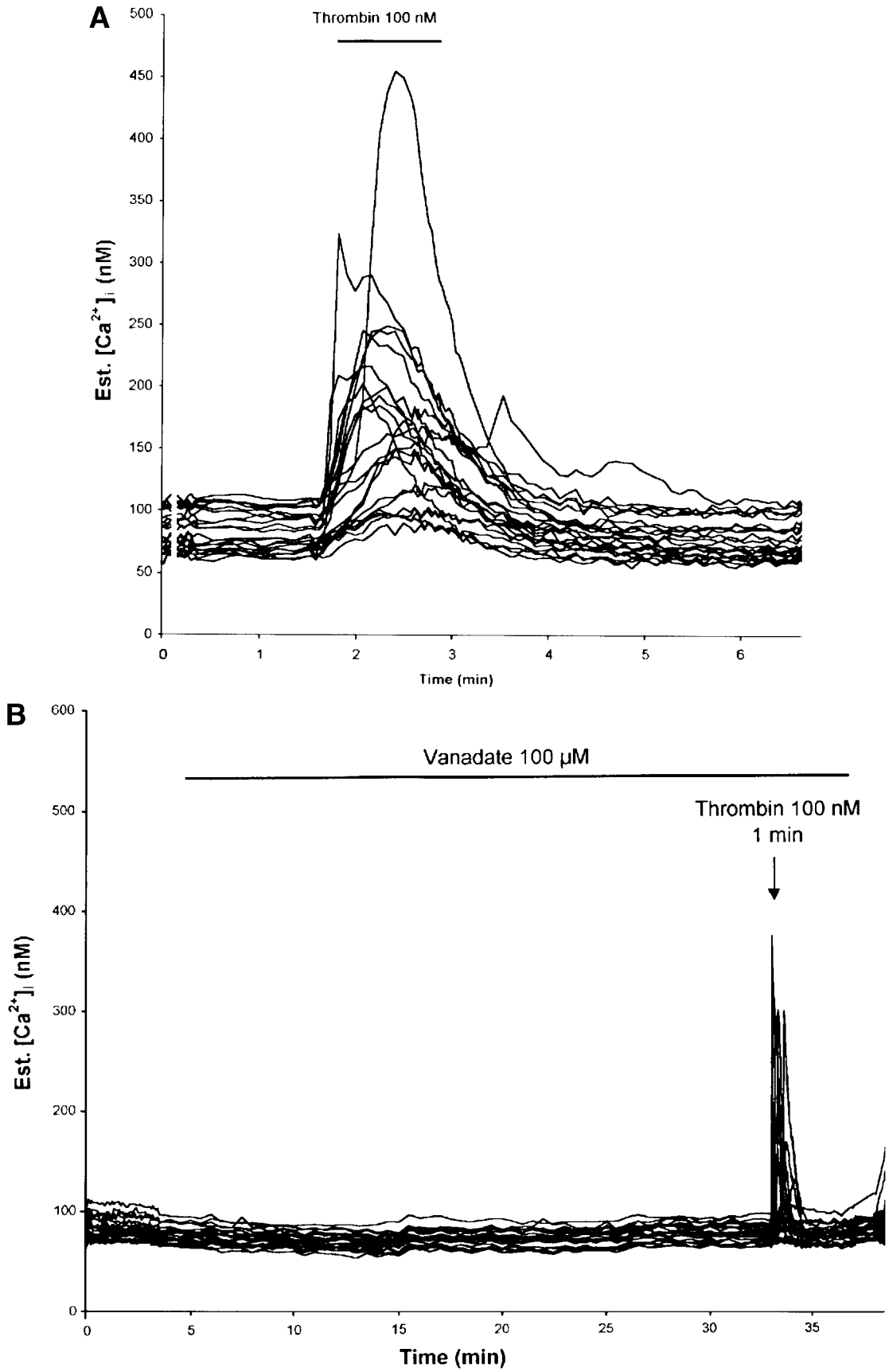

Fig. 4. Effect of vanadate on thrombin-induced Fura-2 fluorescence. Intracellular Ca^{2+} was measured with the Ca^{2+}-sensitive fluorescent indicator fura-2 as described in Materials and Methods. Data shown are from a representative experiment showing the effect of vanadate and vanadate plus thrombin on $[Ca^{2+}]_i$ in 26 cells. Each line represents the calculated $[Ca^{2+}]_i$ of a single cell (see Materials and Methods). **A:** The expected rapid rise in cytosolic $[Ca^{2+}]_i$ after 100 nM thrombin. **B:** Vanadate (100 µM fails to either directly alter EC $[Ca^{2+}]$ or affect the thrombin-induced Ca^{2+} response.

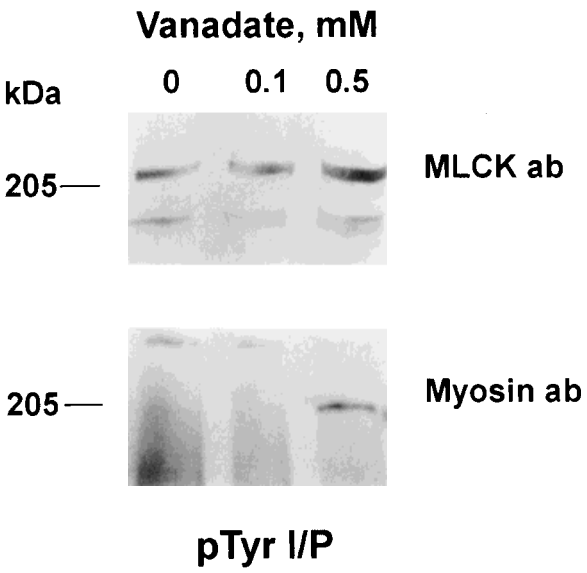

Fig. 5. Effect of vanadate on endothelial cell MLCK phosphotyrosine content. BPAEC monolayers were treated with vehicle or vanadate for 15 min and then used for phosphotyrosine immunoprecipitation. Resulting samples were separated by 4–15% gradient SDS-PAGE, transferred to nitrocellulose, and reacted with anti-MLCK and myosin antibodies. Vanadate treatment increased the total phosphotyrosine protein content in the MLCK and myosin, providing an increase in the amount of MLCK and myosin in phosphotyrosine immunoprecipitates.

TABLE II. Effect of Vanadate on Kinase Activity Present in EC MLCK Immunoprecipitates[a]

	MLCK activity (% of control)
Vanadate (100 μM)	124 ± 22
Genistein (100 μM)	23 ± 16*
Thrombin (100 nM)	190 ± 31*

[a]EC monolayers were pretreated with vehicle (DMSO), vanadate (10 min), genistein (1 h), or thrombin (2 min) and EC MLCK activity determined using MLC as a substrate in the presence of γ-^{32}P-ATP as described in Materials and Methods. MLCK activity is expressed as a percentage change ± SD from control, vehicle-treated (30 min) monolayers. Genistein significantly reduced kinase activity in EC MLCK immunoprecipitates, whereas thrombin and, to a lesser extent, vanadate stimulated MLCK activity.
*Significant difference ($P < 0.05$) from control level of MLCK activity.

muscle contraction [Abebe et al., 1995; Jin et al., 1995; Stephanie et al., 1996; Sauro et al., 1996; DiSalvo et al., 1994]. The regulation of EC contraction by tyrosine kinases is complex, however, as the tyrosine kinase inhibitor and erbstatin analogue, 2,5 DHC, not only failed to attenuate thrombin-induced MLC phosphorylation and permeability but directly reduced EC electrical resistance [Shi et al., in press]. Given that the extent of tyrosine phosphorylation represents a balance of tyrosine kinase and tyrosine phosphatase activities, we have utilized the tyrosine phosphatase inhibitor (vanadate) to directly assess the role of tyrosine phosphorylation in EC contractile responses. Interestingly, vanadate also produced a dose-dependent decrease in tyrosine kinase activity. As this activity was not completely abolished by vanadate, it is likely that only specific tyrosine kinases are susceptible to vanadate-mediated and inhibition enhanced autophosphorylation [Mustelin et al., 1989]. Nevertheless, the cumulative effect of vanadate to increase the abundancy of phosphotyrosine proteins resulting in significant EC barrier disruption as reflected by albumin clearance and monolayer electrical resistance. Given the direct association of EC contraction with MLC phosphorylation and EC permeability [Garcia et al., 1995; Patterson et al., 1994], the observation that vanadate causes an increase in MLC phosphorylation further strengthens the notion that tyrosine phosphorylation participates in the regulation of EC contractile pathways.

It is interesting to note that maximal increases in MLC phosphorylation after both vanadate and thrombin occur early (<10 min), whereas barrier disruption remains sustained. Thrombin maximally increases tyrosine kinase activity at 5 min [Shi et al., 1998], and thus the accumulation of tyrosine phosphoproteins evoked by vanadate and thrombin appears to elicit a "latch state-like" contractile physiologic response, where tension is maintained despite a decline in MLC phosphorylation. Vanadate also prolonged the duration of thrombin-induced MLC phosphorylation, producing an additive effect on EC permeability (Fig. 3) again consistent with an effect of vanadate on EC MLCK or EC myosin-associated phosphatase activities. Although tyrosine phosphorylation has been reported to alter Ser/Thr phosphatase activities in vitro [Johansen et al., 1986], we did not observe a reduction in phosphorylase A Ser/Thr phosphatase activity in vanadate-challenged EC monolayers (Table I), suggesting that the increase in MLC phosphorylation observed after vanadate occurs as a result of sustained MLCK activity. To further explore this notion, we assessed Ca^{2+} transients in vanadate-treated

EC, as vanadate has been reported to increase cytosolic free Ca^{2+} in smooth muscle cells [Sandivasegarane et al., 1995], thereby providing a potential signal for activation of the smooth muscle contractile apparatus. We have previously noted that an increase in intracellular Ca^{2+} precedes thrombin-stimulated EC MLC phosphorylation and is necessary but not sufficient for induction of EC MLCK activity [Garcia et al., 1995]. However, using sensitive single cell Ca^{2+} measurements, we failed to detect any demonstrable effect of vanadate on EC $[Ca^{2+}]_i$ or on subsequent thrombin- induced Ca^{2+} responses (Fig. 4). These studies indicate that an increase in intracellular Ca^{2+} flux does not explain vanadate-mediated activation of EC MLCK and the contractile apparatus.

Our recent studies of the 214 kDa EC MLCK isoform have identified unique consensus sites for phosphorylation which do not overlap with the primary sequence of the smooth muscle MLCK, including several sites for phosphorylation by cAMP/cGMP kinases, tyrosine kinases, proline-directed, tyrosine-regulated MAP kinases, and cdc2 kinases [Garcia et al., 1997a; Verin et al., 1998]. Given the role of protein phosphorylation/dephosphorylation in enzymatic regulation, the examination of these sites as potential participants in the posttranslational modification of MLCK is likely of critical importance in elucidating MLCK regulatory mechanisms. In this regard, we have recently demonstrated that increases in the activity of the cAMP-dependent protein kinase A or inhibition of Ser/Thr phosphatase activities result in enhanced MLCK phosphorylation but a significant reduction in MLCK activity [Garcia et al., 1997a; Verin et al., 1998]. We have now extended our earlier work by demonstrating that the EC MLCK isoform is a phosphotyrosine protein and that vanadate-mediated tyrosine phosphatase inhibition produces distinct increases in MLCK phosphotyrosine content in concert with increased enzymatic activity. Although speculative, we believe that likely targets include Tyr 59, Tyr 464, and Tyr 485 which exist within the NH_2 terminus of the high molecular weight EC MLCK isoform and thus are not found in the smooth muscle cell (SMC) MLCK isoform. Tyr 485 is a consensus site for phosphorylation by $p60^{src}$, and both Tyr 59 and Tyr 464 are present within SH-2 binding motifs, with phosphorylation at these sites providing a mechanism by which src kinases may bind and exert close regulation of MLCK activity. While these data represent the first report of a correlation between phosphotyrosine phosphorylation and MLCK enzymatic activity, our data also demonstrated an increase in the phosphotyrosine content of myosin heavy chains after vanadate stimulation. Thus, in addition to EC MLCK, phosphorylation of myosin tyrosine residues may alter myosin-MLCK interaction in such a manner as to enhance MLCK activity. Further understanding of the mechanism underlying vanadate-mediated MLCK activation awaits purification of the 214 kD kinase, determination of tyrosine phosphorylation sites, and site-directed mutagenesis of Tyr phosphorylation sites—studies which are currently under way.

In summary, our data indicate that tyrosine phosphatase activities are directly involved in the regulation of EC contraction and barrier properties. Inhibition of EC tyrosine phosphatases with vanadate directly increased MLC phosphorylation and EC barrier dysfunction while potentiating the contractile response to thrombin, a potent permeability-inducing agonist which rapidly increases tyrosine kinase activities. The vanadate-mediated increase in MLC phosphorylation does not appear to involve either increases in cytosolic Ca^{2+} or alterations in Ser/Thr phosphatase activites but rather appears to be evoked by enhanced MLCK activity possibly related to significant increases in MLCK phosphotyrosine content. Taken together, these data suggest that tyrosine kinase and phosphatase activities are significant participants in the regulation of the EC contractile apparatus and barrier.

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