Journal of Cellular Biochemistry 79:496–505 (2000)

Endotoxin Causes Phosphorylation of MARCKS in Pulmonary Vascular Endothelial Cells

Ying Zhao[1]* and Harold W. Davis[2]

[1]Department of Internal Medicine (Pulmonary/Critical Care Medicine), University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0564
[2]Molecular and Cellular Physiology, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0564

Abstract Protein kinase C (PKC) has been implicated in lipopolysaccharide (LPS)-induced endothelial cell (EC) monolayer permeability. Myristoylated alanine-rich C kinase substrate (MARCKS), as a specific PKC substrate, appears to mediate PKC signaling by PKC-dependent phosphorylation of MARCKS and subsequent modification of the association of MARCKS with filamentous actin and calmodulin (CaM). Therefore, in the present study, we investigated LPS-induced MARCKS phosphorylation in bovine pulmonary artery EC (BPAEC). LPS potentiated MARCKS phosphorylation in BPAEC in a time- and dose-dependent manner. The PKC inhibitor, calphostin C, significantly decreased LPS-induced phosphorylation of MARCKS. In addition, downregulation of PKC with phorbol 12-myristate 13-acetate (PMA) did not affect the LPS-induced MARCKS phosphorylation, suggesting that LPS and PMA activate different isoforms of PKC. Pretreatment with SB203580, a specific inhibitor of p38 MAP kinase, or genistein, a tyrosine kinase inhibitor, prevented LPS-induced MARCKS phosphorylation. Phosphorylation at appropriate sites will induce translocation of MARCKS from the cell membrane to the cytosol. However, LPS, in contrast to PMA, did not generate MARCKS translocation in BPAEC, suggesting that MARCKS translocation may not play a role in LPS-induced actin rearrangement and EC permeability. LPS also enhanced both thrombin- and PMA-induced phosphorylation of MARCKS, suggesting that LPS was able to prime these signaling pathways in BPAEC. Because the CaM-dependent phosphorylation of myosin light chains (MLC) results in EC contraction, we studied the effect of LPS on MLC phosphorylation in BPAEC. LPS induced diphosphorylation of MLC in a time-dependent manner, which occurred at lower doses of LPS, than those required to induce MARCKS phosphorylation. In addition, there was no synergism between LPS and thrombin in the induction of MLC phosphorylation. These data indicate that MLC phosphorylation is independent of MARCKS phosphorylation. In conclusion, LPS stimulated MARCKS phosphorylation in BPAEC. This phosphorylation appears to involve activation of PKC, p38 MAP kinase, and tyrosine kinases. Further studies are needed to explore the role of MARCKS phosphorylation in LPS-induced actin rearrangement and EC permeability. J. Cell. Biochem. 79:496–505, 2000.

Key words: endotoxin; lipopolysaccharide; MARCKS; protein kinase C; pulmonary endothelial cells

The endothelial cell (EC) lining of blood vessels provides integrity and a selective barrier to fluid and solute flux across the vascular wall. Breakdown of this barrier by bacterial endotoxin (lipopolysaccharide, LPS) during sepsis results in increased EC monolayer permeability in the lungs and development of adult respiratory distress syndrome (ARDS). EC, like other cells, contain filamentous (F)-actin, myosin and associated proteins involved in cellular motility [Shasby et al., 1982]. Morphological studies of LPS-exposed pulmonary EC monolayers have demonstrated actin reorganization, intercellular gap formation, and increased permeability [Goldblum et al., 1993]. In addition, phosphorylation of the regulatory light chain of myosin by the Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase, is shown to be another important mechanism for F-actin-induced myosin ATPase activation and subsequent contraction and permeability of EC [Wysolmerski and Lagunoff, 1990; Garcia et al., 1995]. However, the signal transduction mechanisms for LPS-induced EC permeability are not clear.

Grant sponsor: American Lung Association; Grant sponsor: National Institutes of Health; Grant number: HL 53203.

*Correspondence to: Ying Zhao, Department of Internal Medicine, Division of Immunology, P.O. Box 670563, University of Cincinnati Medical Center, Cincinnati, OH 45267-0563.

Received 13 March 2000; Accepted 30 May 2000

The EC dysfunction during sepsis can be either direct, by binding of LPS to the EC or via release of cytokines from LPS-stimulated myeloid cells [Pugin et al., 1995]. The mechanism for recognition of EC by LPS has been well studied, and the results demonstrate that optimal activation of EC by LPS requires the presence of serum in which soluble CD14 (sCD14) and LPS-binding proteins (LBP) are the key proteins mediating the EC response [Pugin et al., 1993; Goldblum et al., 1994]. While the signaling mechanisms distal to LPS binding are poorly understood, protein kinase C (PKC) has been implicated as a significant mediator for transducing the LPS signal [McKenna et al., 1994; McKenna et al., 1995].

Myristoylated alanine-rich C kinase substrate (MARCKS) is one of the most prominent intracellular substrates for PKC. It was originally described as a protein (termed "80–87K") transiently phosphorylated upon treatment of cells with phorbol esters [Wu et al., 1982]. MARCKS was subsequently shown to be an F-actin- and CaM-binding protein [Hartwig et al., 1992] and has been implicated in numerous cell functions such as secretion [Wu et al., 1982], cell motility [Thelen et al., 1991], and differentiation [Chakravarthy et al., 1995]. F-actin and CaM both bind MARCKS in the PKC phosphorylation site domain (PSD), so they bind reciprocally and are released on phosphorylation at this site [Aderem, 1992]. Our previous studies have shown that the MARCKS phosphorylation induced by either thrombin or phorbol 12-myristate 13-acetate (PMA) in bovine pulmonary artery EC (BPAEC) [Zhao and Davis, 1996] is partially dependent on PKC activation. We therefore hypothesized that MARCKS may be involved in the LPS-induced PKC signaling pathway, which results in actin rearrangement and cell contraction, and subsequently, the development of EC permeability. The present study was undertaken to investigate the MARCKS phosphorylation in LPS-treated ECs.

MATERIALS AND METHODS

Reagents

BPAEC cultures (CCL209, ATCC, Rockville, MD) were maintained in Dulbecco's Modified Eagle Medium (DMEM; GIBCO-BRL, Grand Island, NY) supplemented with 20% (vol/vol) fetal bovine serum (Irvine Scientific, Santa Ana, CA) and 1% antibiotic and antimycotic (penicillin, 10,000 U/ml; streptomycin, 10 μg/ml and amphotericin B, 25 μg/ml; GIBCO-BRL). LPS (*Escherichia coli*, 026:B6), bovine thrombin, and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). Calphostin C and bisindolylmaleimide I were purchased from Calbiochem Corp. (La Jolla, CA). SB203580 and genistein were purchased from Upstate Technology and GIBCO-BRL, respectively. PMA, calphostin C, bisindolylmaleimide I, SB203580, and genistein were prepared as dimethyl sulfoxide (DMSO) stock solutions. The final concentration of DMSO in the cells was $<0.3\%$.

A polyclonal antibody to recombinant MARCKS was raised in rabbits by traditional methods. Recombinant, tagged MARCKS was prepared with the pPROEX-1 expression vector (GIBCO-BRL, Gaithersburg, MD). The MARCKS coding sequence from pBB80K2A (provided by Drs. Perry Blackshear and Deborah Stumpo, Duke University School of Medicine; [Stumpo et al., 1989]) was removed with AlwI, blunted, and ligated into the blunted EcoRI site of pPROEX-1. BL-21 *E. coli* were transformed with this vector, and MARCKS was purified by the manufacturer's instructions, except that a subsequent step of CaM-agarose chromatography was used [Adelstein and Klee, 1981].

Antibodies to p38 and activated (phosphorylated) p38 were obtained from New England Biolabs (Beverly, MA). An enhanced chemiluminescence kit from Amersham (Piscataway, NJ) was used to develop the immunoblots.

Bovine Pulmonary Artery Endothelial Cell Culture

BPAEC were obtained frozen at 16 passages and were used at passages 19–25. Cells were cultured, as described previously [Stasek et al., 1992], in complete media and maintained at 37°C in a humidified atmosphere of 5% CO_2–95% air. The ECs 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 into 25-cm^2 flasks for MARCKS phosphorylation and translocation studies or 24-well plates for myosin light-chain phosphorylation study. Before treatment, the BPAEC were rinsed and incubated in DMEM containing 1% fetal bovine serum and buffered with either $NaHCO_3$ or 20 mM HEPES.

MARCKS Phosphorylation and Translocation

Confluent BPAEC in T-25 flasks were incubated with $^{32}PO_4$ (0.1 mCi/ml) and treated with agonists for a total of 4 h. MARCKS phosphorylation was determined by immunoprecipitation of the MARCKS protein with a specific MARCKS antibody. Briefly, after treatment the cells were lysed in MARCKS extraction buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 1% Igepal CA-630 (Sigma Chemical Co., St. Louis, MO). Equal quantities of protein from each sample were boiled for 5 min and then centrifuged. The heat-stable proteins (including MARCKS) were subjected to immunoprecipitation with the MARCKS antibody overnight, then protein A-agarose for 2 h at 40°C. The samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and MARCKS phosphorylation was quantified by densitometry of the autoradiographs and expressed as a percentage of control (no treatment).

MARCKS translocation from membrane to cytosol was assessed by ultracentrifugation of cell homogenates to separate the membrane and cytosolic fractions, followed by SDS-PAGE and immunoblotting. Briefly, control or treated confluent BPAEC were scraped into buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 330 mM sucrose, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 0.5 mM phenylmethylsulphonylfluoride). The cell homogenates were subjected to ultracentrifugation at 100,000*g* for 90 min at 4°C. The supernatants (cytosolic fraction) were collected and the pellets (membrane fraction) were solubilized in lysis buffer containing 0.5% Triton X-100 in buffer B (buffer A without sucrose) and then centrifuged at 100,000*g* for 40 min. Both cytosol and membrane fraction were subjected to 10% SDS-PAGE, transferred to nitrocellulose, and probed with the MARCKS antibody. The quantity of MARCKS protein in the membrane and cytosolic fractions was determined by densitometry and expressed as a percentage of total MARCKS.

Myosin Light-Chain Phosphorylation

Confluent BPAEC on 24-well plates were incubated in 1% serum DMEM buffered with 20 mM HEPES and treated with LPS. After termination of the reactions with 10% trichloroacetic acid, the cells were scraped into tubes and centrifuged. Myosin light-chain phosphorylation was examined by the method of Persechini et al. [1986]. Briefly, the pellets were solubilized in urea gel sample buffer and run on urea-glycerol gels. These gels separate native myosin light chains from monophosphorylated and diphosphorylated myosin light chains. The proteins were then transferred to nitrocellulose and blotted with a myosin light-chain antibody (provided by Dr. C. S. Packer, Indiana University School of Medicine). Phosphorylation was quantified by densitometry and expressed as percentage of total myosin light chain.

Statistics

Data are expressed as mean ± SEM. Student's *t*-test was used to compare means between two groups. Statistically significant differences were assumed at $P < 0.05$.

RESULTS

A major phosphoprotein band of ~87 kDa on SDS-PAGE was detected when $^{32}PO_4$-loaded BPAEC were immunoprecipitated with anti-MARCKS antibodies. The dose–response and time-course of MARCKS phosphorylation in BPAEC in response to LPS stimulation is shown in Figure 1. Low concentrations of LPS (0.01–0.025 μg/ml) had no effect on MARCKS phosphorylation, but higher concentrations, beginning at 0.05 μg/ml, dose-dependently increased MARCKS phosphorylation, reaching a maximal increase of approximately twofold at 1 μg/ml. LPS also produced a time-dependent phosphorylation of MARCKS in BPAEC. At both 1 and 10 μg/ml, LPS induced a sustained increase of phosphorylation, starting after 60 min, reaching a maximum phosphorylation of approximately twofold above basal at 90 min and remaining elevated for at least 180 min. This phosphorylation was not caused by an alteration of MARCKS protein levels because there was no increase in MARCKS protein as determined by immunoblotting (data not shown).

Figure 2 shows the effects of inhibition of PKC on LPS-induced MARCKS phosphorylation in BPAEC. LPS-induced MARCKS phosphorylation was inhibited significantly by the PKC inhibitor, calphostin C. However, another PKC inhibitor, bisindolylmaleimide I, did not

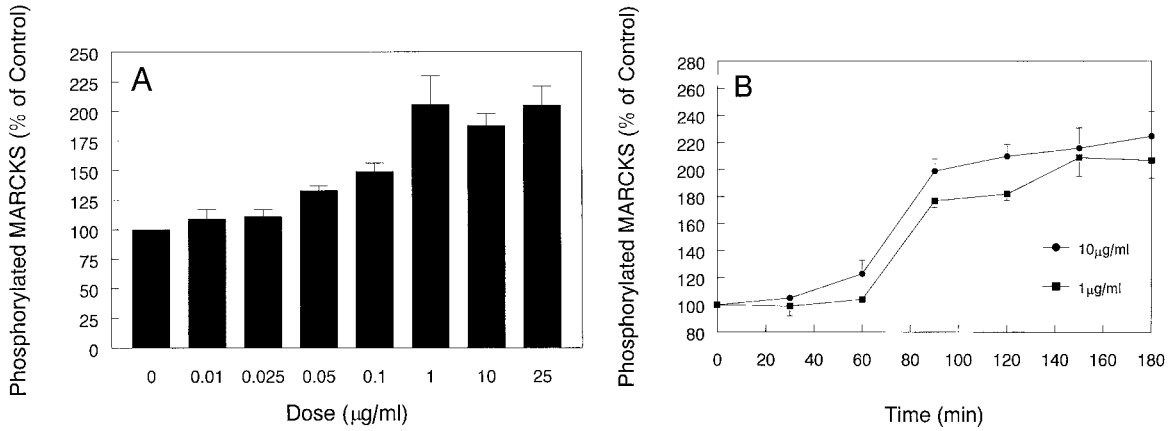

Fig. 1. Dose–response and time-course of LPS-induced phosphorylation of MARCKS in BPAEC. BPAEC were treated with either various concentrations of LPS (0–25 μg/ml) for 2 h (**A**), or 1 or 10 μg/ml LPS for various periods of time (0–180 min, **B**), and MARCKS was immunoprecipitated and quantified as described in Materials and Methods. The mean percentage changes in phosphorylated MARCKS are shown as bar graphs or line graphs (n = 3–8). The increase of MARCKS phosphorylation by LPS was both dose–dependent and time–dependent.

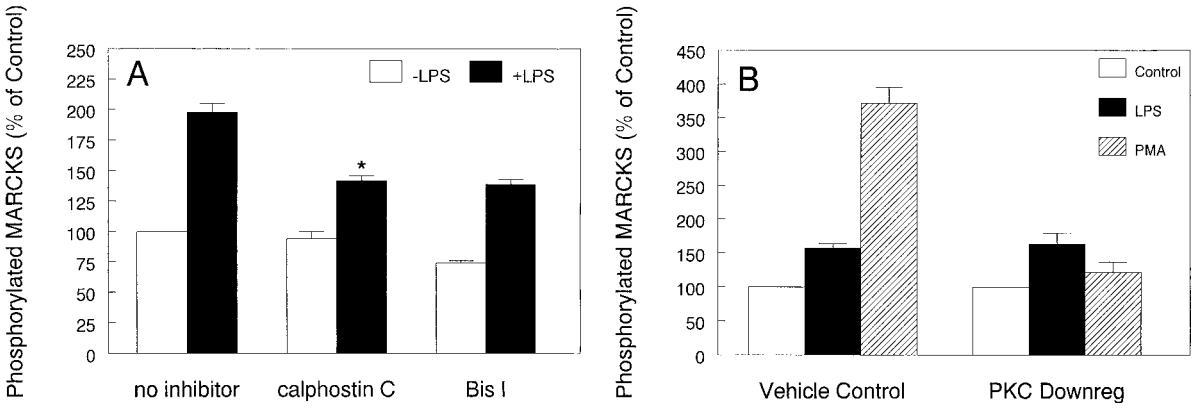

Fig. 2. Effect of inhibition of PKC activity on LPS-induced phosphorylation of MARCKS in BPAEC. BPAEC were treated with 1 μg/ml LPS for 2 h in the absence or presence of PKC inhibitors, 1 μM calphostin C, or 2 μM bisindolylmaleimide I (Bis I) (**A**, n = 3–4). In (**B**), the cells were pretreated with 1 μM PMA for 20 h to downregulate PKC activity (n = 3). The MARCKS phosphorylation induced by LPS was greatly attenuated by calphostin C (*$P < 0.05$, compared with the cells treated with LPS in the absence of the inhibitors), but not by downregulating PKC activity (PMA- but not LPS-induced phosphorylation is abolished by PMA pretreatment), suggesting that LPS-induced MARCKS phosphorylation is caused by the activation of PKC isoforms different from those activated by PMA. PMA treatment for 20 h increased basal MARCKS phosphorylation to 250% of the vehicle control, but these data were normalized to 100% for no LPS treatment, because it was our intent to show the effects of PKC downregulation on LPS-induced MARCKS phosphorylation.

greatly decrease LPS-induced MARCKS phosphorylation when the reduced basal phosphorylation level was taken into account (Fig. 2A). In parallel experiments, these two PKC inhibitors also decreased the PMA (1 μM)-induced MARCKS phosphorylation (630% of control for PMA versus 435% or 131% of control for PMA plus calphostin C or PMA plus Bis I, respectively). Downregulation of PKC activity by PMA treatment for 20 h increased the basal level of MARCKS phosphorylation to 250 ± 14% of the vehicle control but did not prevent phosphorylation of MARCKS by LPS, although it abolished subsequent PMA-induced MARCKS phosphorylation (Fig. 2B). Although we have no data to suggest a mechanism for this increase in basal MARCKS phosphorylation by prolonged PMA treatment, it may be because of the inhibition of a phosphatase or the activation of a kinase that is normally regulated by a downregulated PKC isoform.

As shown in Figure 3, treatment of BPAEC with LPS resulted in activation of p38 MAP kinase in a time-dependent manner. p38 is ac-

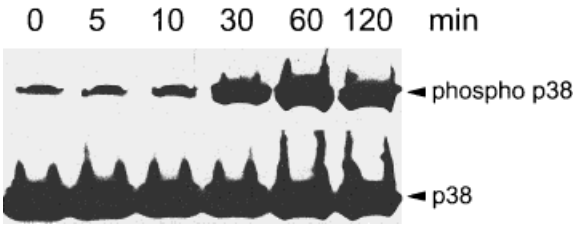

Fig. 3. Activation of p38 MAP kinase by LPS. BPAEC proteins were isolated, separated by SDS-PAGE, and transferred to nitrocellulose as described in Materials and Methods. p38 is activated by dual phosphorylation, so the nitrocellulose membranes were probed with either an antibody specific for the phosphorylated (activated) form of p38 or an antibody that recognizes total p38 (to show equal loading). The blots were developed with chemiluminescence. LPS induced a time-dependent activation of p38 in BPAEC.

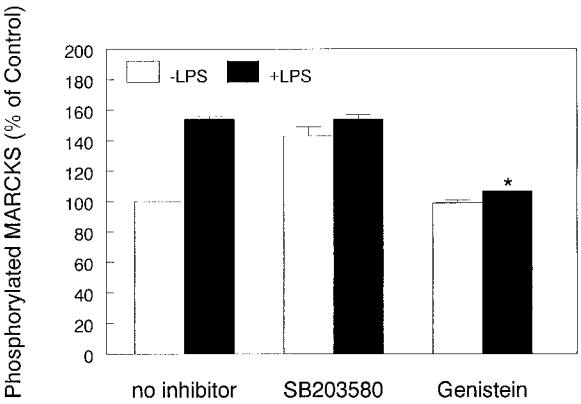

Fig. 4. Effect of inhibition of MAP kinase and tyrosine kinase activity on LPS-induced phosphorylation of MARCKS in BPAEC. BPAEC were treated with 1 μg/ml LPS for 2 h in the absence or presence of the p38 MAP kinase inhibitor, SB203580 (10 μM), or the tyrosine kinase inhibitor, genistein (10 μg/ml). The MARCKS phosphorylation induced by LPS was nearly abolished by inhibition of either p38 MAP kinase or tyrosine kinase activity (n = 3–7, $*P < 0.05$, compared with cells treated by LPS in the absence of the inhibitor). However, inhibition of p38 MAP kinase increased the basal level of MARCKS phosphorylation.

tivated in response to LPS by 30 min and remains activated for at least 120 min, which is consistent for a role in MARCKS phosphorylation. Conversely, p44/42 was not activated by LPS (data not shown). Therefore, we examined the effects of inhibition of p38 MAP kinase on LPS-induced MARCKS phosphorylation. As shown in Figure 4, SB203580, a specific inhibitor of p38 MAP kinase, increased the basal phosphorylation of MARCKS but blocked further phosphorylation of MARCKS in response to LPS. MAP kinases are activated by the dual phosphorylation of tyrosine and threonine residues, and inhibition of tyrosine kinases has

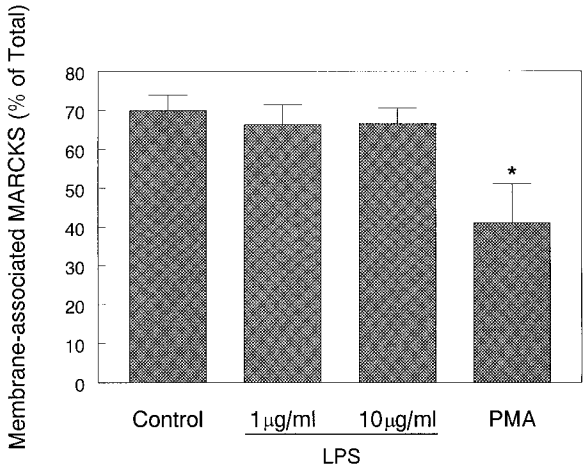

Fig. 5. MARCKS translocation between membrane and cytosol in BPAEC in response to LPS stimulation. Cells were treated with either 1 or 10 μg/ml LPS for 2 h, or 10 μM PMA for 10 min. MARCKS translocation was measured by ultracentrifugation of cell homogenates to separate the membrane and cytosol fractions, followed by SDS-PAGE and immunoblotting as described in Materials and Methods (n = 4). The MARCKS protein bound to the membrane was not significantly altered by LPS, indicating that MARCKS phosphorylation did not correlate with the translocation during this period. In contrast, PMA decreased the MARCKS proteins associated with the membrane ($*P < 0.05$, compared with control cells).

been shown to interfere with MAP kinase activation [Guan, 1994]. Therefore, we examined the role of tyrosine kinase activity on MARCKS phosphorylation. The data in Figure 4 demonstrate that inhibition of tyrosine kinase activity with genistein abolishes LPS-induced MARCKS phosphorylation without altering the basal level of phosphorylation. Because MARCKS contains no tyrosine residues, these data indicate that tyrosine kinase activation mediates LPS-signaling upstream of MARCKS phosphorylation in BPAEC.

MARCKS binds the plasma membrane via the PSD and an N-terminal myristoyl group. In some cell types, phosphorylation of MARCKS by LPS results in translocation from the cell membrane to cytosol [Aderem, 1992]. Data from the present study indicate that translocation of MARCKS did not occur in response to treatment with LPS, even though there was a twofold increase in MARCKS phosphorylation (Fig. 5). In contrast, PMA caused significant MARCKS translocation from membrane to cytosol. We had previously reported that MARCKS was evenly distributed between the membrane and cytosol fractions (in the present study, 70% of MARCKS is associated with the membrane) and that PMA caused only a mod-

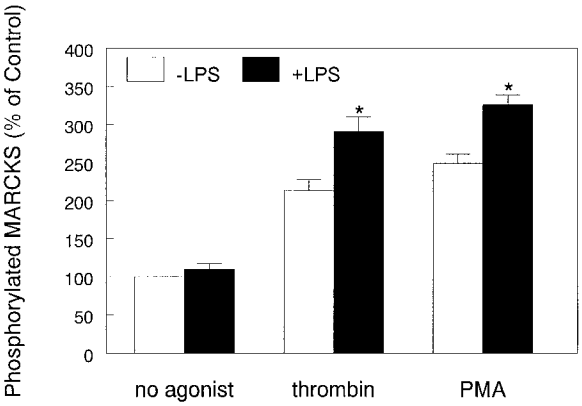

Fig. 6. Effects of LPS on thrombin- and PMA-induced MARCKS phosphorylation in BPAEC. BPAEC were exposed to 25 μg/ml LPS for 60 min and treated with either 0.1 μM thrombin for 2 min or 1 μM PMA for 10 min (n = 4–6). LPS greatly potentiated the phosphorylation of MARCKS induced by both thrombin and PMA (*$P < 0.05$, compared with cells in the absence of LPS), suggesting that LPS was priming the signaling pathways for the other agonists.

est (20%) translocation [Zhao and Davis, 1996]. Although the former and current data are qualitatively similar, they are somewhat different quantitatively. The differences may be reconciled by the fact that in the previous study, the cells were incubated in serum-free media, whereas in the present study, the cells were incubated in media containing 1% fetal bovine serum (to allow for appropriate LPS effects). Serum may induce a dephosphorylation of MARCKS that allows more binding to the membrane and allows more PKC-catalyzed phosphorylation, resulting in translocation. Nevertheless, the current results indicate that MARCKS phosphorylation and translocation are uncoupled in LPS-treated BPAEC.

Exposure of BPAEC to concentrations of LPS up to 25 μg/ml for 1 h had no effect on MARCKS phosphorylation. However, the MARCKS phosphorylation induced by either thrombin or PMA was greatly potentiated when the cells were pretreated for 1 h with 25 μg/ml LPS (Fig. 6). Similarly, Rosen et al. [1989] have demonstrated that LPS enhanced PMA-induced MARCKS phosphorylation in macrophages. These results suggest that LPS primes signaling pathways for other agonists.

As mentioned, nonphosphorylated MARCKS binds CaM and may serve as a CaM sink. Upon phosphorylation by PKC, the released CaM may become available for CaM-dependent processes such as phosphorylation of myosin light chains by myosin light-chain kinase [Hartwig et al., 1992], an important signaling pathway for cell contraction. We have, therefore, investigated the effect of LPS on myosin light chain phosphorylation, and the data are shown in Figure7. LPS at 1 μg/ml induced sustained diphosphorylation of myosin light chains, starting at 60 min and reaching the plateau at 120 min with a maximal increase of twofold (Fig. 7B). However, diphosphorylation of myosin light chains was maximal by 0.01 μg/ml LPS (Fig. 7A) and was not further enhanced as MARCKS was phosphorylated. Consequently, the CaM that is released during MARCKS phosphorylation is not necessary for myosin light chain kinase activation in LPS-stimulated BPAEC. In support of this conclusion, LPS did not affect thrombin-induced myosin light chain phosphorylation (Fig. 8), although LPS pretreatment did enhance thrombin-induced MARCKS phosphorylation (Fig. 6), further suggesting a diverse mechanism for MARCKS phosphorylation and myosin light chain phosphorylation by LPS.

DISCUSSION

Gram-negative sepsis is the most common setting in which the abnormality in lung function called ARDS develops [Newman, 1985]. Lipopolysaccharide, a component of the gram-negative bacteria wall, has been shown to cause the pulmonary edema and the subsequent development of ARDS associated with sepsis both in humans and animal models [Esbenshade et al., 1982; Bone, 1991]. It has been demonstrated that LPS can cause EC monolayer permeability via direct binding of EC [Pugin et al., 1995]. Identification of a specific LPS-binding protein (LBP) and the subsequent interaction of the LPS–LBP complex with an sCD14 in the serum provide a receptor basis for LPS action [Ulevitch and Tobias, 1995]. However, LPS signaling can also occur by LBP- and sCD14-independent mechanisms. This usually requires higher, supraphysiological concentrations because sCD14 is reported to enhance recognition of LPS and responses to LPS but is not absolutely required for signaling [Ulevitch and Tobias, 1995]. Currently, there are few definitive data that have delineated the postreceptor molecular events of LPS action, although a number of studies indicate that PKC, MAP kinases, and tyrosine kinases are activated by LPS [Novogrodsky et al., 1994; McKenna et al., 1994, 1995; DeFranco et al., 1995].

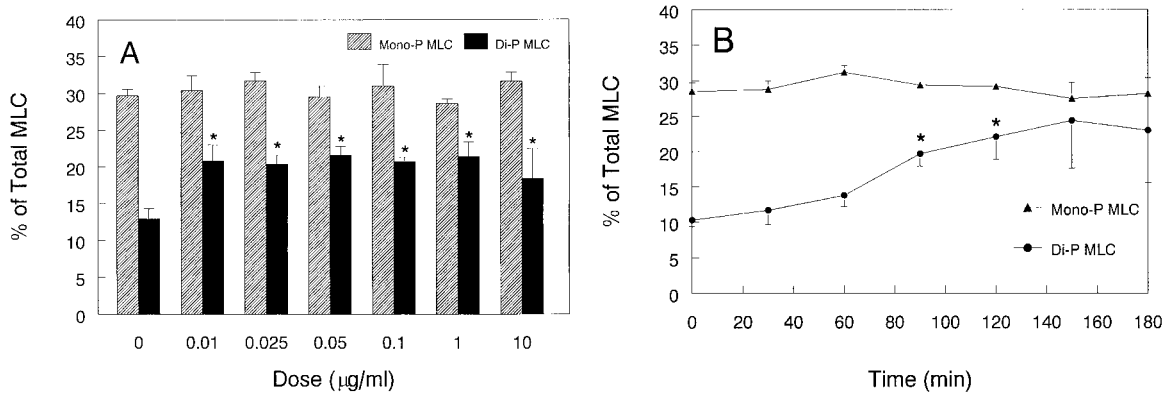

Fig. 7. Dose–response and time–course of LPS-induced phosphorylation of myosin light chains in BPAEC. BPAEC were treated with various concentrations of LPS (0–10 μg/ml) for 2 h (**A**) or with 1 μg/ml LPS for various periods of time (0–180 min, **B**). The mono- and diphosphorylated myosin light chains (MLC) were quantified as described in Materials and Methods (n = 3–6, $*P < 0.05$, compared with the cells in the absence of LPS). LPS at 0.01–10 μg/ml had no effect on monophosphorylated MLC levels, but significantly increased diphosphorylation of MLC without dose dependence. However, LPS-induced diphosphorylation of MLC time-dependent was reaching a maximum twofold increase at 120 min and maintaining that for 180 min.

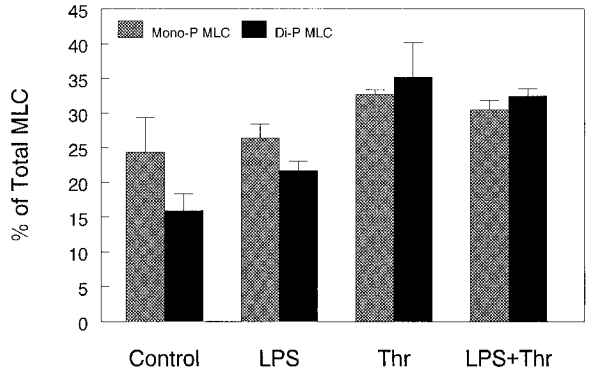

Fig. 8. Effects of LPS on thrombin-induced phosphorylation of myosin light chains (MLC) in BPAEC. BPAEC were exposed to 25 μg/ml LPS for 60 min and then treated with 0.1 μM thrombin for 2 min (n = 3). LPS did not affect either the mono- or diphosphorylation of MLC induced by thrombin.

LPS has been shown to increase the PKC activity in a variety of cells, including EC [McKenna et al., 1994, 1995]. Moreover, Aderem and colleagues [Rosen et al., 1989; Thelen et al., 1991] have reported that MARCKS is phosphorylated in response to LPS in macrophages and neutrophils via activation of PKC. In the present study, we demonstrated that MARCKS is phosphorylated by LPS in BPAEC in a time- and dose-dependent manner and that this phosphorylation is mediated, in part, by PKC activation. Although our results with calphostin C indicated that PKC activation mediates LPS-induced MARCKS phosphorylation in BPAEC, treatment with bisindolylmaleimide I and downregulation of PKC with PMA did not affect this phosphorylation.

PKC is a family of at least 12 kinases encoded by different genes or, in the case of PKC βI and II, by alternative splicing. These isoforms differ in tissue distribution, cofactor dependency, and substrate specificity. Based on their enzymatic properties, the isozymes can be classified into three groups: the conventional (cPKC), Ca^{2+}- and diacylglycerol-dependent forms (α, βI, βII, and γ); the novel (nPKC), Ca^{2+}-independent forms (δ, ϵ, η, θ, and μ) and the atypical PKC forms that require neither Ca^{2+} nor diacylglycerol (ι, λ, and ζ). All of the isoforms require phosphatidylserine for optimal activation. BPAEC have been reported to express mainly α, βII, δ, ϵ, ζ, ι, λ, and μ [Stasek and Garcia, 1993]. The results from the PKC inhibitors and prolonged PMA treatment experiments strongly suggest that LPS and PMA may activate different isoforms of PKC. Calphostin C inhibits PKC isoforms by blocking the diacylglycerol-binding site, whereas bisindolylmaleimide I inhibits by blocking the ATP-binding site. These differences in inhibition may account for the different results. In fact, our data suggest that basal phosphorylation of MARCKS is due, partially, to a bisindolylmaleimide I-sensitive PKC isoform but not a calphostin C-sensitive isoform. This isoform appears to be different from the PKC isoform that phosphorylates MARCKS in response to LPS. In addition, our data suggest that LPS induces MARCKS phosphorylation via an isoform(s) that is not substantially downregulated by PMA. Indeed, Stasek and Garcia [1993] have demonstrated that prolonged treatment with

PMA resulted in substantial downregulation of only the α and βII isoforms. PKC δ and η were only minimally downregulated and the others were unaffected.

Inhibition of tyrosine kinase activity with genistein nearly abolished LPS-induced MARCKS phosphorylation, indicating that tyrosine phosphorylation is involved in this signaling pathway. Because tyrosine kinase activation also appears to participate in MARCKS phosphorylation in response to thrombin and PMA [Zhao and Davis, 1996], it may be speculated that this is a general mechanism for MARCKS phosphorylation. However, because MARCKS does not contain any tyrosine residues, the effect of tyrosine kinase inhibition is via attenuation of upstream enzymes, which activate kinases that phosphorylate MARCKS. Two possible explanations can be provided from these data. First, PKC may be activated via tyrosine phosphorylation. Nakai et al. [1999] recently reported that stimulation of microglia by amyloid beta protein induced tyrosine phosphorylation and the subsequent activation of PKCδ, resulting in MARCKS phosphorylation. As mentioned above, PKCδ is not downregulated by prolonged PMA treatment of BPAEC. Therefore, because LPS-induced MARCKS phosphorylation is not inhibited by prolonged PMA treatment, but is inhibited by genistein, it is possible that tyrosine phosphorylation and subsequent activation of PKCδ could be involved in LPS-induced MARCKS phosphorylation in BPAEC.

Second, tyrosine phosphorylation may lead to MARCKS phosphorylation via activation of p38 MAP kinase. It has been reported that MARCKS is phosphorylated on serines adjacent to prolines outside the PKC-phosphorylation domain [Taniguchi et al., 1994] and MARCKS is phosphorylated in vitro by at least two proline-directed kinases, cdc2 kinase and tau protein kinase at the same sites [Yamamoto et al., 1995]. MAP kinases are a family of proline-directed (the phosphorylate serines or threonines with prolines on the C-terminal side) protein kinases that are activated as an early response to a variety of stimuli involved in cellular growth, transformation, and differentiation [Guan, 1994]. Tyrosine kinase activation in BPAEC in response to LPS or PMA leads to tyrosine phosphorylation and subsequent activation of p38 (activated by LPS) or p44/42 (activated by PMA, data not shown) MAP kinases. Our data indicate that p44/42 MAP kinase is not involved in LPS-induced MARCKS phosphorylation because it was not activated by LPS in BPAEC and because PD98059 (a specific inhibitor of p44/42 MAP kinase) could not prevent the LPS-induced MARCKS phosphorylation (data not shown). Conversely, MAP kinase p38 is activated by LPS in a timely manner, and SB203580 prevented the LPS-induced increase in MARCKS phosphorylation. A likely explanation for the increased basal level of MARCKS phosphorylation by SB203580 is that inhibition of p38 either allowed another kinase to phosphorylate MARCKS to high levels or resulted in the inhibition of a phosphatase that normally maintains low MARCKS phosphorylation. The lack of further increase in the phosphorylation of MARCKS induced by LPS in the presence of SB203580 could be interpreted in one of two ways: 1) LPS-induced phosphorylation of MARCKS is catalyzed by p38 kinase and thus directly inhibited by SB303580; or 2) less likely, the high basal phosphorylation of MARCKS may prevent further phosphorylation in the presence of LPS because all of the sites phosphorylated in response to LPS are already modified (but not by p38). Our current data cannot distinguish between these two scenarios, but to our knowledge, this is the first report that p38 may be involved in MARCKS phosphorylation.

Nonphosphorylated MARCKS is associated with the cell membrane in many cells [Swierczynski and Blackshear, 1995]. It is targeted to the membrane by an amino-terminal myristoyl group and a basic 25-amino-acid domain (PSD). Phosphorylation of MARCKS in the PSD has been shown to be accompanied by its translocation from the membrane to the cytosol [Thelen et al., 1991]. Nonphosphorylated MARCKS also crosslinks actin, so it is likely that the actin network associated with the membrane via MARCKS is relatively rigid. Phosphorylated, cytosolic MARCKS still associates with actin filaments, but does not crosslink them. The F-actin linked to phosphorylated MARCKS is likely to be spatially separated from the membrane and be more flexible. When MARCKS is dephosphorylated, it returns to the membrane, where it can, once again, crosslink F-actin. However, the results from the present study indicate that LPS, unlike PMA, does not cause translocation of MARCKS, although it significantly increases phosphorylation of MARCKS. This suggests that MARCKS translocation between membrane and cytosol does not contribute to the LPS-induced F-actin reorganization and the

changes in BPAEC morphology. Phosphorylation of MARCKS outside of the PSD (i.e., by MAP kinases or specific isoforms of PKC) could explain the lack of translocation and the inability of MARCKS to contribute CaM to myosin light-chain kinase (see below).

MARCKS has also been shown to bind CaM in a Ca^{2+}-dependent manner, and this is prevented by PKC-catalyzed phosphorylation in the PSD [Graff et al., 1989]. Phosphorylation of the PSD decreases its affinity for CaM by ~200-fold, and rapidly disrupts preexisting peptide–CaM complexes [Mcllroy et al., 1991], suggesting that MARCKS could serve as reservoir for CaM in resting cells, perhaps releasing CaM on PKC activation. In support of this, we have shown that thrombin-induced MARCKS phosphorylation in BPAEC is potentiated in the presence of the CaM antagonist, W7, which also increases basal phosphorylation [Zhao and Davis, 1996]. In addition, we have previously demonstrated that diphosphorylation of myosin light chain by the CaM-dependent myosin light chain kinase plays an important role in regulating EC contraction and monolayer permeability [Garcia et al., 1995]. Therefore, we investigated the effects of LPS on myosin light-chain phosphorylation in BPAEC. LPS caused a sustained increase in diphosphorylated myosin light chains in a time-dependent manner. However, a low concentration (0.01 μg/ml) of LPS induced myosin light-chain phosphorylation, without affecting MARCKS phosphorylation, suggesting that myosin light-chain phosphorylation, independent of MARCKS phosphorylation, is an early event in LPS action and may play an important role in mediating LPS-induced EC contraction and permeability.

Our results also demonstrate that LPS enhances MARCKS phosphorylation induced by either thrombin or PMA in BPAEC. This is consistent with studies on macrophages reported by Rosen et al. [1989] in that LPS potentiated PMA-induced MARCKS phosphorylation. They concluded that LPS promotes phosphorylation on novel sites as well as stimulating phosphorylation in the PSD. These results could be explained by the following scenarios: 1) LPS, thrombin, and PMA each activate different protein kinases (such as PKC or MAP kinases), or different isoforms of PKC, resulting in the confluence of signaling pathways; 2) LPS induces a modification of MARCKS itself, because it has been reported that LPS promotes the myristoylation and membrane association of MARCKS [Aderem, 1988], where MARCKS becomes more closely associated with activated PKC; or 3) LPS may exert its effects by inhibition of a protein phosphatase.

In summary, the present study has established MARCKS phosphorylation in BPAEC in response to LPS stimulation. Our results demonstrate that LPS induces a sustained phosphorylation of MARCKS with both time dependence and dose dependence, which is mediated by both PKC and tyrosine kinase (and possibly p38 MAP kinase) activation. In addition, LPS increases myosin light-chain phosphorylation in BPAEC, which may play a role in LPS-induced EC permeability via cell contraction. Our data suggest that MARCKS phosphorylation may mediate LPS-induced PKC signal transduction, but further studies are needed to determine the role of MARCKS phosphorylation in LPS-induced actin reorganization and EC permeability.

ACKNOWLEDGMENTS

The authors thank Drs. Perry Blackshear and Deborah Stumpo for the MARCKS cDNA constructs (pCMV80K1.2 and pBB80K2A) and Dr. Subah Packer for the myosin light chain antibody. We also thank Ms. Bonnie Neltner for maintaining the BPAEC in culture. This work was supported by the American Lung Association (Y.Z.) and the National Institutes of Health (HL 53203; H.W.D.).

REFERENCES

Adelstein RS, Klee CB. 1981. Purification and characterization of smooth muscle myosin light chain kinase. J Biol Chem 256:7501–7509.

Aderem A. 1988. Protein myristoylation as an intermediate step during signal transduction in macrophages: its role in arachidonic acid metabolism and in responses to interferon. J Cell Sci Suppl 9:151–167.

Aderem A. 1992. Signal transduction and the actin cytoskeleton: the roles of MARCKS and profilin. Trends Biochem Sci 17:438–443.

Bone RC. 1991. The pathogenesis of sepsis. Ann Intern Med 115:457–469.

Chakravarthy BR, Isaacs RJ, Morley P, Durkin JP, Whitfield JF. 1995. Stimulation of protein kinase C during Ca^{2+}-induced keratinocyte differentiation: selective blockade of MARCKS phosphorylation by calmodulin. J Biol Chem 270:1362–1368.

DeFranco AL, Hambleton J, McMahon M, Weinstein SL. 1995. Examination of the role of MAP kinase in the response of macrophages to lipopolysaccharide. Proc Clin Biol Res 392:407–420.

Esbenshade AM, Newman J, Lams P, Jolles B, Brigham K. 1982. Respiratory failure after endotoxin infusion in sheep: lung mechanics and lung fluid balance. J Appl Physiol 53:967–976.

Garcia JGN, Davis HW, Patterson CE. 1995. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. J Cell Physiol 163:510–522.

Goldblum SE, Ding X, Brann TW, Campbell-Washington J. 1993. Bacterial lipopolysaccharide induces actin reorganization, intercellular gap formation and endothelial barrier dysfunction in pulmonary vascular endothelial cells: concurrent F-actin depolymerization and new actin synthesis. J Cell Physiol 157:13–23.

Goldblum SE, Brann TW, Ding X, Pugin J, Tobias PS. 1994. Lipopolysaccharide (LPS)-binding protein and soluble CD14 function as accessory molecules for LPS-induced changes in endothelial barrier function in vitro. J Clin Invest 93:692–702.

Graff JM, Stumpo DJ, Blackshear PJ. 1989. Characterization of the phosphorylation sites in the chicken and bovine myristoylated alanine-rich C kinase substrate protein, a prominent cellular substrate for protein kinase C. J Biol Chem 264:11912–11919.

Guan KL. 1994. The mitogen activated protein kinase signal transduction pathway from the cell surface to the nucleus. Cell Signal 6:581–589.

Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature 356:618–622.

McIlroy BK, Walters JD, Blackshear PJ, Johnson JD. 1991. Phosphorylation-dependent binding of a synthetic MARCKS peptide to calmodulin. J Biol Chem 266:4959–4964.

McKenna TM, Clegg JM, Williams TJ. 1994. Protein kinase C is a mediator of lipopolysaccharide-induced vascular suppression in the rat aorta. Shock 2:84–89.

McKenna TM, Li S, Tao S. 1995. PKC mediates LPS- and phorbol-induced cardiac cell nitric oxide synthase activity and hypocontractility. Am J Physiol 269:H1891–H1898.

Nakai M, Hojo K, Saito N, Taniguchi T, Terashinma A, Kawamata T, Hashimoto T, Maeda K, Gechwendt M, Yamamoto H, Miyamota E, Tanaka C. 1999. Amyloid beta protein (25-35) phosphorylates MARCKS through tyrosine kinase-activated protein kinase C signaling pathway in microglia. J Neurochem 72:1179–1186.

Newman JH. 1985. Sepsis and pulmonary edema. Clin Chest Med 6:371–391.

Novogrodsky A, Vanichkin A, Patya M, Gazit A, Osherov N, Levitzki A. 1994. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosin kinase inhibitors. Science 264:1319–1322.

Persechini A, Kamm KE, Stull JT. 1986. Different phosphorylated forms of myosin in contracting tracheal smooth muscle. J Biol Chem 261:6293–6299.

Pugin J, Schurer-Muly CC, Leturcq D, Moriarty A, Ulevitch RJ, Tobias PS. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci USA 90:2744–2748.

Pugin J, Ulevitch RJ, Tobias PS. 1995. Activation of endothelial cells by endotoxin: direct versus indirect pathways and the role of CD14. Proc Clin Biol Res 392:369–373.

Rosen A, Nairn AC, Greengard P, Cohn ZA, Aderem A. 1989. Bacterial lipopolysaccharide regulates the phosphorylation of the 68K protein kinase C substrate in macrophages. J Biol Chem 264:9118–9121.

Shasby DM, Shasby SS Sullivan JM, Peach MJ. 1982. Role of endothelial cell cytoskeleton in control of endothelial permeability. Circ Res 51:657–661.

Stasek JE, Garcia JGN. 1993. Phorbol ester differentially modulates protein kinase C isotypes in vascular endothelium. Clin Res 41:281A.

Stasek JE, Patterson CE, Garcia JGN. 1992. Protein kinase C phosphorylates caldesmon and vimentin and enhances albumin permeability across cultured bovine pulmonary artery endothelial cell monolayers. J Cell Physiol 153:62–75.

Stumpo DJ, Graff JM, Albert KA, Greengard P, Blackshear PJ. 1989. Molecular cloning, characterization and expression of a cDNA encoding the 80- to 87-kDa myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. Proc Natl Acad Sci USA 86:4012–4016.

Swierczynski SL, Blackshear PJ. 1995. Membrane association of the myristoylated alanine-rich C kinase substrate (MARCKS) protein. Mutational analysis provides evidence for complex interactions. J Biol Chem 270: 13436–13445.

Taniguchi H, Manenti S, Suzuki M, Titani K. 1994. Myristoylated alanine-rich C kinase substrate (MARCKS), a major protein kinase C substrate, is an in vivo substrate of proline-directed protein kinase(s). J Biol Chem 269:18299–18302.

Thelen M, Rosen A, Nairn AC, Aderem A. 1991. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. Nature 351:320–322.

Ulevitch RJ, Tobias PS. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Ann Rev Immunol 13:437–457.

Wu WCS, Walaas SI, Nairn AC, Greengard P. 1982. Calcium/phospholipid regulates phosphorylation of an 87 kilodalton substrate protein in brain synaptosomes. Proc Natl Acad Sci USA 79:5249–5253.

Wysolmerski RB, Lagunoff D. 1990. Involvement of myosin light-chain kinase in endothelial cell retraction. Proc Natl Acad Sci USA 87:16–20.

Yamamoto H, Arakane F, Ono T, Tashima K, Okumura E, Yamada K, Hisanaga S, Fukunaga K, Kishimoto T, Miyamoto E. 1995. Phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) by proline-directed protein kinases and its dephosphorylation. J Neurochem 65:802–809.

Zhao Y, Davis HW. 1996. Thrombin-induced phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) protein in bovine pulmonary artery endothelial cells. J Cell Physiol 169:350–357.