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CYCLIC STRAIN STIMULATES DEPHOSPHORYLATION OF THE 20kDa REGULATORY MYOSIN LIGHT CHAIN IN VASCULAR SMOOTH MUSCLE CELLS

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The role of cyclic strain in the regulation of 20 kDa myosin light chain phosphorylation (MLC20) in cultured smooth muscle cells (SMC) is unknown. The objective of this study was to determine whether cyclic strain stimulates the dephosphorylation of MLC20 in serum-fed SMC displaying a high basal level of phosphorylation. Confluent bovine aortic SMC were subjected to 10% average strain at 60 cycles per minute for 30 and 60 minutes. Basal MLC20 phosphorylation (N=non,M=mono,D=di) of serum-fed SMC was as follows: N=34%:M=27%:D=39%. After 60 min of cyclic strain, both mono and diphosphorylated MLC20 were decreased to 21 and 15%, respectively. The strain-induced dephosphorylation of MLC20 was partially inhibited by the protein phosphatase 1/2A inhibitor, calyculin A (5 nM). However, phosphorylase a phosphatase activities in Triton-soluble and insoluble fractions of SMC were unaffected by cyclic strain. The data suggest that cyclic strain causes dephosphorylation of MLC20 in SMC which may be partially due to activation of MLC20 phosphatase and/or inhibition of MLC20 phosphorylation. © 1994 Academic Press, Inc.

Vascular smooth muscle cell structure and function are markedly influenced by cyclic strain (1,2). In the acute setting, previous studies conducted in our laboratory (3) and in others (4,5) have demonstrated that both static and cyclic strain modulates second messenger pathways in cultured SMC involving both Ca^{++} and cyclic AMP. In one such study, Bialecki et al. (4) demonstrated elevations in both calcium influx and efflux in cultured pulmonary arterial SMC subjected to acute, static 20% stretch. However, little is known concerning the downstream events influenced by these mechanosignaling events.

Phosphorylation of the 20kDa regulatory myosin light chain (MLC20) is the seminal event in the initiation of vascular smooth muscle cell contraction (6). The phosphorylation of MLC20 is regulated by the action of both myosin light chain kinase and myosin light chain phosphatase. MLCK catalyzes the phosphorylation of serine 19 and threonine 18 which stimulates the actin-activated MgATPase activity of myosin and subsequently leads to contraction (7,8). Recent data suggest that dephosphorylation of MLC20 is accomplished via a type 1 phosphatase (9). In addition,

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inhibition of this phosphatase by a G-protein dependent mechanism may play a role in the regulation of SMC contraction by increasing the sensitivity of the contractile apparatus to calcium (10).

The specific objective of this study was to determine whether cyclic strain affects MLC20 phosphorylation in SMC. Barany et al. (11) had previously reported a decrease in stretch-induced phosphorylation of MLC20 over 30-60 min in carotid arteries.

MATERIALS AND METHODS

Cell culture: Bovine aortas were obtained from freshly killed calves at a local slaughterhouse. Vascular smooth muscle cells were obtained from explants and utilized at less than 5 passages. Bovine aortic smooth muscle cells (SMC) were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin and 2.8 mM glutamine. SMC were confirmed by positive immunofluorescence with a monoclonal antibody directed against smooth muscle derived α-actin (Sigma; Cat #A2547).

Application of cyclic strain in vitro: SMC were seeded at a density of 60,000 cells/cm^2 well (six-well plates) on collagen coated rigid or flexible bottomed wells (Flexcell Corporation, McKeesport, PA). At confluence, plates were placed in a stress unit (Flexercell Strain Unit, Flexcell Corp.) which consists of a vacuum manifold in a tissue culture incubator (5%CO_2, 37°C) which is regulated by a solenoid valve controlled by a computer with a timer program (12). The membrane bottoms were deformed by a known percentage elongation by application of a precise vacuum. Upon release of the vacuum, the bottoms returned to their original conformation. Since cells adhere to the flexible surface of the culture well, it is presumed that the cell experiences the same force that is applied to the culture well. Force analysis of the strain on the flexible well during stretch at various vacuum levels (i.e., increasing levels of deformation) have been calculated mathematically by finite element analysis and empirically by measuring with a micrometer the distance between concentric circles (radial strain) or diametric axes (axial strain) marked on the membrane (13,14). Very little change is observed in the latter; hence, the force on the attached cells is uniaxial.

In the present experiments, SMC were subjected to an average strain of 10% at 60 cycles/minute (0.5 sec of strain alternating with 0.5 sec in neutral conformation) for the indicated times. Unstretched SMC served as control. In some experiments, the phosphatase inhibitor calyculin A or vehicle (0.1% ethanol) was added to SMC 10 minutes prior to the initiation of cyclic strain.

MLC20 phosphorylation: At the conclusion of a cyclic strain regimen, media was aspirated from the wells. SMC were treated with 90% acetone, 10% TCA and 10 mM DTT. SMC were mechanically harvested, and centrifuged at 10,000 x g for 10 min at 4°C. The pellet was washed 3 times. The dried pellet was resuspended in urea sample buffer containing 8 M urea, 18.5 mM Tris, 20.4 mM glycine, 10 mM DTT, 50 μl/ml saturated sucrose, and 0.004% bromophenol blue. The pellet was incubated in sample buffer and sonicated prior to electrophoresis.

Samples were subjected to urea/glycerol/PAGE electrophoresis as described by Hathaway and Haeberle (15). Following a pre-electrophoresis for 1 hour, samples were electrophoresed at 5 mA constant current for approximately 4 hours in a minigel apparatus (Hoefer Scientific, San Francisco, CA). Lower tank buffer contained 20 mM Tris/22 mM glycine. The upper tank buffer was equivalent to the lower tank buffer with 2.4 mM DTT and 2.9 mM thioglycolate. After electrophoresis, protein was transferred to nitrocellulose overnight at 0.1 A in transfer buffer containing Tris/glycine/methanol in a Transblot cell (Hoefer Scientific). Nitrocellulose blots were exposed to MLC20 antisera obtained from rabbits injected with recombinant MLC20 (rMLC20) from a cDNA library derived from human umbilical artery (16). MLC20 antisera was specific for smooth muscle MLCK phosphorylated MLC20 (non, monophosphorylated and diphosphorylated

species) and does not recognize non muscle isoforms of MLC20 (17). This was confirmed by the following: 1) 2D analysis of MLC20 mono and diphosphorylated species (H. Davis, University of Cincinnati, personal communication) and C.S. Packer (preliminary data), 2) corresponding increase in phosphorylation of the mono- and di- bands by the Ca^{++} ionophore ionomycin in quiescent SMC (preliminary data, not shown), 3) SMC-MLC20 -specific antibody raised to chicken gizzard MLC20 (gift of Dr. David Hathaway) displayed identical immunogenicity (17) and 4) no effect of the PKC inhibitor, calphostin C, on MLC20 phosphorylation levels (preliminary data, not shown). MLC20 was detected by the ECL Chemiluminescent Assay (Amersham Corp., Arlington Heights, Ill.) and quantitated with a Visage 2000 densitometer (BioImage, Ann Arbor, MI).

Protein phosphatase assay: Protein phosphatase activity was measured as described (18). Briefly, SMC were washed and lysed with 0.1% Triton X-100 buffer A (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 100 μM PMSF, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The supernatants were collected as Triton-soluble fractions. Triton-permeabilized cells were scraped and washed. The suspensions of SMC cytoskeletal protein were sonicated for 5 sec twice and centrifuged. The supernatants were prepared for Triton-insoluble fractions (myofilaments). Activities of phosphatase 1 and 2A in both the Triton-soluble and -insoluble fractions were measured with ^{32}P-labeled phosphorylase a. Protein concentration was determined by the method of Bradford (19).

Statistics: Data are presented as the mean ± SE. Statistical analysis was performed by a paired t-test with a p value of <0.05 considered significant.

RESULTS

In the presence of 10% fetal bovine serum, SMC exhibited a high basal level of MLC20 phosphorylation. The ratio of phosphorylated species to total MLC20 (nonphosphorylated+ monophosphorylated+diphosphorylated) was approximately 65% with 40% in the diphosphorylated state (Figure 1).

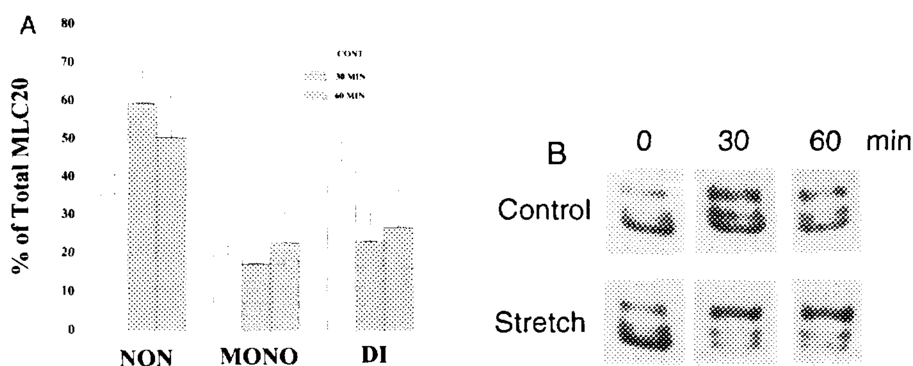

Figure 1. Dephosphorylation of MLC20 in serum-fed SMC subjected to cyclic strain. A. Densitometric analysis of MLC20. SMC in 10% FBS were stretched for 30 and 60 minutes as described in Methods. The ratio of phosphorylated species to total MLC20 (non+mono+di) is shown. Data shown represent the mean ± SE of five experiments (*=P<0.05 by paired t-test). B. Representative immunoblot.

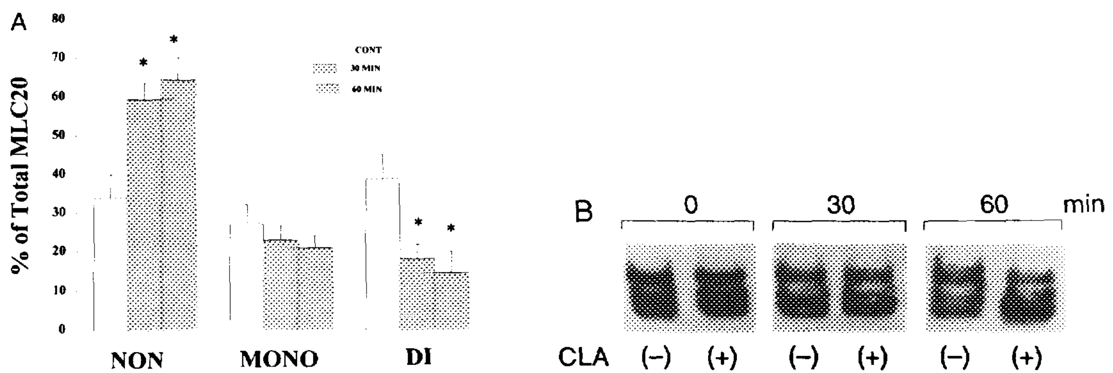

Figure 2. Partial inhibition by calyculin A on stretch-induced dephosphorylation of MLC20 in SMC. Calyculin A (5 nM) was added for 10 minutes prior to cyclic strain. SMC in 10% FBS were stretched for 30 and 60 minutes as described in Methods. The ratio of phosphorylated species to total MLC20 (non+mono+di) is shown. Data shown represent the mean ± SE of five experiments. B. Representative immunoblot.

The high basal MLC20 phosphorylation level was decreased by cyclic strain of 10% average at 60 cycles per minute for 30 minutes (Figure 1). Under these conditions, the level of diphosphorylated MLC20 was inhibited by nearly 50% ($P<0.05$) with a corresponding increase in the nonphosphorylated species. Monophosphorylated MLC20 was also decreased but to a lesser extent. Strain-induced dephosphorylation of MLC20 was slightly more pronounced after 60 minutes of cyclic strain. Unstretched SMC showed no statistically significant change in MLC20 phosphorylation levels (data not shown).

In the presence of calyculin A (5 nM), added 10 minutes prior to the initiation of cyclic strain to inhibit types 1 and 2A phosphatases, the strain-induced dephosphorylation of MLC20 was partially prevented (Figure 2). As shown in the representative immunoblot, MLC20 was heavily diphosphorylated in the presence of calyculin A as compared to SMC incubated in the absence of the inhibitor.

As shown in Table 1, cyclic strain of 30 and 60 minutes did not stimulate SMC phosphorylase a phosphatase activity in Triton-soluble and insoluble fractions. Type 1 phosphatase activity, resistant to inhibition by 1 nM okadaic acid, was also unaffected by cyclic strain.

DISCUSSION

As reported by others (20,21), we have observed that the basal phosphorylation of MLC20 is markedly affected by culture conditions. In the presence of serum, we have observed a high level of basal MLC20 phosphorylation in cultured SMC. Kawamoto and Adelstein (20) also found high levels of phosphorylated MLC20 constituting 50% of the total MLC20 distribution of

unphosphorylated and phosphorylated forms. In addition, they reported a greater percentage of diphosphorylated versus monophosphorylated MLC20 confirming our data presented in this study.

In serum-fed SMC, we have demonstrated cyclic strain-induced dephosphorylation of MLC20. This response was subacute, requiring 30 to 60 minutes to develop. The time course of MLC20 dephosphorylation that we observed is similar to that shown by Barany et al. (11) in stretched carotid arteries from pigs. In their study, stretch-induced phosphorylation decreased from 74% to 64% at 30 seconds and to 39% at 30 minutes. However, they found that the rate of decay of stretch-induced phosphorylation was not influenced if the stretch on the muscle was maintained, slowly slackened or quick-released. In our study, we have measured dephosphorylation under maintained cyclic strain.

The decay in stretch-induced Ca^{++} influx is also temporally consistent with our findings. Bialecki et al. (4) measured calcium transients in pulmonary artery smooth muscle cells exposed to sustained 20% stretch. Stretched-induced calcium influx was a rapid response that peaked by 1 minute and returned to baseline by 60 minutes.

The ability of calyculin A to partially prevent strain-induced dephosphorylation of MLC20 implicates the involvement of type 1 and /or 2A phosphatases in this response. This was examined at a concentration of calyculin A (5 nM) that blocks the activity of both types 1 and 2A phosphatase. Since MLC20 is thought to be dephosphorylated by a type 1 phosphatase (9), the inhibitor data would suggest that cyclic strain activates type 1 phosphatase thereby causing dephosphoryation of MLC20. Consistent with this hypothesis, at a dose of okadaic acid (5 nM) known to inhibit type 2A phosphatases, the strain-induced dephosphorylation was not blocked. Inhibition of type 1 phosphatases, with okadaic acid (1 μM), was precluded by its toxic effect on smooth muscle cells.

However, cyclic strain did not stimulate phosphatase activity in cultured SMC. This may be due to the utilization of phosphorylase a as a phosphatase substrate in these studies. A preferred substrate to examine MLC20-associated phosphatase activity may be heavy meromyosin. Alternatively, the observation of strain-induced dephosphorylation of MLC20 may involve either the activation of a novel phosphatase and/or inhibition of MLC20 phosphorylation.

In summary, the major observation of this study is cyclic strain-induced dephosphorylation of MLC20 in cultured serum-fed SMC. This response was partially reversed by calyculin A suggesting the involvement of phosphatases. However, data obtained with phosphorylase a as a phosphatase substrate failed to demonstrate strain-induced activation of phosphatase activity.

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