The involvement of protein kinase C in myosin phosphorylation and force development in rat tail arterial smooth muscle

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Myosin light-chain phosphorylation is the primary mechanism for activating smooth-muscle contraction and occurs principally at Ser-19 of the 20 kDa light chains of myosin (LC₂₀). In some circumstances, Thr-18 phosphorylation may also occur. Protein kinase C (PKC) can regulate LC₂₀ phosphorylation indirectly via signalling pathways leading to inhibition of myosin light-chain phosphatase. The goal of this study was to determine the relative importance of myosin light-chain kinase (MLCK) and PKC in basal and stimulated LC₂₀ phosphorylation in rat tail arterial smooth-muscle strips (RTA). Two MLCK inhibitors (ML-9 and wortmannin) and two PKC inhibitors (chelerythrine and calphostin C) that have different mechanisms of action were used. Results showed the following: (i) basal LC₂₀ phosphorylation in intact RTA is mediated by MLCK; (ii) α_1 -adrenoceptor stimulation increases LC₂₀ phosphorylation via MLCK and PKC; (iii) Ca²⁺-induced LC₂₀ phosphorylation in Triton X-100-demembranated RTA is catalysed exclusively by MLCK, consistent

with the quantitative loss of PKCs α and β following detergent treatment; (iv) very little LC₂₀ diphosphorylation (i.e. Thr-18 phosphorylation) occurs in intact or demembranated RTA at rest or in response to contractile stimuli; and (v) the level of LC₂₀ phosphorylation correlates with contraction in intact and demembranated RTA, although the steady-state tension–LC₂₀ phosphorylation relationship is markedly different between the two preparations such that the basal level of LC₂₀ phosphorylation in intact muscles is sufficient to generate maximal force in demembranated preparations. This may be due, in part, to differences in the phosphatase/kinase activity ratio, resulting from disruption of a signalling pathway leading to myosin light-chain phosphatase inhibition following detergent treatment.

Key words: contraction, myosin light-chain phosphorylation, protein kinase, vascular smooth muscle.

INTRODUCTION

The primary mechanism of activation of smooth-muscle contraction involves phosphorylation of the 20 kDa light chains of myosin (LC₂₀) catalysed by myosin light-chain kinase (MLCK) [1]. Contractile stimuli lead to LC₂₀ phosphorylation primarily at Ser-19 in both intact and permeabilized smooth muscle (e.g. [2–4]). LC₂₀ monophosphorylated at Ser-19 can be phosphorylated further by MLCK at Thr-18 to produce diphosphorylated LC₂₀; this has been demonstrated *in vitro* using high levels of MLCK [5,6] and, in some instances, in intact [2] and permeabilized smooth muscle [3]. Diphosphorylated myosin exhibits a greater actin-activated MgATPase activity than myosin phosphorylated only at Ser-19 [5], but the velocity of movement of both forms of myosin in the *in vitro* motility assay is the same [7]. Protein kinase C (PKC) can phosphorylate LC₂₀ at Ser-1, Ser-2 and Thr-9 *in vitro* [8], but this does not increase the actin-activated myosin MgATPase activity [8] or evoke movement in the *in vitro* motility assay [7]. Furthermore, phosphorylation of myosin at PKC-specific sites has not been observed in intact smooth muscle in response to agonists that activate PKC (e.g. [2,4,9,10]). PKC may, however, indirectly cause increases in LC₂₀ phosphorylation at Ser-19 and Thr-18 via inhibition of myosin light-chain phosphatase (myosin-associated type-1 phosphatase;

PP1_M), leading to contraction [11–13]. Recently, regulation of PP1_M has been shown to involve a PKC substrate, CPI-17, that in the phosphorylated state is a potent inhibitor of the phosphatase [14–16]. Phosphorylation of CPI-17 occurs in intact arterial smooth muscle in response to agonist stimulation [17]. This protein is expressed in smooth-muscle tissues, but little expression was detected in heart, skeletal muscle or non-muscle tissues [18]. An atypical PKC isoform activated by arachidonic acid may also inhibit PP1_M activity [19]. Furthermore, the 130 kDa myosin-binding subunit of PP1_M (MYPT) can be phosphorylated, and the phosphatase activity inhibited, by Rho-associated kinase (ROK) [20] or an unidentified, chelerythrine-sensitive but calphostin C-insensitive kinase [21]. ROK is also capable of directly phosphorylating LC₂₀, primarily at Ser-19 [22,23], although this does not appear to occur *in situ* [24–26]. Recently, ROK has been shown to phosphorylate CPI-17 at Thr-38 (the PKC site) with a marked increase in PP1_M inhibition [27]. Finally, a Ca²⁺-independent kinase distinct from MLCK but catalysing phosphorylation of LC₂₀ at Ser-19 and/or Thr-18 was recently detected in rat tail arterial and chicken gizzard smooth muscles [28].

The goals of the present study were: (i) to determine the relative importance of MLCK and PKC in phosphorylation of LC₂₀ during unstimulated, and agonist- and Ca²⁺-stimulated

Abbreviations used: DTT, dithiothreitol; LC₂₀, 20 kDa light chain of myosin; P₁-LC₂₀, monophosphorylated LC₂₀; P₂-LC₂₀, diphosphorylated LC₂₀; MLCK, myosin light-chain kinase; PKC, protein kinase C; PP1_C, catalytic subunit of type-1 protein phosphatase; PP1_M, myosin light-chain phosphatase; MYPT, 130 kDa myosin-binding subunit of PP1_M; ROK, Rho-associated kinase; [Ca²⁺]_i, intracellular Ca²⁺ concentration.

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conditions in intact or demembranated rat tail arterial smooth-muscle strips; (ii) to determine the relationship between LC_{20} phosphorylation and steady-state force development in intact and demembranated rat tail arterial smooth muscle; and (iii) to determine the involvement of LC_{20} diphosphorylation in contraction of this vascular smooth muscle. LC_{20} phosphorylation was quantified by urea/glycerol PAGE and immunoblotting using three antibodies: one that recognizes all forms of LC_{20} (phosphorylated or unphosphorylated) [4], one that recognizes only LC_{20} phosphorylated at Ser-19, and one that recognizes only LC_{20} phosphorylated at both Ser-19 and Thr-18 [29]. Four kinase inhibitors were used to investigate the kinases involved in LC_{20} phosphorylation: two MLCK inhibitors (ML-9 and wortmannin) and two PKC inhibitors (chelerythrine and calphostin C). In addition, the type-1 and -2A phosphatase inhibitor microcystin was used to inhibit $PP1_M$ in demembranated arterial muscle strips.

MATERIALS AND METHODS

Materials

Hepes, Tes and PMSF were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Triton X-100 was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Microcystin-LR from *Microcystis aeruginosa*, ML-9 [1-(5-chloronaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine], wortmannin, calphostin C and chelerythrine were purchased from Calbiochem (La Jolla, CA, U.S.A.). Microcystin specifically inhibits type-1 and -2A protein serine/threonine phosphatases with an IC_{50} of 100 nM [30]. ML-9 is a selective inhibitor of MLCK (K_i 3.8 μ M) that is competitive with respect to ATP [31]. Wortmannin is well known as a potent inhibitor of phosphatidylinositol 3-kinase (IC_{50} < 10 nM) [32] but is also commonly used as a MLCK inhibitor (IC_{50} 0.17 μ M); it acts as a non-competitive inhibitor with respect to ATP [33]. Chelerythrine (IC_{50} 0.66 μ M) is a selective, competitive inhibitor of PKC with respect to the protein substrate and a non-competitive inhibitor with respect to ATP [34]. Calphostin C (IC_{50} 50 nM), on the other hand, inhibits PKC by direct interaction with the regulatory domain, competing at the binding site of diacylglycerol and phorbol esters [35]. Stock solutions were prepared in water for microcystin, in 50% (v/v) ethanol for ML-9 and in DMSO for wortmannin, calphostin C and chelerythrine. Calmodulin was isolated as described previously [36]. Cirazoline was provided generously by Dr C. R. Triggle (University of Calgary, Calgary, Alberta, Canada). Anti-PKC ζ (a polyclonal antibody raised against the C-terminal sequence GFEYNPLLLSAEESV) was purchased from Life Technologies. Anti-caldesmon [37] and anti-calponin [38] were characterized previously. Anti-MLCK, anti-MYPT and anti- $PP1_c$ (raised against the catalytic subunit of type-1 protein phosphatase) were provided generously by Dr David Hartshorne (University of Arizona, Tucson, AZ, U.S.A.). All other reagents were of analytical grade or better and were purchased from VWR-CanLab (Edmonton, Alberta, Canada) or Sigma-Aldrich Canada (Mississauga, Ontario, Canada).

Tissue isolation and contractility measurement

Male Sprague-Dawley rats (300–500 g) were anaesthetized by inhalation of a lethal dose of halothane according to a research protocol consistent with the standards of the Canadian Council on Animal Care and approved by the local Animal Care Committee of the Medical Research Council of Canada. Rat tail arteries were cleaned and the endothelial cell layer removed mechanically. Helical strips (≈ 1 mm $\times$ 5–7 mm) were cut and

tied between a solid support and a force transducer, either horizontally on a bubble plate or vertically in a cuvette. Solution changes were effected rapidly by rotation of the bubble plate or replacement of the solution in the cuvette with a pipette. A resting tension of 50 mg was applied to all strips, which were equilibrated for 30–45 min in Hepes-Tyrode buffer (10 mM Hepes/137 mM NaCl/2.7 mM KCl/1 mM $MgCl_2$ /1.8 mM $CaCl_2$ /5.6 mM glucose, pH 7.4). All buffers were at room temperature (20 °C) and those used for intact strips were pre-oxygenated with 100% O_2 .

Intact strips

An initial reference contraction to 0.3 μ M cirazoline was obtained. After 3×15 min washes in Hepes-Tyrode buffer, relaxed strips were treated with vehicle (water, DMSO or ethanol) or inhibitors of MLCK or PKC for 30 min. For basal studies, strips were frozen immediately after the 30 min incubation with the inhibitor. For studies with cirazoline, strips were then exposed to the same inhibitor in the presence of 0.3 μ M cirazoline and rapidly frozen at the plateau of the sustained tonic component of the contraction.

Demembranated strips

While still intact, strips were contracted repeatedly with 117 mM KCl in Hepes-Tyrode buffer (with equimolar NaCl removed) until similar levels of force were obtained. The strips were demembranated in the bath with 1% (w/v) Triton X-100 in 30 mM Tes/50 mM KCl/5 mM K_2EGTA /150 mM sucrose/0.5 mM dithioerythritol, pH 7.4, for 2 h at room temperature. Solutions for experiments with demembranated arterial smooth-muscle strips contained 3.2 mM MgATP, 2 mM free Mg^{2+} , 12 mM phosphocreatine, 0.5 mM NaN_3 , 15 units/ml creatine kinase, 1 μ M calmodulin and 30 mM Tes (pH 6.9), with ionic strength adjusted to 150 mM with potassium propionate. Desired free Ca^{2+} concentrations (expressed as $-\log[Ca^{2+}]$ or pCa) were obtained by mixing stock solutions containing K_2EGTA and $K_2CaEGTA$ [39]. Tissues were incubated with inhibitors of MLCK or PKC in a pCa 9 solution for 30 min, followed by addition of the same inhibitor in a pCa 6 solution. Strips were frozen at the plateau of force development.

For analysis of the efficiency of demembranation, intact and Triton X-100-demembranated rat tail arterial smooth-muscle strips (17 strips, each ≈ 6 cm long) were lyophilized, weighed (16.9 and 16.8 mg of dry weight, respectively) and homogenized with a Tissue Tearor model 985-370 (BioSpec Products, Bartlesville, OK, U.S.A.) for 3×10 s in 1 ml of 20 mM Tris/HCl, pH 7.5/2 mM EDTA/2 mM EGTA/1 mM dithiothreitol (DTT)/0.1 mM PMSF/10 μ g/ml leupeptin/1 μ g/ml pepstatin/1% (w/v) SDS/0.1 mg/ml soya bean trypsin inhibitor. The homogenate was transferred to a centrifuge tube, the homogenizer blade and homogenization tube rinsed with 6×250 μ l of homogenization buffer and combined with the initial homogenate prior to centrifugation at 100 000 g for 1 h at 20 °C in a Beckman TL-100 table-top ultracentrifuge. Samples were subjected to SDS/PAGE (7.5–20% polyacrylamide gradient gels) and stained with Coomassie Brilliant Blue as described previously [38] or subjected to Western blotting (see below).

Measurement of LC_{20} phosphorylation

Muscle strips were frozen by immersion in a slurry of solid CO_2 with 10% (w/v) trichloroacetic acid/10 mM DTT in acetone for 1 min initially, then for another 10 min after removal from the force transducer and support. Strips were washed in 10 mM

DTT in acetone, lyophilized overnight and stored at -80°C until LC_{20} extraction. Proteins were extracted from tissue strips in $100\ \mu\text{l}$ of 6 M urea/200 mM Tris/220 mM glycine/10 mM DTT/10 mM EGTA/1 mM EDTA/1 mM PMSF/0.6 M KI/0.1% (w/v) Bromphenol Blue by constant rotation in a microcentrifuge tube for 3 h at 20°C . After filtration through a $0.45\ \mu\text{m}$ Centricon filter (Millipore, Bedford, MA, U.S.A.), unphosphorylated LC_{20} (LC_{20}), monophosphorylated LC_{20} ($\text{P}_1\text{-LC}_{20}$) and diphosphorylated LC_{20} ($\text{P}_2\text{-LC}_{20}$) were separated by urea/glycerol PAGE using a modification of the method of Sobieszek and Jertschin [40] using 0.75 mm-thick mini slab gels and $30\ \mu\text{l}$ of sample/lane. Electrophoresis was carried out at 6 mA/gel for 5 h. Proteins were transferred to $0.2\ \mu\text{m}$ nitrocellulose membranes in 10 mM sodium cyclohexylaminopropane sulphonic acid (pH 11.0) in a large transblot cell at 20 V and 4°C for 16 h. Western blotting was performed using three different antibodies: anti- LC_{20} (a polyclonal antibody raised in rabbits against chicken gizzard LC_{20} that recognizes all phosphorylated and unphosphorylated forms of LC_{20}) was used at 1:5000 dilution [4]; antibody pLC1 (a monoclonal antibody that recognizes LC_{20} phosphorylated only at Ser-19) was used at 1:200 dilution [29]; and antibody pLC2 (a peptide-directed polyclonal antibody raised in rabbits that specifically recognizes LC_{20} phosphorylated at both Ser-19 and Thr-18) was used at 1:5000 dilution [29]. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse, both purchased from Boehringer Mannheim and used at 1:5000 dilution) and developed with the SupersignalTM CL-HRP Substrate System (Pierce Chemical Co., Rockford, IL, U.S.A.) as described by the manufacturer. LC_{20} phosphorylation was quantified by densitometric scanning using a Pharmacia Image Master Desktop Scanning System. In separate experiments we established the linear range for the relationship between LC_{20} signal intensity ($A \times \text{mm}^2$) and protein amount. The specificity of these antibodies has been characterized previously [4,29].

Western blotting

Proteins separated by SDS/PAGE were transferred to nitrocellulose membranes ($0.2\ \mu\text{m}$) in 25 mM Tris/192 mM glycine/20% (v/v) methanol/0.1% (w/v) SDS, pH 8.3, at 40 V and 4°C for 16 h. Subsequent procedures were as described previously [28] using polyclonal antibodies raised in rabbits (anti-PKC ζ , $5\ \mu\text{g}/\text{ml}$; anti-calponin, 1:5000 dilution; anti-caldesmon, 1:10000 dilution; anti-MLCK, 1:500 dilution; and anti-PP1 α , 1:2000 dilution) or a monoclonal antibody to MYPT (1:2000 dilution). Secondary antibodies and enhanced chemiluminescence detection were as described above.

Intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) measurements

Involved rings of rat tail artery were threaded on to glass capillaries (0.35 mm diameter) and loaded with $16\ \mu\text{M}$ fura 2 acetoxymethyl ester (fura 2/AM) for 3–4 h at room temperature. They were then mounted in a perfusion bath on top of an inverted microscope for recording of epifluorescence at 510 nm with excitation at 340 and 380 nm as described previously [41]. The perfusion inlet was directed on to the tissue at a distance of $<1\ \text{mm}$ and solution from pre-equilibration reservoirs was changed by switching a magnetic valve. Fura 2 measurements were carried out at room temperature.

Data calculation and statistics

LC_{20} phosphorylation was quantified by Western blotting using anti- LC_{20} that recognizes all species of LC_{20} . Monophosphory-

lation levels are expressed as the percentage of total LC_{20} , i.e. $[\text{P}_1\text{-LC}_{20}/(\text{LC}_{20} + \text{P}_1\text{-LC}_{20} + \text{P}_2\text{-LC}_{20})] \times 100\%$. Vehicle treatment (ethanol and/or DMSO at $\leq 0.5\%$, v/v) of either intact or demembranated rat tail arterial smooth-muscle strips did not significantly alter contractile or LC_{20} phosphorylation responses compared with untreated controls; these control groups were therefore combined. Values are presented as means $\pm$ S.E.M. Data were compared by one-way analysis of variance, followed by modified Bonferroni posteriori tests, as appropriate. A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Basal LC_{20} phosphorylation

The primary purpose of this work was to determine the relative importance of MLCK and PKC in regulating myosin phosphorylation at Ser-19 and Thr-18 under basal conditions and during α_1 -adrenoceptor stimulation of vascular smooth muscle.

The basal level of $\text{P}_1\text{-LC}_{20}$ in intact rat tail artery was $18.4 \pm 2.2\%$ ($0.184 \pm 0.022\ \text{mol of P}_1/\text{mol of LC}_{20}$, $n = 6$; Figure 1). $\text{P}_2\text{-LC}_{20}$ was also detected in intact rat tail arterial smooth muscle under resting conditions using antibody pLC2, which recognizes LC_{20} only when phosphorylated at both Ser-19 and Thr-18 (Figure 2D, lane 2). However, the stoichiometry of diphosphorylation was very low (estimated to be $<5\%$) since $\text{P}_2\text{-LC}_{20}$ could not be detected using anti- LC_{20} (Figure 2C, lane 2), which recognizes all three forms of LC_{20} (unphosphorylated, mono- and diphosphorylated; Figure 2C, lane 1). We used two MLCK inhibitors and two PKC inhibitors with different mechanisms of action to identify the kinase(s) responsible for basal phosphorylation of LC_{20} . Figure 1 shows that basal LC_{20} monophosphorylation was significantly reduced by the MLCK inhibitors ML-9 and wortmannin, but not by the PKC inhibitors chelerythrine or calphostin C, indicating that MLCK is responsible for basal monophosphorylation.

Agonist-induced LC_{20} phosphorylation in intact muscle

The α_1 -adrenergic agonist cirazoline ($0.3\ \mu\text{M}$) induced a biphasic contraction of de-endothelialized rat tail arterial smooth-muscle strips, consisting of a rapid increase in force in the first 30 s after stimulation followed by a slower increase in force to a sustained

Figure 1 Effects of inhibitors of MLCK and PKC on the basal level of LC_{20} monophosphorylation in intact rat tail artery

Rat tail arterial smooth-muscle strips were treated with vehicle (C), 10, 30 or $300\ \mu\text{M}$ ML-9 (1, 2 and 3, respectively), $10\ \mu\text{M}$ wortmannin (4), $10\ \mu\text{M}$ chelerythrine (5) or $1\ \mu\text{M}$ calphostin C (6), and LC_{20} monophosphorylation was quantified by immunoblotting using anti- LC_{20} . The horizontal line represents the basal level of LC_{20} monophosphorylation in untreated rat tail arterial smooth-muscle strips. Values represent means $\pm$ S.E.M. ($n = 3-6$). *Significantly different from corresponding untreated response ($P < 0.05$).

Figure 2 Contraction and myosin mono- and diphosphorylation in rat tail arterial smooth-muscle strips in response to α_1 -adrenoceptor stimulation or K^+ -induced depolarization

Time courses of contraction ($\circ$) and monophosphorylation of LC_{20} ($\bullet$) in response to 0.3 μM cirazoline (**A**) or 117 mM KCl (**B**). Representative Western blots using anti- LC_{20} (**C**, lanes 1–8), antibody p LC_1 (**C**, lanes 9–12) and antibody p LC_2 (**D**) are shown under basal conditions (lanes 2), stimulated with 117 mM KCl for 15 s, 30 s, 1 min and 5 min (lanes 3–6, respectively) or with 0.3 μM cirazoline for 20 s and 5 min (lanes 7 and 8, respectively). (**C**) Lanes 9–12 show blots of strips treated with 117 mM KCl for 15 and 30 s (lanes 9 and 10, respectively) or 0.3 μM cirazoline for 20 s and 5 min (lanes 11 and 12, respectively). Lane 1 shows demembrated rat tail arterial smooth muscle treated with 10 μM microcystin at pCa 9 for 60 min as a control to demonstrate that anti- LC_{20} recognizes unphosphorylated, mono- and diphosphorylated LC_{20} (see also Figure 5 in [27]).

steady-state level (Figure 2A). A representative Western blot to determine LC_{20} phosphorylation at 30 s and 5 min, using an antibody that recognizes all forms of LC_{20} , is shown in Figure 2(C), lanes 7 and 8, and quantification of LC_{20} phosphorylation in response to α_1 -adrenoceptor stimulation is shown in Figure 2(A). The tonic and phasic components of cirazoline-induced contraction were both accompanied by an increase in LC_{20} monophosphorylation (Figure 2A), which occurred at Ser-19 (Figure 2C, lanes 11 and 12). A very low level of LC_{20} diphosphorylation was detected following cirazoline treatment (Figure 2D, lanes 7 and 8).

For comparison, Figure 2(B) shows the contractile response of de-endothelialized rat tail arterial smooth-muscle strips to a maximal depolarizing KCl stimulus. Contraction peaked at ≈ 30 s and declined slowly with time. K^+ depolarization evoked a rapid ≈ 4 -fold increase in LC_{20} monophosphorylation that correlated with the rapid increase in force, and then LC_{20} monophosphorylation declined to resting levels by 5 min while

force declined more slowly: 56% of maximal K^+ -induced force was maintained at 5 min. A representative Western blot showing the time course of LC_{20} phosphorylation evoked by K^+ depolarization is shown in Figure 2(C), lanes 3–6. $\text{P}_1\text{-LC}_{20}$ was recognized by antibody p LC_1 , specific for LC_{20} phosphorylated at Ser-19 (Figure 2C, lanes 9 and 10). It is evident from the Western blot in Figure 2(C), lanes 3–6, that very little diphosphorylation of LC_{20} occurred in response to K^+ -induced depolarization, since no immunoreactive band corresponding to $\text{P}_2\text{-LC}_{20}$ was detected with anti- LC_{20} . We estimate that we should have been able to detect 5% $\text{P}_2\text{-LC}_{20}$ with this antibody. However, K^+ -induced LC_{20} diphosphorylation was clearly visualized using the very sensitive antibody p LC_2 , recognizing only LC_{20} phosphorylated at both Ser-19 and Thr-18 (Figure 2D, lanes 3–6). The time course of LC_{20} diphosphorylation was very similar to that of monophosphorylation (compare Figure 2D, lanes 2–6 with Figure 2B), but clearly the stoichiometry of diphosphorylation was very low.

Figure 3 Effects of inhibitors of MLCK on the cirazoline-induced contractile response and LC₂₀ monophosphorylation in intact rat tail artery

Contractions were elicited with 0.3 μM cirazoline in the absence (C) or presence of ML-9 (10 and 30 μM; 1 and 2, respectively) or wortmannin (10 μM; 3). (A) Force measurements. Responses are shown at the tonic contractile maximum. (B) LC₂₀ monophosphorylation was quantified by immunoblotting using anti-LC₂₀. The horizontal line represents the basal level of LC₂₀ monophosphorylation in untreated rat tail arterial smooth-muscle strips. Values represent means ± S.E.M. ($n = 3-5$). *Significantly different from corresponding tonic response to cirazoline alone ($P < 0.05$).

Cirazoline-induced contraction and LC₂₀ monophosphorylation were sensitive to inhibition by the MLCK inhibitors ML-9 and wortmannin (Figure 3) and the PKC inhibitors chelerythrine and calphostin C (Figure 4), indicating that MLCK and PKC are involved in cirazoline-induced LC₂₀ monophosphorylation and contraction.

Using fura 2-loaded intact rat tail arterial muscle strips, we verified that neither chelerythrine nor calphostin C had any significant effect on resting $[Ca^{2+}]_i$ or the cirazoline-induced increase in $[Ca^{2+}]_i$ (Figure 5). The control traces in Figures 5(A) and 5(B) show typical $[Ca^{2+}]_i$ changes elicited by K⁺ and cirazoline. From Figure 5(C), it is apparent that chelerythrine alone at 10 μM had no significant effect on basal $[Ca^{2+}]_i$ (the change in $[Ca^{2+}]_i$ on addition of chelerythrine was $9.7 \pm 10.9\%$, $n = 3$). $[Ca^{2+}]$ transients induced by cirazoline in the presence of chelerythrine amounted to $101.7 \pm 4.7\%$ ($n = 3$) of the control response to cirazoline alone. Figure 5(D) confirms the lack of effect of chelerythrine in a post-treatment protocol. $[Ca^{2+}]_i$ was $95.5 \pm 3.6\%$ and $88.6 \pm 5.0\%$ ($n = 4$) of the level induced by cirazoline following treatment with 10 or 30 μM chelerythrine, respectively. This slight decline in $[Ca^{2+}]_i$ was also observed in control experiments during prolonged stimulation with cirazoline. $[Ca^{2+}]_i$ declined to $95.9 \pm 3.8\%$ ($n = 4$) of the maximal cirazoline-induced value after 5–10 min and to $89.4 \pm 5.7\%$ ($n = 4$) of the maximal value after a further 10–15 min of cirazoline treatment. Similarly, Figure 5(E) shows that calphostin C had no significant effect on the cirazoline-induced increase in $[Ca^{2+}]_i$, which was $90.1 \pm 5.2\%$ ($n = 4$) of the maximal cirazoline-induced value after 5–10 min incubation with 1 μM calphostin C and $90.9 \pm 6.1\%$ ($n = 4$) after a further 10–15 min treatment with 3 μM calphostin C.

Figure 4 Effects of inhibitors of PKC on the cirazoline-induced contractile response and LC₂₀ monophosphorylation in intact rat tail artery

Contractions were elicited with 0.3 μM cirazoline at the indicated concentrations of chelerythrine (○) or calphostin C (●). (A) Force measurements. Responses are shown at the tonic contractile maximum. (B) LC₂₀ monophosphorylation was quantified by immunoblotting using anti-LC₂₀. Values represent means ± S.E.M. ($n = 3-5$). * and #, significantly different from corresponding tonic response to cirazoline alone ($P < 0.05$) for chelerythrine and calphostin C, respectively.

Characterization of intact and demembranated rat tail arterial smooth muscle

Prior to conducting experiments with Triton X-100-demembranated rat tail arterial strips, we compared the protein contents of this preparation and of intact muscle strips. The efficiency of demembranation and the protein composition of intact and demembranated rat tail arterial smooth-muscle strips are shown in Figure 6. Figure 6(A) shows that the cytoskeletal, contractile and regulatory proteins are completely retained following demembranation with Triton X-100 and that there is no evidence of proteolysis following Triton X-100 treatment. Closer inspection reveals the loss of several proteins, presumably cytosolic and membrane-bound proteins, following detergent treatment; some are indicated by arrowheads. The Western blots in Figures 6(B)–6(G) demonstrate the efficiency of extraction of soluble and detergent-solubilized proteins more clearly: PKC isoenzymes ζ , α and β are completely removed (Figure 6B) while the actin-binding proteins calponin, caldesmon and MLCK (Figures 6C–6E) and the myosin-binding PP1_M [both the 130 kDa myosin-binding subunit (MYPT or M130) and the 38 kDa catalytic subunit (PP1_C)] are quantitatively retained (Figures 6F and 6G). We have shown previously that Triton X-100-demembranated rat tail arterial smooth-muscle strips generate comparable force in response to Ca²⁺ as intact strips in response to K⁺ depolarization [28]. We conclude, therefore, that Triton X-100 treatment effectively removes cytosolic and membrane proteins without affecting the contractile machinery.

Figure 5 $[Ca^{2+}]_i$ measurements in intact rat tail artery during stimulation with K^+ and cirazoline and the effect of PKC inhibitors

(A) $[Ca^{2+}]_i$ transients in response to K^+ (117 mM, 5 min, white bars) and cirazoline (0.3 μ M, 5 min, black bars). (B) A $[Ca^{2+}]_i$ transient induced by K^+ (117 mM, 5 min, white bar) was followed by repetitive stimulation with cirazoline (0.3 μ M, 5 min, black bars). (C) A $[Ca^{2+}]_i$ transient was elicited with cirazoline (0.3 μ M, black bars) in the presence (hatched bar) or absence of chelerythrine (10 μ M). (D) A $[Ca^{2+}]_i$ transient was elicited with cirazoline (0.3 μ M, black bar), and then chelerythrine (10 μ M for 5–10 min and 30 μ M for 10–15 min) was added (hatched bar). (E) A $[Ca^{2+}]_i$ transient was elicited with cirazoline (0.3 μ M, black bar), and then calphostin C (1 μ M for 5–10 min and 3 μ M for 10–15 min) was added (hatched bar). Breaks in the records represent 20 min periods during which excitation and data acquisition were turned off. Neither chelerythrine nor calphostin C autofluoresced at 510 nm at the concentrations used, as tested by focusing the microscope on the medium beside the muscle before and after perfusion of the chamber with the PKC inhibitors. Values indicate ratios of fluorescence emission at 340 nm to that at 380 nm.

Ca^{2+} -induced LC_{20} phosphorylation in demembranated muscle

In Triton X-100-demembranated rat tail arterial smooth muscle, in contrast to intact rat tail artery, no basal (i.e. at pCa 9) LC_{20} phosphorylation was detectable with either anti- LC_{20} or antibody pLC2 (lanes 1 in Figures 7B and 7C, respectively). Elevation of the free $[Ca^{2+}]_i$ to 1 μ M (pCa 6) resulted in contraction and an increase in P_1 - LC_{20} (Figures 7A and 7B, lane 2), but only a small increase in P_2 - LC_{20} (Figure 7C, lane 2). The stoichiometry of LC_{20} monophosphorylation in demembranated muscle strips at the plateau of force development in response to pCa 6 (0.19 ± 0.06 mol of P_1 /mol of LC_{20} , $n = 5$) was similar to the basal level of LC_{20} phosphorylation in intact muscle strips (Figure 1) and markedly less than the levels determined at the peak of contraction in intact strips stimulated with 117 mM KCl (0.61 ± 0.06 mol of P_1 /mol of LC_{20} , $n = 4$), despite reaching similar levels of tension [246 ± 17 mg ($n = 5$) and 255 ± 26 mg ($n = 7$), respectively]. At pCa 4.5, there were further increases in LC_{20} monophosphorylation (Figure 7B, lane 3) and diphosphorylation (Figure 7C, lane 3), but increasing the free $[Ca^{2+}]_i$ to pCa 4.5 at the plateau of force development at pCa 6 caused only a slight increase in force (results not shown). Inhibition of

PP1_M by microcystin at pCa 9 caused a marked increase in LC_{20} monophosphorylation (Figure 7B, lane 4) and diphosphorylation (Figures 7B and 7C, lanes 4), while steady-state force was comparable with that evoked at pCa 6 [28]. LC_{20} mono- and diphosphorylation in response to K^+ depolarization are shown for comparison in Figures 7(B) and 7(C), lanes 5.

Contraction of demembranated muscle strips and LC_{20} monophosphorylation at pCa 6 were inhibited significantly by ML-9 and wortmannin, but not by chelerythrine or calphostin C (Figure 8), indicating that MLCK and not PKC is responsible for this Ca^{2+} -induced LC_{20} monophosphorylation. This is consistent with the loss of PKCs α and β following detergent treatment (Figure 6B).

Tension- LC_{20} phosphorylation relationships

The relationships between tension and LC_{20} phosphorylation for intact and demembranated rat tail arterial smooth muscle are shown in Figure 9. In intact muscle strips there is a sigmoidal relationship between LC_{20} monophosphorylation and tension with increases in force occurring over the range of ≈ 0.3 – 0.5 mol of P_1 /mol of LC_{20} (Figure 9A). In demembranated muscle strips, on the other hand, the tension- LC_{20} monophosphorylation relationship is hyperbolic with maximal force achieved at < 0.2 mol of P_1 /mol of LC_{20} (Figure 9A). Contractions induced in demembranated muscle strips by the phosphatase inhibitor microcystin in the absence of Ca^{2+} displayed a tension- LC_{20} monophosphorylation relationship intermediate between intact and demembranated strips treated with cirazoline and Ca^{2+} , respectively (Figure 9A). No relationship was observed between LC_{20} diphosphorylation and tension (Figure 9B).

DISCUSSION

It is well accepted that MLCK triggers smooth-muscle contraction via LC_{20} monophosphorylation at Ser-19. However, questions remain to be answered regarding the involvement of other kinases (PKC, ROK, Ca^{2+} -independent LC_{20} kinase and others) in the regulation of contraction. We have used two MLCK inhibitors and two PKC inhibitors to investigate the roles of these kinases in LC_{20} phosphorylation under basal and agonist-stimulated conditions. In intact rat tail arterial smooth-muscle strips, basal LC_{20} monophosphorylation is due to MLCK. α_1 -Adrenoceptor stimulation increases LC_{20} monophosphorylation via MLCK and PKC activation. Inhibition of PKC by chelerythrine or calphostin C had no significant effect on resting $[Ca^{2+}]_i$ or the increase in $[Ca^{2+}]_i$ evoked by α_1 -adrenoceptor stimulation. It should be emphasized that ROK may also be activated in response to α_1 -adrenoceptor stimulation and may contribute to LC_{20} phosphorylation via inhibition of PP1_M [42].

In demembranated rat tail arterial smooth-muscle strips, MLCK mediated the Ca^{2+} -induced contraction and LC_{20} monophosphorylation. It is of interest that cirazoline-induced contraction of intact rat tail arterial smooth-muscle strips is much more sensitive to ML-9 than is Ca^{2+} -induced contraction of demembranated muscle strips (compare Figures 3A and 8A). This is probably due to the different tension- LC_{20} phosphorylation relations in intact and demembranated strips (see below). Very low levels of P_2 - LC_{20} were detected in demembranated muscle strips contracted at pCa 6.

During the course of this work, we observed a relationship between LC_{20} phosphorylation and contractile force similar to those in studies with other smooth-muscle tissues (e.g. [43–47]). The results of the present study show that no clear relationship exists between steady-state tension and the very low levels of

Figure 6 Analysis of the efficacy of demembration of rat tail arterial smooth muscle

(A) Lanes 1–4, intact rat tail artery (loading levels corresponded to 337, 202, 67 and 34 μ g of tissue dry weight, respectively); lane 5, molecular-mass markers; lanes 6–9, Triton X-100-demembrated rat tail artery (loading levels as for lanes 1–4). (B–G) Western blots of intact (I) and Triton X-100-demembrated (skinned, S) rat tail arteries with anti-PKC ζ (B), anti-calponin (CaP, C), anti-caldesmon (CaD, D), anti-MLCK (E), anti-MYPT (M130; F) and anti-PP1 c (G). The immunoreactive band above PKC ζ in (B) is PKCs α and β , which are also recognized by this antibody. Two faint bands of lower molecular mass than PKC ζ are also detected by the antibody, but represent non-specific bands since they were also seen in the presence of the competing peptide antigen. Slight proteolysis of MLCK and PP1 c is evident in (E) and (G), respectively. SM22 is a 22 kDa smooth-muscle protein of unknown function [52].

LC₂₀ diphosphorylation detected in either intact or demembrated rat tail arterial smooth muscle (Figure 9B). We conclude, therefore, that Ser-19 phosphorylation plays a critical role in contraction whereas Thr-18 phosphorylation has no apparent role in the contractile response in normal rat tail arterial smooth muscle in response to membrane depolarization or α_1 -adrenoceptor stimulation. Diphosphorylation of LC₂₀ may however play a role in other smooth-muscle tissues, in response to other stimuli, or in pathophysiological conditions such as coronary artery spasm [48–50].

Another interesting aspect of the present study is the large difference in the relationship between LC₂₀ monophosphorylation and steady-state tension in intact compared with demembrated rat tail arterial smooth-muscle strips. Demembrated muscle strips showed a steep relationship that reached a plateau in tension at $\approx 20\%$ phosphorylated LC₂₀, in good agreement with previous studies of permeabilized smooth muscle (e.g.

[44,46]) and as predicted by the 'latch bridge' model of Hai and Murphy [43]. In intact muscle strips, on the other hand, there is a significant basal level of LC₂₀ phosphorylation and a more gradual increase in tension with increases in LC₂₀ phosphorylation. The latch bridge model assumes that slowly cycling, dephosphorylated cross-bridges (latch bridges) arise from the activity of PP1 M on phosphorylated cross-bridges and contribute to steady-state tension. Under conditions of high phosphatase activity, more latch bridges will form, leading to a steeper relationship between tension and LC₂₀ phosphorylation. On the other hand, under conditions of low phosphatase activity, the relationship will be less steep. Based on the latch bridge model, the results of the present study suggest that there is a high level of phosphatase activity in demembrated compared with intact rat tail artery, perhaps due to the loss of the phosphatase inhibitor, CPI-17. A previous study demonstrated preferential extraction of PP1 M over MLCK upon treatment of Triton X-100-

Figure 7 Contraction and LC₂₀ monophosphorylation in demembrated rat tail artery in response to Ca²⁺

(A) Demembrated rat tail arterial smooth-muscle strips in relaxing solution (pCa 9) were stimulated to contract by increasing the [Ca²⁺] to 1 μ M (pCa 6) at time 0. Values represent means $\pm$ S.E.M. ($n = 5$). Representative Western blots using anti-LC₂₀ (B) or antibody pLC2 (C) are shown for demembrated rat tail arterial smooth-muscle strips exposed for 15 min to pCa 9 (lanes 1), pCa 6 (lanes 2), pCa 4.5 (lanes 3) or pCa 9 + 10 μ M microcystin (lanes 4), and for intact strips exposed for 1 min to 117 mM KCl (lanes 5).

Figure 8 Effects of inhibitors of MLCK and PKC on pCa 6-induced contraction and LC₂₀ monophosphorylation in demembrated rat tail arterial smooth-muscle strips

Contractions were elicited by increasing [Ca²⁺] from pCa 9 to pCa 6 in the absence (C) or presence of ML-9 (300 μ M; 1), wortmannin (10 μ M; 2), calphostin C (10 μ M; 3) or chelerythrine (100 μ M; 4). (A) Contractile responses. (B) LC₂₀ monophosphorylation levels. Values represent means $\pm$ S.E.M. ($n = 3-7$). *Significantly different from corresponding control value ($P < 0.05$).

Figure 9 Relationships between contraction and LC₂₀ mono- and diphosphorylation in intact and demembrated rat tail arterial smooth-muscle strips under various conditions

(A) Tension–LC₂₀ monophosphorylation relationship. (B) Tension–LC₂₀ diphosphorylation relationship. Key to symbols: $\blacktriangledown$, intact strips under basal conditions and stimulated with cirazoline; $\bullet$, demembrated strips at pCa 9, 6 or 4.5; $\circ$, demembrated strips treated with 10 μ M microcystin at pCa 9. Data points shown include the peak of K⁺-induced contractions, the phasic and tonic maxima for cirazoline-induced contractions, and intermediate and plateau levels of force induced by microcystin at pCa 9 in the absence and presence of kinase inhibitors. Curves in (A) show lines of best fit: dashed line is the best fit to $\blacktriangledown$; solid line is best fit to $\bullet$.

demembrated chicken gizzard fibres for 10 h at 37 °C in glycerol-containing relaxing solution [46]. The method of demembration employed in the present study was relatively mild, involving treatment with 1 % Triton X-100 at 20 °C for only 2 h in the absence of glycerol. In fact, Western blots revealed that MLCK and the catalytic and myosin-binding subunits of PP1_M were retained in demembrated rat tail arterial strips (Figures 6E–6G). Thus the possibility that preferential removal of the phosphatase led to an artificially high ratio of kinase/phosphatase activity in demembrated strips seems unlikely with the protocol used in the present study. Loss of additional proteins such as calponin and SM22 has been suggested to contribute to the steep relationship between LC₂₀ phosphorylation and contraction [46]. However, calponin was also retained in demembrated arterial strips (Figure 6C). We did not blot for SM22, but from Coomassie Brilliant Blue-stained gels of intact and demembrated arterial strips, it was also retained (Figure 6A). The most likely explanation for the difference in relationship between intact and demembrated rat tail arteries is a large increase in phosphatase activity after demembration that may be related to the loss of, for example, a PKC isoenzyme and/or CPI-17. In support of this, inhibition of PP1_M with microcystin at pCa 9 (Figure 9A) produced a tension–LC₂₀ phosphorylation relationship intermediate between those observed for intact and demembrated rat tail arteries. This observation suggests that differences in phosphatase activity

between intact and demembranated arteries is a major contributor to the difference in the tension- LC_{20} phosphorylation relationship. This is in contrast to results obtained with permeabilized rabbit portal vein [51] or mesenteric artery [47], in which treatment with a phosphatase inhibitor (okadaic acid or calyculin A, respectively) did not alter the relationship between maximal force output and LC_{20} phosphorylation, but is similar to the rightward shift in the tension- LC_{20} phosphorylation relationship observed on treatment of Triton X-100-demembranated chicken gizzard smooth-muscle strips with okadaic acid [46].

In conclusion, the results of the present study show that: (i) basal LC_{20} monophosphorylation in intact rat tail arterial smooth muscle is due to MLCK; (ii) α_1 -adrenoceptor stimulation increases LC_{20} monophosphorylation via MLCK and PKC activation; (iii) in demembranated rat tail arterial smooth-muscle strips, Ca^{2+} -induced LC_{20} monophosphorylation is effected by MLCK, consistent with the loss of PKCs α and β following detergent treatment; (iv) very little LC_{20} diphosphorylation occurs in intact or demembranated muscle strips at rest or in response to contractile stimuli; (v) the steady-state tension- LC_{20} monophosphorylation relationship is markedly different between intact and demembranated rat tail arterial smooth muscle. This difference supports the latch-bridge hypothesis and could be explained by a higher phosphatase/kinase activity ratio in demembranated compared with intact arteries. This may be due to disruption of a signalling pathway leading to $PP1_M$ inhibition upon Triton X-100 treatment, e.g. loss of an important PKC isoenzyme and/or CPI-17 that lead to phosphorylation and inhibition of $PP1_M$.

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