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ORIGINAL ARTICLE

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Myosin light chain diphosphorylation is enhanced by growth promotion of cultured smooth muscle cells

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Abstract The characteristics of actively growing smooth muscle cells (a variant, SM-3) were compared with those of growth-arrested cells with regard to response of myosin light chain (MLC) phosphorylation. Augmented MLC phosphorylation, in particular diphosphorylation, was observed in actively growing cells when stimulated with 30 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The maximum level of diphosphorylation in growing cells was significantly higher than that in growth-arrested cells. The MLC diphosphorylation was sensitive to protein kinase C down-regulation by phorbol dibutylate and pretreatment by the protein kinase inhibitors, staurosporine (30 nM) and isoquinoline sulphonamide HA1077 (20 μM). The actively growing cells contained larger amounts of protein kinase C than growth-arrested cells. The phosphorylation sites of mono- and diphospho-MLC were determined to be MLC kinase-dependent sites (Thr^{18}, Ser^{19}). The $PGF_{2\alpha}$ concentration/response curves of MLC diphosphorylation were shifted to the left and upwards in the presence of the protein phosphatase inhibitor calyculin A. These results suggest that $PGF_{2\alpha}$ stimulation of actively growing SM-3 cells augments MLC kinase-dependent MLC diphosphorylation. Protein kinase C is involved indirectly in this reaction, possibly through MLC phosphatase-sensitive regulatory mechanisms.

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Introduction

Coronary vasospasm and re-stenosis after angioplasty can occur in regions of artherosclerotic lesions [1, 5]. These abnormal responses may result mostly from local hyperreactivity in the arterial wall, as related to dysfunction of the endothelium, hypersensitivity of the vascular smooth muscle, the release of several vasoactive substances from the associated leukocytes or combinations of these [15, 17, 19].

We have reported recently that rabbit carotid arteries, in which hyperplasia was induced by balloon injury, showed a hyperreactive contraction associated with an enhanced and sustained phosphorylation, in particular diphosphorylation, of myosin light chain 20 kDa (MLC) [33]. We attributed the hyperreactivity to alteration of smooth muscle cells (SMC) in portions with intimal thickening. Campbell and Campbell [2] and Ross have proposed that, with passage and growth promotion of SMC in culture, there is a phenotype modulation to a synthetic state. Phenotype modulation may cause alterations in agonist receptors [7], contractile protein isoforms [6, 18, 24] and the extracellular matrix, with loss of contractile ability. Phenotype modulation can occur also in intimal neoplastic regions of arteriosclerotic arteries [22]. The concept of phenotype modulation contradicts the hyperreactive characteristics of SMCs in the intimal thickening artery. An important problem is whether phenotype modulation of the intimal SMCs is involved in the contractile potentiation related to some types of vasospasm.

In an attempt to clarification this question we used actively growing SMC, a variant SM-3 cell isolated from medial SMC of the rabbit aorta [26], to

characterize these cells' contractile responses whilst focussing on mechanisms of MLC phosphorylation and dephosphorylation.

Materials and methods

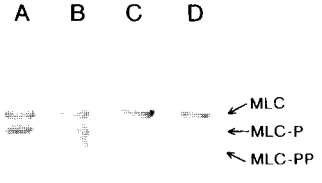
Smooth muscle cells SM-3

Rabbit aortic smooth muscle cells (line SM-3) were cultured as described elsewhere [26]. SM-3 cells are characterized on the basis of biochemical, histochemical and pharmacological factors as a reversible cell line between contractile and synthetic phases [26, 28]. We used 0.5% fetal calf serum (FCS)-minimum essential medium (MEM) for growth-arrested cells and 10% FCS-MEM for growing cells. The growth-arrested cells were prepared as follows: the 5-day culture of SM-3 cells after confluence in 10% FCS-MEM, referred to as the 5-day postconfluent culture, was gently dispersed and replated at a density of 3.7×10^6 cells/dish (100 mm diameter) containing 0.5% FCS-MEM for 48 h. Under these conditions the growth of cells is completely arrested [20] and the SM-3 cells show α-type of actin dominantly [26]. The actively growing cells (more than three passages) were cultured in 10% FCS-MEM for 30–35 h at an initial cell density of 1.6×10^6 cells/dish (100 mm). Under these conditions, β and γ-isoforms of actin are dominant [26]. The resulting cells were rinsed with 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES)-buffered MEM (pH 7.1; 37° C) and placed for another 30–60 min in a CO_2 incubator.

Determination of MLC phosphorylation

Measurements of non-, mono- and diphosphorylated MLC were made using glycerol-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis using an anti-MLC antibody, as previously described [27]. This method has been validated in previous studies with [^{32}P]adenosine triphosphate ([^{32}P]ATP) incorporation into separated MLC bands [27] and phosphomyosin-specific phosphatase [30]. The urea extracts of growing SM-3 cells before and after 30 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulation contained non-, mono-, and diphosphorylated MLC (Fig. 1, lanes A and B). Urea extracts were diluted ten fold (v/v) with distilled water and then incubated with a protein phosphatase for 2 h resulting in the disappearance of the phosphates (Fig. 1, lanes C and D). The phosphatase fraction was obtained from an extract of porcine aorta according to the method of Yoshida and Yagi [38] with minor modification and use of diethylaminoethyl (DEAE)-cellulose, red-agarose and phosphothioester-MLC column chromatography. MLC phosphorylation responses to $PGF_{2\alpha}$ were determined dose and time dependently as described else where [28].

Fig. 1 Immunostaining of the myosin light chain (*MLC*) in actively growing SM-3 cells. Actively growing SM-3 cells were stimulated with 30 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) for 5 min followed by termination of the reaction with 6.5% trichloroacetic acid. MLC was extracted as described in Materials and methods. Cell extracts before (*lane A*) and after (*lane B*) stimulation were treated with myosin phosphatase and then used for the MLC detrmination process (*lanes C* and *D* respectively) (*MLC-P* monophosphorylated MLC, *MLC*-PP *diphosphorylated MLC*)



Down-regulation of protein kinase C and determination of fractional content of protein kinase C

Protein kinase C in SM-3 cells was down-regulated by adding 50 ng/ml phorbol dibutylate (PDBu) followed by 28 h incubation according to the method of Kariya et al. [13]. Immunoblot analysis and determination of protein kinase C in the cellular extracts were done [28]. After the rinsed cell pellet had been sonicated in 20 mM *n*-octyl-β-glucoside, the resulting cell extract was chromatographed on a DEAE-cellulose column and the protein kinase C fraction eluted with 0.3 M NaCl. All eluates of the SM-3 cell preparations reacted with the antibody specific to isozyme type α of protein kinase C, but not with isozymes type β or γ. Khalil et al. [16] have identified the Ca^{2+}-independent protein kinase C isoforms ε and ξ in smooth muscle cells although the functional role of these isoforms is still unclear. In this report, we focussed on the protein kinase C isozyme type α which is sensitive to agonist-mediated changes in intracellular [Ca^{2+}] and diacylglyceride.

Two-dimensional phosphopeptide

Purified tracheal smooth muscle MLC was phosphorylated by gizzard MLC kinase to obtain mono- and diphosphorylated MLC standards, as previously described [4]. Growing SM-3 cells were labelled in phosphate-free physiological salt solution containing 100 μCi/ml [^{32}P]H_3PO_4 for 2 h before stimulation. Cells were then stimulated with 30 μM $PGF_{2\alpha}$ for 5 min. The cellular reaction was terminated with cold solution of 10% trichloroacetic acid, 10 mM dithiothreitol and 2 mM ethylenglycol-*bis*(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). MLC was extracted and purified using an immunoprecipitation method as described previously [4]. Purified MLC derived from SM-3 and standards of monophospho-MLC and diphospho-MLC were subjected to sodium dodecylsulphate (SDS)-PAGE on a 15% polyacrylamide. Segments of gel containing MLC were excised and incubated in 1 ml 25 mM NH_4HCO_3 (pH 8.4) and 25 μg tosylphenylalanyl chloromethyl ketone-treated trypsin for 2 h at 37° C. At the end of digestion, gel slices were removed and the remaining solution was lyophilized. After adding H_2O, the samples were dried in vacuo. Samples were resuspended in electrophoresis buffer and subjected to high-voltage, thin-layer electrophoresis and chromatography as described previously [4]. We used autoradiography to locate the ^{32}P-phosphopeptides.

Statistics

Each set of data was expressed as a mean ± SD. Student's *t*-test was used to determine the significance of differences between the means and $P < 0.05$ was considered as statistically significant.

Results

With 30 μM $PGF_{2\alpha}$ stimulation, MLC phosphorylation in the growth-arrested cells was apparent with maximum responses of 44% and 14% for mono- and diphosphorylation respectively (Fig. 2-A). Similarly, the MLC in actively growing cells was 36% monophosphorylated and 27% diphosphorylated at 2 min (Fig. 2-B). The

fractional content of diphosphorylated MLC and the phosphorylation pattern in growing cells were characteristically different from those in growth-arrested cells. The increase was rapid and a maximum was reached at 2 min, followed by a sustained high level of 19% at 15 min.

We and others have reported that protein kinase C is involved in enhancement of MLC phosphorylation or sensitization of MLC phosphorylation to Ca^{2+} [10, 30], but the related molecular mechanisms are unknown [34]. Figure 3A and B shows effects of the protein kinase inhibitors, staurosporine (30 nM) [29] and HA1077 (20 μM) [32] on MLC phosphorylation in actively growing cells. Pretreatment with these inhibitors for 15 min decreased MLC phosphorylation responses. The concentrations required for 50% inhibition (IC_{50}) of HA1077 for mono and diphosphorylation of MLC were 23 and 8 μM respectively. For staurosporine for mono and diphosphorylation of MLC the IC_{50}s were 40 and 10 nM respectively. MLC diphosphorylation was more sensitive to these kinase inhibitors than MLC monophosphorylation. Nifedipine (0.05–2.0 μM) did not decrease the MLC phosphorylation responses or the phospho-MLC basal levels (data not shown). Furthermore, MLC phosphorylation in both actively growing and growth-arrested SM-3 cells stimulated by $PGF_{2\alpha}$ was not influenced by the withdrawal of extracellular Ca^{2+} from the medium (data not shown). To clarify further the involvement of protein kinases in MLC phosphorylation, we down-regulated-protein kinase C [13, 28]. We have reported previously that the PDBu treatment (50 ng/ml for 28 h) completely suppresses the type α protein kinase C in growth-arrested SM-3 as shown by immunoblot analyses [28]. Treatment of growing cells with 50 ng/ml PDBu for 28 h suppressed the MLC diphosphorylation, but little or none of the monophosphorylation (Fig. 3-C). The MLC diphosphorylation curve was similar to that of the intact, growth-arrested cells stimulated with $PGF_{2\alpha}$ (cf. Fig. 2A). These results suggest that protein kinase C may be involved in the MLC diphosphorylation system in $PGF_{2\alpha}$-stimulated cells.

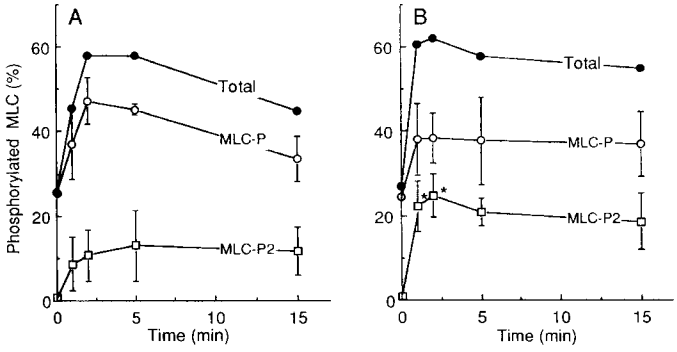


Fig. 2 Time course of MLC phosphorylation in growth-arrested (**A**) and growing (**B**) SM-3 cells. Cell were stimulated with 30 μM $PGF_{2\alpha}$ for the time shown in a minimum essential medium containing 20 mM 4,2-(hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.1) at 37° C. The degree of phosphorylation is expressed as a % of the total extracted MLC. ○, monophosphorylated MLC □, diphosphorylated MLC, ●, total phosphorylated MLC. Data are mean ± SD from five independent experiments $^*P < 0.05$ (Student's *t*-test) vs growth-arrested cells

We determined the fractional content of protein kinase C in actively growing and growth-arrested SM-3 cells. Figure 4 shows that the actively growing cells contained larger amounts of protein kinase C determined in an immunoblot analysis using an antibody specific to type α protein kinase C, than the post-confluent and growth-arrested cells (5- and 12-day post-confluent cultures). However, these cell homogenates did not immunoreact with other antibodies specific for types β and γ. We did not check for novel types of protein kinase C in the cell homogenates.

To determine whether protein kinase C involvement is direct or indirect, we examined the sites of mono-and diphosphorylation of MLC in $PGF_{2\alpha}$-stimulated SM-3 cells. Purified tracheal smooth muscle MLC was phosphorylated by smooth muscle MLC kinase from chicken gizzard to obtain mono- and diphosphorylated MLC standards, as previously described [4]. Two- dimensional phosphopeptide mapping of mono- and diphosphorylated standards revealed a single major spot (Fig. 5A and B

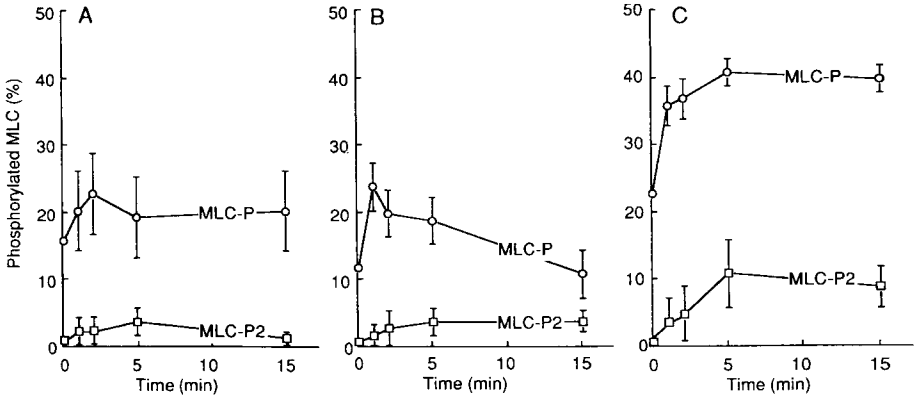


Fig. 3 Effect of the kinase inhibitors (*Staurosporine* or *HA1077*) down-regulation of protein kinase C by phorbol dibutylate (*PDBu*) on MLC phosphorylation in actively growing SM-3 cells. **A** Effect of 30 nM staurosporine or **B** 20 μM HA1077 for 15 min or **C** 50 ng/ml PDBu for 28 h with subsequent stimulation with 30 μM $PGF_{2\alpha}$. Time courses of mono (○) and diphosphorylation (□) of MLC are shown as means ± SD from five independent experiments

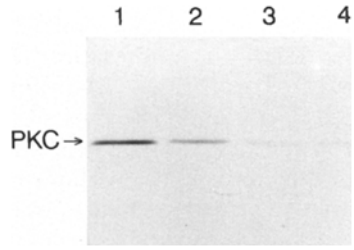


Fig. 4 Immunoblot analysis of type α protein kinase C in actively gorwing and growth-arrested SM-3 cells. The detergent extract of SM-3 cells (7×10^6 cells) and rabbit brain (140 μg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophore and electrophoretic transfer to a nitrocellulose membrane. Immunoblot analysis was made using a monoclonal antibody againist type α isozyme of protein kinase C. *Lane 1* rabit brain extract, *lane 2* actively growing SM-3 cell extract, *lane 3* growth-arrested SM-3 cells 5 days after confluent stage, *lane 4* growth-arrested SM-3 cells 12 days after confluent stage

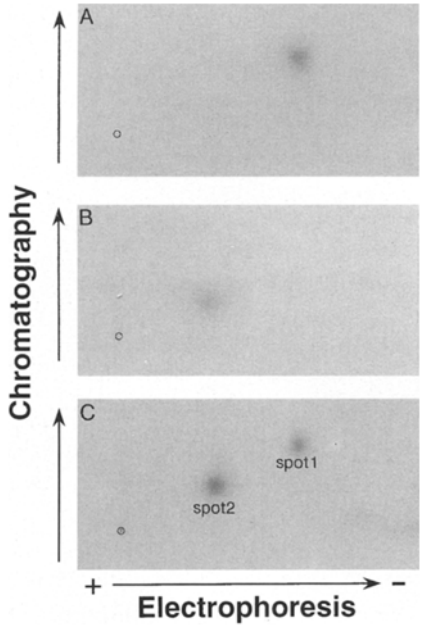


Fig. 5A–C Two-dimensional phosphopeptide mapping of mono-, diphosphorylated MLC standards and extract of actively growing SM-3 cells. Purified tracheal smooth muscle MLC was phosphorylated by smooth muscle MLC kinase to make mono- and diphosphorylated MLC standards as described in Materials and methods. Two-dimensional phosphopeptide mapping of **A** mono- and **B** diphosphorylated MLC standards demonstrated a single major spot. That of phosphorylated MLC from $PGF_{2\alpha}$-stimulated SM-3 cells (**C**) showed two major spots (*spot1* and *spot2*)

respectively). The mono-phosphorylated peptide consistently migrated farther in both electrophoretic and chromatographic directions than did the diphosphorylated peptide, as previously described [4]. Two-dimensional phosphopeptide maps of phosphorylated MLC from $PGF_{2\alpha}$-stimulated growing SM-3 cells showed two major spots (Fig. 5C, spot 1 and spot 2), the minor spots in Fig. 5C represent less than 5% of the total radioactivity. The mobilities of spots 1 and 2 were similar to the mobilities of mono- and diphosphorylated MLC standards respectively. The phosphopeptide maps of smooth muscle MLC phosphorylated by protein kinase C shows three major spots which migrated much farther in the electrophoretic direction with no migration in the chromatographic direction relative to phosphopeptides obtained from MLC phosphorylated by MLC kinase [4]. These results suggest that phosphopeptides 1 and 2 correspond to MLCs mono- and diphosphorylated by MLC kinase respectively. Using a radioanalytical imaging system, the ratio of radioactivity of spot 1 to spot 2 was calculated to be 1:1.43. The ratio of mono- to diphosphorylated MLC in $PGF_{2\alpha}$-stimulated SM-3 extracts before digestion determined by the glycerol-PAGE and an immunoblot method was 1:1.41. Mono- and diphosphorylated MLC in $PGF_{2\alpha}$-stimulated SM-3 cells is thus catalysed probably by MLC kinase, but not by protein kinase C.

On the other hand, the involvement of protein kinase C in the activity of MLC phosphatase was noted in smooth muscle, through the phosphorylation of MLC phosphatase inhibitors [3]. The MLC phosphorylation level is regulated by a balance between MLC phosphorylation by MLC kinase and dephosphorylation by MLC phosphatases. To investigate the effect of MLC phosphatase activity on MLC diphosphorylation in $PGF_{2\alpha}$-stimulated SM-3, the phosphatase inhibitor calyculin A was used. The concentration/response curves of MLC phosphorylation 2 min after the addition of various concentrations of calyculin A are shown in Fig. 6A. Calyculin A (10 nM) slightly increased the amount of MLC monophosphorylation, but did not affect that of MLC diphosphorylation. At concentrations exceeding 100 nM, calyculin A greatly increased the amount of MLC diphosphorylation but decreased that of MLC monophosphorylation. The concentration/response curves of MLC diphosphorylation 2 min after stimulation with various concentrations of $PGF_{2\alpha}$ in the presence or absence of 10 nM calyculin A are shown in Fig. 6B. Calyculin (10 nM) A shifted the $PGF_{2\alpha}$ concentration/response curves of MLC diphosphorylation to the left and upwards.

Discussion

Monical et al have reported that the SM and nonmuscle (NM) isoforms of the MLC are expressed in cultured SMC and have demonstrated that changes in the SM MLC isoform content are inversely correlated with cell growth [21]. However, they also have mentioned that no differences in either the magnitude or the kinetics of phosphorylation are observed for SM vs. NM isoforms [21]. In the present study we thus did not analyse separately the phosphorylation of SM and NM MLC isoforms.

The basal level of MLC phosphorylation in unstimulated SM3 cells is rather higher (23–25%) than that in

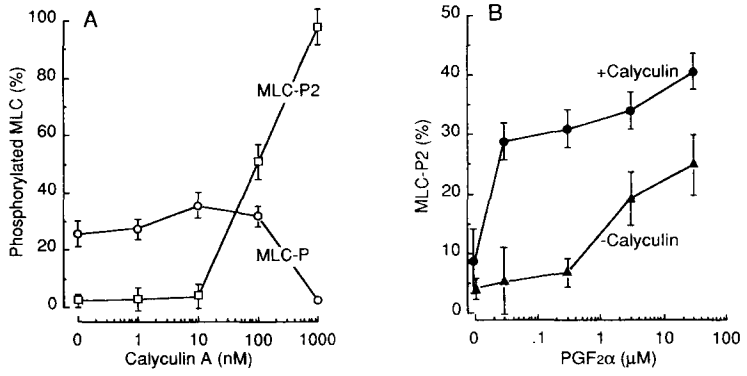


Fig. 6 Concentration/response curves for MLC phosphorylation in response to calyculin A alone (**A**) and calyculin A with $PGF_{2\alpha}$ (**B**). In **A**, growing SM-3 cells were stimulated with various concentrations of calyculin A (0, 1, 10, 100 and 1000 nM) and the extent of mono (O) and diphosphorylated MLC (□) 2 min after stimulation with calyculin A shown as means ± SD from four independent expeiments. In **B**, growing SM-3 cells were stimulated with various concentrations of $PGF_{2\alpha}$ in the presence (●) or in the absence (▲) of 10 nM calyculin A. The extent of diphosphorylated MLC 2 min after stimulation is shown as means ± SD from four independent experiments

isometrically mounted rabbit aortic strips (2–5%) [30]. Some degree of MLC phosphorylation may be necessary for cultured smooth muscle cells to adhere to the substratum.

With $PGF_{2\alpha}$ stimulation, the extent of MLC monophosphorylation in actively growing SM-3 cells was much the same as that in growth-arrested cells. However, MLC diphosphorylation in actively growing SM-3 cells was significantly greater than that in growth-arrested cells. We have reported previously that the diphosphorylation form in the rabbit aorta strips is specifically induced by $PGF_{2\alpha}$ but not by K^+ or histamine, even at a high dose which results in an approximately equal amount of incorporated phosphate as with $PGF_{2\alpha}$ stimulation [31]. The amount of MLC diphosphorylation may not simply correlate with the amount of MLC monophosphorylation (the substrate for diphosphorylation) in smooth muscle cells. Interestingly, MLC diphosphorylation was more sensitive to down-regulation of protein kinase C and to pretreatment with staurosporine and HA1077 than MLC monophosphorylation. These observations suggest that the $PGF_{2\alpha}$-induced MLC diphosphorylation pathway involves protein kinase C activation. Although MLC is phosphorylated by both MLC kinase and protein kinase C in vitro [8, 9], our two-dimensional phosphopeptide maps showed that mono- and diphosphorylated MLCs in $PGF_{2\alpha}$-stimulated SM-3 cells were catalysed by MLC kinase and not by protein kinase C. These findings are compatible with those of Colburn et al. [4] and Kamm et al. [11] who used intact bovine trachea tissues. Protein kinase C may regulate indirectly the formation of diphospho-MLC .

The level of MLC phosphorylation is regulated by a balance between MLC phosphorylation by MLC kinase and dephosphorylation by MLC phosphatases. One possible interpretation for the enhanced and protein kinase C-sensitive MLC diphosphorylation is that MLC kinase is modified (should be phosphorylated by protein kinase C), and consequently its activity increased, in growing cells. However, although stimulation of smooth muscle tissue with phorbol ester increases MLC kinase phosphorylation, there is no apparent change in its activity [36]. Although purified protein kinase C phosphorylates MLC kinase, the phosphorylation reduces rather than increase the activity of the enzyme [12]. Thus, it is unlikely that MLC kinase is modified by protein kinase C in growing cells.

Another interpretation is that activity of the phosphatases is impaired in growing SM-3 cells. We have demonstrated that the phospho-MLC levels in the isolated rabbit carotid artery with intimal thickening are higher than in normal carotids in which intimal thickenings contain large numbers of actively growing smooth muscle cells [33]. Treatment with calyculin A alone increased the MLC phosphorylation in the growing SM-3 cells. Calyculin A concentrations below 10 nM induced some accumulation of monophospho- but not diphospho-MLC. Higher concentrations (10–100 nM) increased MLC diphosphorylation without any apparent effect on levels of MLC monophosphorylation. Concentrations over 100 nM gradually increased MLC diphosphorylation but decreases in monophosphorylation. Furthermore, calyculin A alone did not increase intra-cellular $[Ca^{2+}]$ (data not shown). Some Ca^{2+}- and stimulant-independent MLC kinase(s) may exist in SM-3 cells. Morgan and colleagues [14, 37] have speculated that $PGF_{2\alpha}$ stimulation of isolated smooth muscle could inhibit MLC phosphatase activity. If the $PGF_{2\alpha}$ stimulation were to inhibit MLC phosphatase activity in growing SM-3 cells, the phosphatase inhibition by an exogenous inhibitor such as calyculin A should enhance further MLC diphosphorylation. Indeed, calyculin A at 10 nM, a concentration which alone does not increase MLC diphosphorylation, enhanced the MLC diphosphorylation reaction in the SM-3 cells exposed to

$PGF_{2\alpha}$. Augmented MLC diphosphorylation in growing cells may thus involve attenuation of MLC phosphatases activity.

The activity of MLC phosphatases is thought to be regulated through activation and inactivation cycles of endogenous protein phosphatase inhibitors [3]. Somlyo and colleagues [35] have suggested that these inhibitors are activated through phosphorylation catalysed by protein kinase C. Since the fractional content of protein kinase C in growing cells is larger than that in growth-arrested ones, this would contribute to the modulation of MLC phosphatases activation and inactivation cycles, resulting in augmented accumulation of MLC diphosphorylation in growing SM-3 cells.

We have noted previously that a hyperreactive contraction associated with augmented MLC phosphorylation, in particular diphosphorylation, occurs in the rabbit carotid artery in which hyperplasia is induced by balloon injury [33]. Association of actively growing SMCs with the thickened intima of the early phase of arteriosclerosis and vasospasm is of great significance. Second site phosphorylation of MLC by MLC kinase is reported to increase further the actin-activated Mg^{2+}-ATPase activity of myosin in an vitro system [8] and, from studies on the rabbit aorta, has been suggested to increase further the rate of force generation [31]. Thus, the contractile potential of growing SMC in hyperplastic arteries, in which a higher extent of MLC diphosphorylation is induced by stimulation, may be augmented compared with that of growth-arrested cells in media. Therefore, part of the hyperreactive contraction of the initial arteriosclerotic plaque may be due to unique properties of actively growing SMCs. However, the biochemical or pharmacological characteristics of SMCs in culture are not necessarily identical to those of SMCs in intact arteries. Studies in which the distribution of MLC diphosphorylation in the hyperplastic artery is visualized by an immunohistochemical method with a specific antibody against diphospho-MLC are in progress [25].

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