Diphosphorylation of myosin light chain in smooth muscle cells in culture

Possible involvement of protein kinase C

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Prostaglandin (PG) $F_{2\alpha}$ (30 μM) stimulated both monophosphorylation and diphosphorylation of myosin light chain (MLC) in a smooth muscle cell line (SM-3). The diphosphorylation was significantly decreased by treatment with the protein kinase C inhibitor staurosporine (30 nM, 30 min) from 20.1% of total MLC to 4.5%. The protein kinase C down-regulation treatment of SM-3 cells with phorbol dibutyrate suppressed to 8.7% the MLC diphosphorylation activity in the SM-3 cells. This down-regulation treatment had little effect on the monophosphorylation. We propose that the MLC diphosphorylation in $PGF_{2\alpha}$-stimulated SM-3 cells in culture may be regulated through mechanisms sensitive to protein kinase C.

Myosin light chain; Protein kinase C; Down-regulation; Staurosporine; Smooth muscle cell line

1. INTRODUCTION

Ikebe et al. reported that myosin light chain kinase (MLCK) catalyzed the rapid phosphorylation of isolated gizzard myosin light chain (MLC) at serine-19 and that the second site (threonine-18) was phosphorylated by an excess amount of MLCK [1,2]. On the other hand, the Ca^{2+}-activated phospholipid-dependent protein kinase C can also phosphorylate gizzard smooth muscle MLC at sites identified to be threonine-9, serine-1, and serine-2 [3,4], thereby decreasing the actin-activated Mg-ATPase activity and the contractile force [5,6]. Much of this evidence was obtained using in vitro systems or chemically permeable smooth muscle fibers. Thus, it is not clear whether multiple sites phosphorylation of MLC in intact contracting smooth muscle tissues or cells is the result of both MLCK and protein kinase C activities or MLCK activity alone.

We reported that the prostaglandin (PG)$F_{2\alpha}$-induced contraction of rabbit aortic smooth muscle tissue [7] and cells [8] is associated with both MLC monophosphorylation and diphosphorylation; when cultured SM-3 cells were stimulated with $PGF_{2\alpha}$ (30 μM), a relatively high level of the disphosphorylation occurred within 5 min. We have now used a biopharmacological method to determine whether the $PGF_{2\alpha}$-induced MLC diphosphorylation in contracting smooth muscle cells in culture is associated with the activity of MLCK or of protein kinase C.

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Abbreviations: MLC, myosin light chain; SM-3, smooth muscle cell line; MLCK, myosin light chain kinase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PDBu, phorbol dibutyrate; Str, staurosporine

2. MATERIALS AND METHODS

2.1. *Smooth muscle cells SM-3*

Rabbit aortic smooth muscle cells (line SM-3) were cultured as described [8]. The contractile response to vasoactive substances was monitored with an Olympus phase-contrast microscope and determined with a planimeter, as described [9]. All the experiments were carried out with quiescent phase SM-3 cells: the postconfluent and growth-arrested cells were used as the seed culture, and the cells were plated and cultured at 3.5×10^6 cells/100 mm dish containing 0.5% FCS-MEM for 2 days. The resulting cells were stimulated with $PGF_{2\alpha}$ in 20 mM Hepes-buffered MEM (pH 7.1) at 37°C.

2.2. *Down-regulation of protein kinase C*

The down-regulation of protein kinase C in SM-3 cells was performed by adding 50 ng/ml phorbol dibutyrate (PDBu) followed by 28 h incubation in a CO_2 incubator, according to the method of Kariya et al. [10]. After washing 3 times with warmed 20 mM Hepes-buffered MEM (pH 7.1), the cells were used for each experiment. Immunoblot analysis of the protein kinase C isozymes was carried out with a minor modification of the method of Hidaka et al. [11] involving DEAE cellulose column chromatography.

2.3. *Determination of MLC phosphorylation*

Separation and determination of non-, mono-, and diphosphorylated MLC were carried out by the method of Taylor and Stull [12] with minor modification, as described [7,8,13], a procedure which involves a glycerol-PAGE and a immunoblot analysis using anti-MLC_{20} antibody. After the $PGF_{2\alpha}$ stimulation, the cellular reaction was terminated with cold 10% trichloroacetic acid/10 mM dithiothreitol/2 mM EGTA solution. The resulting cell pellet (3.5×10^6 cells) was washed twice with acetone/10 mM dithiothreitol,

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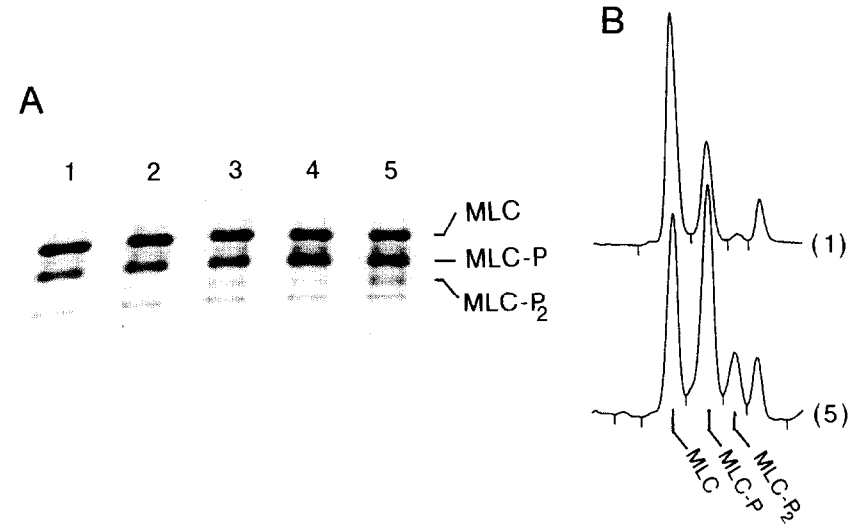

Fig. 1. Immunoblots of myosin light chain phosphorylation in $PGF_{2\alpha}$-stimulated SM-3 cells. (A) Quiescent SM-3 cells were stimulated with 0 μM (1), 1 μM (2), 3 μM (3), 10 μM (4), 30 μM (5) $PGF_{2\alpha}$ in 20 mM Hepes-buffered MEM (pH 7.1) at 37°C, and the stimulation was terminated by adding 10% TCA solution. Nonphosphorylated (MLC), monophosphorylated (MLC-P), and diphosphorylated (MLC-P2) forms of light chain were separated and analyzed by glycerol-PAGE/immunoblotting as described in section 2. (B) Immunoblot scanning of nonphosphorylated and phosphorylated myosin light chains. Densitometry of these blots and quantitation of absorbance peaks were carried out with a Densitron PAN-FV (Jookoo, Japan) equipped with a recording integrator. (1) Nonstimulated SM-3 fraction; (5) 30 μM $PGF_{2\alpha}$-stimulated SM-3 fraction.

and then dried. The dried acetone powder was extracted with 35 μl of glycerol-PAGE sample buffer containing 8 M urea, then was passed through a 0.45 μm membrane filter. The urea solubilized samples were subjected to glycerol-PAGE/immunoblot analysis. In our analysis, mono- and diphosphorylated MLCs were converted into the nonphosphorylated form by phosphomyosin-specific phosphatase, as described [7,13]. We confirmed these bands in [^{32}P]ATP experiments: as ^{32}P-labeled SM-3 cells in culture were stimulated with $PGF_{2\alpha}$, labelled MLC-P was phosphorylated only on the serine residue, and labelled MLC-P2 was phosphorylated on the serine and threonine residues in a molar ratio of 1 : 1. Thus, the mono- and diphosphorylation fractions on immunoblotting paper may not involve any other protein fractions such as 28 kDa protein as pointed out by Colburn et al. [14].

3. RESULTS AND DISCUSSION

Upon stimulation with $PGF_{2\alpha}$ of SM-3 cells, both monophosphorylated and diphosphorylated MLC fractions increased, in a dose- and time-dependent manner (Figs 1A and 2A) and a correlation with increases in cell contraction was evident (data not shown). With 30 μM $PGF_{2\alpha}$ stimulation, $42.9 \pm 0.4\%$ ($n = 4$–5) of monophosphorylation and $20.1 \pm 6.2\%$ ($n = 4$–5) of diphosphorylation were observed at 5 min (Fig. 2). The extent of MLC diphosphorylation appears to be distinctly high, as compared with the results reported by

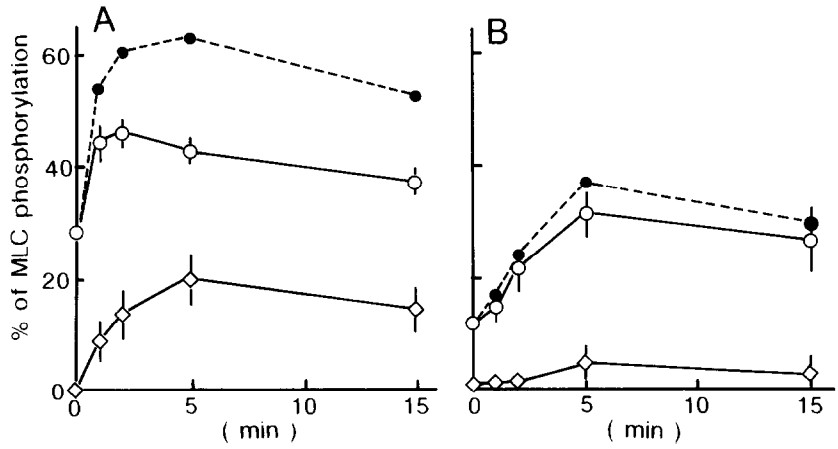

Fig. 2. Time course of MLC phosphorylation in control (A) or Str-treated (B) SM-3 cells. (A) Quiescent SM-3 cells were stimulated with 30 μM $PGF_{2\alpha}$, for the time shown, in 20 mM Hepes-buffered MEM (pH 7.1) at 37°C. (B) Quiescent SM-3 cells were exposed to 30 μM Str for 30 min at 37°C prior to $PGF_{2\alpha}$ stimulation. The figures represent the mean SE of 4–5 independent experiments. Phosphorylation levels are expressed as a % of the total extracted MLC. Symbols: (○) monophosphorylated MLC; (◇) diphosphorylated MLC; (●) total phosphorylated MLC.

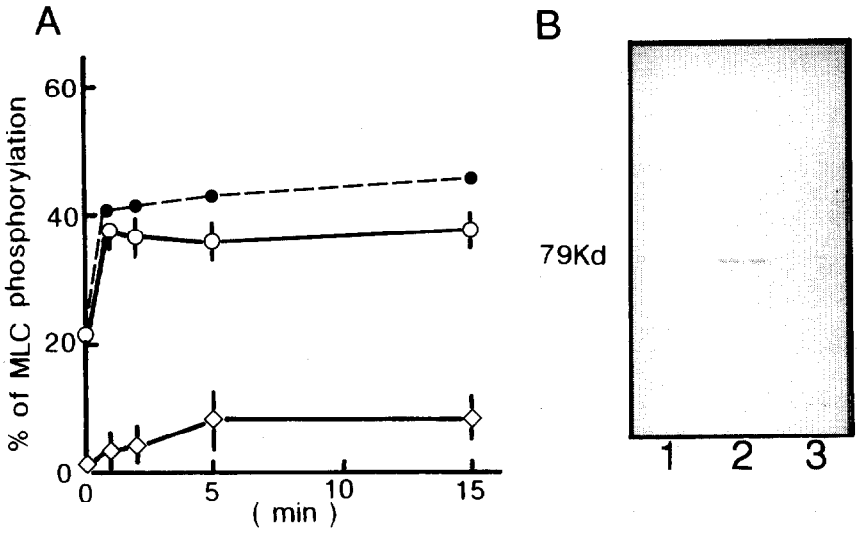

Fig. 3. Myosin light chain phosphorylation in PDBu-treated SM-3 cells and immunoblot analysis of protein kinase C. (A) Time course of MLC phosphorylation induced by $PGF_{2\alpha}$ in PDBu-treated SM-3 cells. Quiescent culture of SM-3 cells were treated with 50 ng/ml PDBu for 28 h. Then the cells were stimulated with 30 μM $PGF_{2\alpha}$ for the time shown. (B) Immunoblot analysis of type III protein kinase C. The detergent extract of SM-3 cells (7×10^6 cells) and of rabbit brain (140 μg) were subjected to sodium dodecyl sulfate-PAGE and electrophoretic transfer to a nitrocellulose filter. Immunoblot analysis was made using the mono-clonal antibody (from Seikagaku Kogyo, Japan) against type III isozyme of protein kinase C. (Lane 1) Rabbit brain extract; (lane 2) non-treated control cells extract; (lane 3) PDBu-treated (50 ng/ml, 28 h) cell extract. Symbols are the same as in Fig. 2.

other investigators [12,14,15]. Like the case of aortic strip stimulation with $PGF_{2\alpha}$ [7], the diphosphorylation occurred even at concentrations over 3 μM (Fig. 1). In contrast to evidence that multiple sites of smooth muscle MLC could be phosphorylated in vitro by both MLCK and protein kinase C with different rates and intensities of the enzyme activities [1-6,16], Colburn et al. proposed that the MLC in carbachol-stimulated intact tracheal smooth muscle may be diphosphorylated by MLCK at the serine and threonine residues but not by protein kinase C [14]. We made use of the protein kinase C inhibitor staurosporine (Str; prepared in Asahi Chem. Ind.) to determine which protein kinase is involved in the formation of diphosphorylation of MLC in SM-3 cells stimulated with $PGF_{2\alpha}$. Pretreatment of the SM-3 cells with Str (30 nM, 30 min) suppressed the formation of diphosphorylated MLC (Fig. 2B), which was significantly lower than that in the control cells ($5.7 \pm 2.3\%$ vs $20.1 \pm 6.2\%$, at 5 min). Treatment with Str reduced the basal level of MLC monophosphorylation ($28.7 \pm 3.4\%$ to $15.4 \pm 3.3\%$, at 0 min), and also 50 μM HA1077, a calcium antagonist [17], reduced the basal level to 5% or less (data not shown). However, it had little affect on the monophosphorylation activity ($31.5 \pm 5.7\%$ vs $42.9 \pm 0.4\%$, at 5 min; $26.6 \pm 5.4\%$ vs $37.7 \pm 2.5\%$, at 15 min). Treatment with high concentration of Str (50 nM, 30 min) reduced the monophosphorylation level of MLC ($42.9 \pm 0.4\%$ to $16.8 \pm 1.6\%$). These results suggest that there is MLC diphosphorylation pathway sensitive to Str treatment in SM-3 cells. We then examined whether or not phorbol ester-induced down-regulation of protein kinase C affects MLC diphosphorylation in the SM-3 cells. In the prolonged PDBu-treated (50 ng/ml, 28 h) SM-3 cells, $PGF_{2\alpha}$ induced MLC monophosphorylation to levels slightly lower than those seen in the control cells, but not significantly (Fig. 3A vs 2A). The PDBu-treatment lowered the basal level of monophosphorylation from $28.7 \pm 3.4\%$ to $21.9 \pm 1.7\%$. In contrast, the $PGF_{2\alpha}$-induced diphosphorylation was suppressed in the PDBu-treated cells, but not completely: $20.1 \pm 6.2\%$ to $8.7 \pm 2.4\%$. Immunoblot analyses with specific monoclonal antibodies against the type III isozyme were carried out to determine the amount of down-regulated protein kinase C. Fig. 3B shows that SM-3 cells contained the type III isozyme but not types I and II (data not shown), whereas the type III isozyme disappeared after a 28 h exposure to 50 ng/ml of PDBu. In this experiment, the PDBu-treatment did not completely suppress the diphosphorylation activity despite the complete disappearance of type III protein kinase C. The residual diphosphorylation activity may be due to an activity of down-regulation insensitive protein kinase C since the protein kinase C activity in the PDBu-treated rabbit aortic smooth muscle cells remained at the level of 30-40% of the control cells [10].

This report provides the first evidence suggesting that MLC diphosphorylation in intact smooth muscle cells may be regulated through a mechanism sensitive to protein kinase C down-regulation and to the protein kinase C inhibitor Str, and different from mechanisms regulating monophosphorylation.

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