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# PHOSPHORYLATION OF CARDIAC MYOSIN LIGHT CHAIN 2 BY PROTEIN KINASE C AND MYOSIN LIGHT CHAIN KINASE INCREASES $Ca^{2+}$-STIMULATED ACTOMYOSIN MgATPase ACTIVITY

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**SUMMARY:** Myosin light chain 2 (MLC2) phosphorylation in rat cardiac whole myosin by cardiac myosin light chain kinase (MLCK) or by protein kinase C (PKC) resulted in increased actin-stimulated myosin MgATPase activity. The phosphorylation also increased $Ca^{2+}$-stimulated myofibrillar MgATPase activity upon substitution of the phosphorylated myosin into myofibrils. In addition, phosphorylation of MLC2 in myofibrils by MLCK increased both the $Ca^{2+}$-sensitivity and maximum activity of the myofibrillar $Ca^{2+}$-stimulated MgATPase activity. The latter effect was inhibited by PKC-phosphorylation of troponin I, troponin T and C-protein. A role for both PKC and MLCK in regulating cardiac myofibrillar activity, *via* phosphorylation of various contractile proteins, is indicated. 

The 19,000-Da regulatory light chain of cardiac myosin (MLC2) can be phosphorylated *in vitro* by $Ca^{2+}$/calmodulin-dependent myosin light chain kinase (MLCK) (1,2). Recently, a potential role for the phosphorylation of cardiac MLC2, like its counterpart in skeletal muscle (3,4), in the regulation of cardiac function and contractility has become apparent (5-8). For example, MLC2 phosphorylation, by addition of MLCK to detergent-skinned ventricular and atrial muscle fibers, has resulted in increased $Ca^{2+}$-sensitivity of force development and MgATPase activity (5-8), an effect apparently enhanced by protein kinase C (PKC) (8). In contrast, PKC phosphorylation of the thin filament regulatory proteins troponin I (TnI) and/or troponin T (TnT) has been shown to inhibit the $Ca^{2+}$-stimulated MgATPase activity of reconstituted actomyosin (9-11). This effect is likely responsible for inhibition of myofibrillar $Ca^{2+}$-stimulated MgATPase activity following phosphorylation of the myofibrillar proteins by PKC *in vitro* (11) and through activation of PKC in intact cardiomyocytes (12). In this paper we show that phosphorylation of MLC2 by PKC or MLCK increases actomyosin MgATPase activity, implicating a role for both PKC and MLCK in regulating myofibrillar activity, in part, *via* MLC2 phosphorylation.

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## MATERIALS AND METHODS

*Materials*: Phosphatidylserine (PS), *sn*-1,2 diolein, leupeptin, pepstatin, and aprotinin were purchased from Sigma; Calyculin A was from LC Services Corp.; [$\gamma$-$^{32}$P]ATP was from ICN Radiochemicals; fresh bovine and porcine hearts and porcine brains were from local slaughterhouses.

*Preparation and phosphorylation of cardiac myosin and myofibrils*: Cardiac myofibrils were prepared from adult rat and bovine hearts using the Triton X-100 extraction procedure of Solaro *et al.* (13) as modified by Murphy and Solaro (14). Protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of leupeptin, 5 μg/ml of pepstatin A, and 5 μg/ml of aprotinin) were included in all solutions during the myofibril preparation. Myosin from the rat and bovine heart myofibrils was prepared according to the method of Siemankowski and White (15) as modified by Hartzell (16) and purified by chromatography on DEAE-Sephacel. Mixed cardiac myosin light chains were also prepared from the bovine myosin according to Perrie and Perry (17). PKC was purified brom porcine brain as described previously (18) and presumably represents a mixture of PKC isozymes found in brain (19). MLCK was purified from porcine heart by the method of Walsh *et al.* (20). Phosphorylation of rat cardiac myofibrils and myosin was achieved by incubation of myofibrils (800 μg) or myosin (1.6 mg) at 30°C in a reaction mixture (1.2 ml) containing 50 mM Tris-HCl (pH 7.5), 30 mM 2-mercaptoethanol, 0.9 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 mM EGTA, 1.0 mM ATP, 0.1 μM of the phosphoprotein phosphatase inhibitor Calyculin A (21), and 50 mM KCl (for myofibrils) or 0.3 M KCl (for myosin). To these mixtures, PKC (40 μg) with PS (30 μg) and diolein (2.4 μg) and/or MLCK (80 μg) with 1 μM calmodulin or heat-inactivated enzymes were added and the incubations carried out for 1 h with additional 1 mM ATP added after 30 min (for myofibrils). Phosphorylation of the myofibrils was terminated by dilution of the mixture in 4 volumes of resuspension buffer (20 mM imidazole/HCl, pH 7.0, containing 50 mM KCl, 20 mM NaF, 0.5 mM EGTA and 0.5 mM dithiothreitol) and centrifugation at 1,100 x g for 10 min at 4°C. The myofibrils were taken up (1 mg/ml) in the resuspension buffer. Incubations with myosin were terminated by 16-h dialysis of the mixture against 1 mM imidazole (pH 7.0), 1.0 mM dithiothreitol, and 10 mM NaF at 4°C. After dialysis, the precipitated myosin was resuspended (4 mg/ml) in dialysis buffer containing 0.6 M KCl. Parallel incubations (0.1 ml) containing [$\gamma$-$^{32}$P]ATP (300 cpm/pmol) were carried out and terminated by addition of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, followed by SDS-PAGE (on 6-15% gradients) and autoradiography (10).

*$Ca^{2+}$-stimulated myofibril and actin-stimulated myosin MgATPase assays*: The enzyme activities were measured according to previously described methods (9,10). Kinetic data for the $Ca^{2+}$-stimulated MgATPase activity of myofibrils were calculated by non-linear least-squares regression analysis using a modified form of the Hill equation:

$$v = V_{max} \cdot [Ca^{2+}]^n/(EC_{50}{}^n + [Ca^{2+}]^n),$$

where v and Vmax represent the observed MgATPase activity and its maximum activity, respectively; $EC_{50}$ is the concentration of $Ca^{2+}$ causing 50% maximum activity; and n is the Hill coefficient, a measure of positive cooperativity. Kinetic constants for phosphorylation of MLC2 and for actin stimulation of myosin MgATPase activity were determined by non-linear least -squares regression analysis of the data, using the Michaelis-Menton equation. All values reported in the text are means ± SEM. Statistical analysis was by paired Student's t tests.

## RESULTS AND DISCUSSION

Incubation of isolated cardiac myofibrils from adult rats with MLCK resulted in the selective phosphorylation of MLC2, with the incorporation of 0.5 mol of phosphate/mol after 60

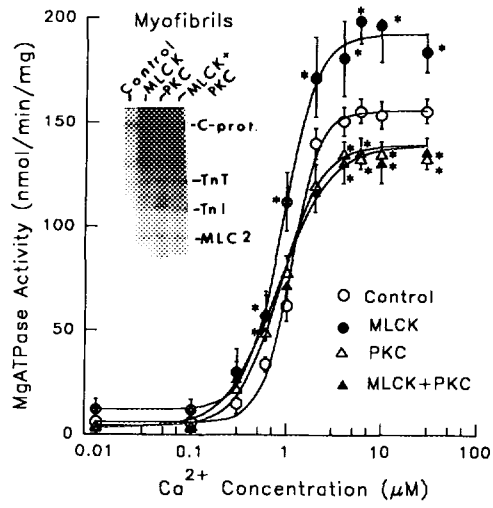


**Figure 1.** Effect of phosphorylation of rat cardiac myofibrillar proteins by MLCK and/or PKC on myofibrillar $Ca^{2+}$-stimulated MgATPase activity. Adult rat myofibrils were incubated with MLCK in the presence of calmodulin and $Ca^{2+}$, with PKC in the presence of PS, diolein and $Ca^{2+}$ or with heat denatured enzyme (control) for 60 min. The MgATPase activity of the myofibrils (50 μg) was assayed in the presence of various $Ca^{2+}$ concentrations (up to 30 μM) as described in "Materials and Methods" and the $Ca^{2+}$-independent activity (30 nmol/min/mg) was subtracted from each data point. Values are means ± SEM from 5 experiments; * denotes significance from control ($p<0.05$). Lines drawn through the data points in this figure and **Fig. 3** were calculated by non-linear least-squares regression using the Hill equation. A typical autoradiogram of a gel from SDS-PAGE of phosphorylated myofibrillar proteins from parallel incubations containing [$\gamma$-$^{32}$P]ATP is also shown (*inset*).

min (Fig. 1, *inset*). As a result, the $Ca^{2+}$ sensitivity and the maximal $Ca^{2+}$-stimulated MgATPase activity of the myofibrils were increased (Fig. 1). The $EC_{50}$ for $Ca^{2+}$ decreased from 1.12 ± 0.05 to 0.90 ± 0.04 μM, while the $V_{max}$ of the enzyme activity increased from 160 ± 3 to 193 ± 4 nmol/min/mg upon phosphorylation of the myofibrils by MLCK. Phosphorylation of the intact myofibrils by PKC was more extensive and resulted in the incorporation of phosphate into C-protein (1.2 mol/mol), TnT (1.0 mol/mol), TnI (0.8 mol/mol), and MLC2 (0.5 mol/mol). MLC2 phosphorylation was complete by 60 min for both enzymes as was phosphorylation of TnI, TnT, and C protein by PKC (data not shown). In contrast to the effects of specific MLC2 phosphorylation by MLCK, phosphorylation of a number of the myofibrillar proteins by PKC caused a decrease (to 140 ± 4 nmol/min/mg) in the overall maximum $Ca^{2+}$-stimulated MgATPase activity (Fig. 1), consistent with our previous reports on myofibrils and reconstituted actomyosin complex (9-11). Furthermore, the combined effects of phosphorylation by both enzymes resulted in decreased maximal $Ca^{2+}$-stimulated MgATPase activity (to 139 ± 6 nmol/min/mg), suggesting that the inhibitory effect of TnI and TnT phosphorylation (by PKC) could overcome the stimulatory effect of MLC2 phosphorylation (by MLCK and PKC).

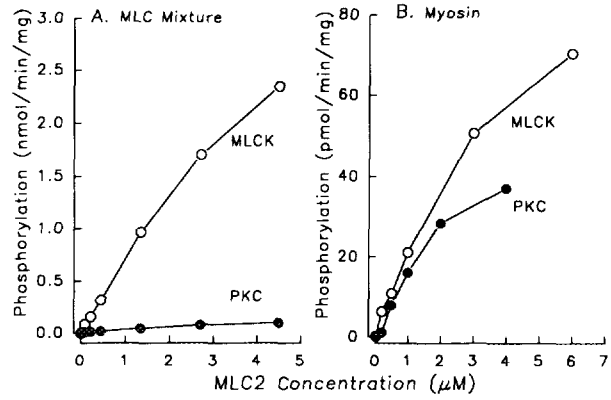


**Figure 2.** Phosphorylation of MLC2 in mixed cardiac myosin light chains and whole myosin by PKC or MLCK. Bovine cardiac mixed myosin light chains (**A**) or adult rat cardiac whole myosin (**B**) were incubated with $Ca^{2+}$, and either PKC (in the presence of PS, diolein, and 50 μM [γ-$^{32}$P]ATP) or with MLCK (in the presence of calmodulin and 1 mM [γ-$^{32}$P]ATP) for 10 min, using the phosphorylation conditions described for myofibrils in "Materials and Methods". Incorporation of $^{32}$P into MLC2 was quantitated by SDS-PAGE of the samples, excision of the bands corresponding to MLC2 from the stained gels, and scintillation counting of the radioactivity. Values are representative of two experiments.

In order to determine the effects of MLC2 phosphorylation by PKC, we investigated the possibility that MLC2, in a mixed light chain preparation or in whole myosin, could be phosphorylated by PKC and then the phosphorylated preparations substituted back into myofibrils. MLC2 in mixed light chains isolated from bovine cardiac whole myosin was not significantly phosphorylated by PKC, whereas MLCK effectively phosphorylated the protein ($K_m$ = 40 μM, $V_{max}$ = 4.1 nmol/min/mg) with maximal incorporation of phosphate to 0.6 mol/mol (Fig. 2A). Nevertheless, both MLCK ($K_m$ = 3.4 μM, $V_{max}$= 128 pmol/min/mg) and PKC ($K_m$ = 4.8 μM, $V_{max}$ = 70 pmol/min/mg) phosphorylated MLC2 present in whole myosin (Fig. 2B). This suggested that a conformational change in MLC2 occurs when it is bound to the myosin heavy chain and is required to expose the PKC phosphorylation sites. Recent work in our laboratory has shown that PKC and MLCK phosphorylate similar sites in cardiac MLC2 in intact myocytes or isolated myofibrils (R.C. Venema and J.F. Kuo, in preparation). Competitive displacement (14,22) of unphosphorylated myosin from isolated myofibrils with excess phosphorylated myosin revealed that phosphorylation of MLC2 by MLCK and/or PKC increased the maximal $Ca^{2+}$-stimulated MgATPase activity, from 84 ± 4 to 104 ± 5 nmol/min/mg, without significantly altering its $Ca^{2+}$ sensitivity (Fig. 3). The excess myosin probably accounted for the lack of any effect of MLC2 phosphorylation on the $Ca^{2+}$ sensitivity of the MgATPase activity, under the experimental conditions.

Since the observed effect of specific MLC2 phosphorylation by PKC and MLCK was to increase the maximum $Ca^{2+}$-stimulated MgATPase activity, we hypothesized that this was due

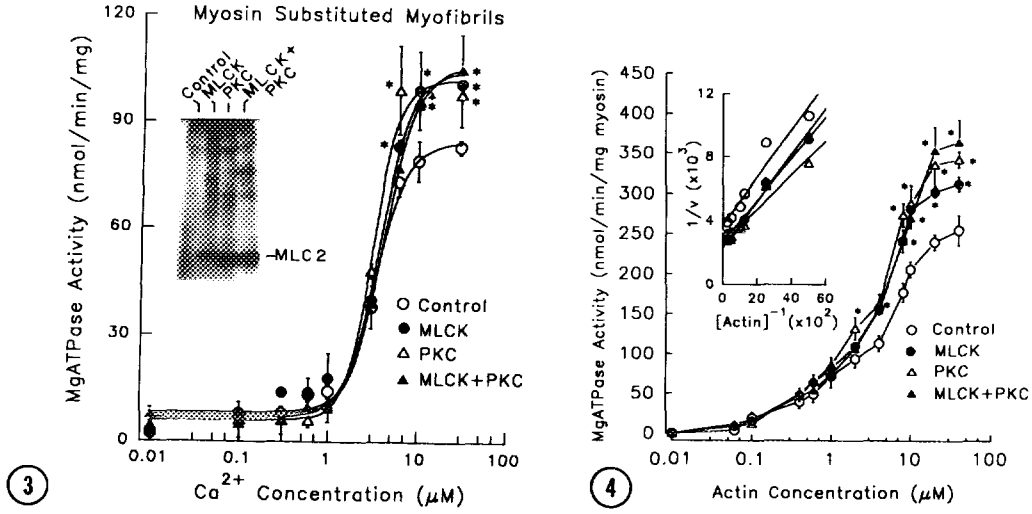


**Figure 3.** Effect of cardiac myosin containing MLCK- and/or PKC-phosphorylated MLC2 on the $Ca^{2+}$-stimulation of the MgATPase activity of myosin-substituted myofibrils. Rat cardiac myosin was incubated with MLCK and/or PKC for 60 min. The phosphorylated or unphosphorylated (control) myosin was then mixed with myofibrils in 0.6 M KCl at a molar ratio of exogenous myosin to myofibrillar myosin equal to 5:1, in order to competitively displace the endogenous myosin. Myofibrillar $Ca^{2+}$-stimulated MgATPase activity was assayed as in **Fig. 1** and the $Ca^{2+}$-independent MgATPase activity (50 nmol/min/mg) was subtracted from all data points. Values are means ± SEM from 4 experiments; * denotes significance from control ($p<0.05$). Autoradiogram showing phosphorylation of MLC2 in whole myosin by PKC and MLCK (*inset*).

**Figure 4.** Actin-stimulated MgATPase activity of cardiac myosin phosphorylated by MLCK and/or PKC. Unphosphorylated (control) or phosphorylated myosin was mixed with various concentrations (up to 40 μM) of actin and the actin-stimulated MgATPase activity was assayed. Enzyme activity due to myosin alone (30 nmol/min/mg) was subtracted from all data points. Values are means ± SEM from 3 experiments; * denotes significance from control ($p< 0.05$). A double-reciprocal plot of the data is also shown (*inset*).

to an enhanced direct interaction of the phosphorylated myosin heads with actin. To test this, we measured the MgATPase activity of whole myosin as a function of actin concentration. Phosphorylation of MLC2 in cardiac myosin increased the $V_{max}$ of the actin-stimulated MgATPase activity from 282 ± 13 (control) to 371 ± 15, 403 ± 23 and 444 ± 23 nmol/min/mg for the MLCK-, PKC- and MLCK plus PKC-phosphorylated myosin preparations, respectively, with little or no change in the affinity ($EC_{50}$ for actin of 4.1 ± 0.5 μM) of the enzyme for actin (Fig. 4). This indicated that the phosphorylated regulatory light chain allowed myosin to interact more rapidly with actin, perhaps by increasing the rate of attachment and/or detachment of the myosin heads to and from actin (4-7). Others have observed that MLCK phosphorylation of MLC2 in skeletal muscle myosin resulted in an increase in the affinity of myosin for actin (23,24). It is possible that our observation of only an increase in the $V_{max}$ for actin activation of the myosin MgATPase activity could be accounted for by differences between cardiac and skeletal myosins (25,26) and the fact that we purified myosin devoid of C protein (16).

The net effects of MLC2 phosphorylation in myofibrils by MLCK were increased $Ca^{2+}$-sensitivity and maximum activity of the myofibrillar $Ca^{2+}$-stimulated MgATPase activity. These results are consistent with those of others (5-8) who have hypothesized that the phosphorylation of cardiac MLC2, like that in skeletal muscle (3,4), allows increased rate of attachment of myosin heads to actin and ultimately $Ca^{2+}$-sensitivity of isometric force. What is an important and new observation here is that PKC can phosphorylate MLC2 in cardiac myosin or myofibrils and activate the MgATPase activity in a manner similar to that of MLCK. Therefore, the inhibitory effects of PKC phosphorylation of TnI and TnT (9-11) and perhaps C protein (this paper and ref. 12) and the stimulatory effects of PKC phosphorylation of MLC2 on MgATPase activity, by acting in a concerted manner, might account, in part, for the observed negative (27-29) and positive (29) inotropic effects of phorbol esters (PKC activators) on various heart preparations.

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## REFERENCES

1. Solaro, R.J. (1986) Protein Phosphorylation in Heart Muscle, CRC Press, Boca Raton.
2. Shabb, J.B. and Corbin, J.D. (1991) In the Heart and Cardiovascular System, Second Edition (H.A. Fozzard, E. Haver, R.B. Jennings, A.M. Katz and H.E. Morgan, Eds.) pp 1539-1562, Raven Press, Ltd., New York.
3. Sweeney, H.L. and Stull, J.T. (1986) Am. J. Physiol. 250, C657-C660.
4. Metzger, J.M., Greaser, M.L and Moss, R.L. (1989) J. Gen. Physiol. 93, 855-883.
5. Morano, I., Hofmann, F., Zimmer, M. and Rüegg J.C. (1985) FEBS Lett. 189, 221-224.
6. Morano, I., Bächle-Stolz, C., Katus, A. and Rüegg, J.C. (1988) Basic. Res. Cardiol. 83, 350-359.
7. Morano, I., Rösch, J., Arner, A. and Rüegg, J.C. (1990) J. Mol. Cell. Cardiol. 22, 805-813.
8. Clement, O., Puceat, M., Walsh, M.P. and Vassort, G. (1992) Biochem. J. 285, 311-317.
9. Noland Jr., T.A. and Kuo, J.F. (1991) J. Biol. Chem. 266, 4974-4978.
10. Noland Jr., T.A. and Kuo, J.F. (1992) Biochem. J. 288, 123-129.
11. Noland Jr., T.A. and Kuo, J.F. (1993) J. Mol. Cell. Cardiol. 25, 53-65.
12. Venema, R.C. and Kuo, J.F. (1993) J. Biol. Chem. 268, 2705-2711.
13. Solaro, R.J., Pang, D.C. and Briggs, F.N. (1971) Biochim. Biophys Acta 245, 259-262.
14. Murphy, A.M. and Solaro, R.J. (1990) Pediatr. Res. 28, 46-49.
15. Siemankowski, R.F. and White H.D. (1984) J. Biol. Chem. 259, 5045-5053.
16. Hartzell, H.C. (1985) J. Mol. Biology 186, 185-195.
17. Perrie, W.T. and Perry, S.V. (1970) Biochem J. 119, 31-38.
18. Girard, P.R. Mazzei, G.J., and Kuo J.F. (1985) J. Biol. Chem 261 , 370-375.
19. Huang, K.-P., Nakabayashi, H. and Huang, F.L. (1986) Proc. Natl. Acad. Sci USA 83, 8535-8539.
20. Walsh, M.P., Vallet, B., Autric, F. and Demaille, J.G. (1979) J. Biol. Chem. 254, 12136-12144.

21. Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato Y., Fusetani, N., Natabe, S., Hashimoto, K., Uemura, D. and Hartshshorne, D.J. (1989) Biochem. Biophys. Res. Commun. 159, 871-877.
22. Lehman, W. and Szent-Györgyi A.G. (1975) J. Gen. Physiol. 66,1-30.
23. Pemrick, S.M. (1980) J. Biol. Chem. 255, 8836-8841.
24. Persechini, A., Stull, J.T. and Cooke, R. (1985) J. Biol. Chem. 260, 7951-7954.
25. Margossian, S.S. (1985) J. Biol. Chem. 260, 13747-13754.
26. Ford, L.E. (1991) Circ. Res. 68, 621-637.
27. Gwathmey, J.K. and Hajjar, R.J. (1990) Circ. Res. 67,744-752.
28. Watson, J.E. and Karmazyn, M. (1991) Circ. Res. 69, 1114-1131.
29. Ward, C.A. and Moffat, M.P. (1992) J. Mol. Cell. Cardiol. 24, 937-948.