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THE MODULATORY ROLE OF MYOSIN LIGHT CHAIN PHOSPHORYLATION IN HUMAN PLATELET ACTIVATION

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Myosin 20 K-Da light chain phosphorylation in human platelets was found to be catalyzed by MLCK in the early phase during collagen activation. The effect of newly synthesized selective inhibitor of MLCK, ML-9, on collagen induced platelet activation was investigated. ML-9 delayed the time course of the myosin 20 K-Da light chain phosphorylation, sequentially led to a delay in aggregation, secretion and phosphorylation of the 40K-Da peptide, in a dose-dependent fashion. It is proposed that the MLCK catalyzed phosphorylation of myosin 20 K-Da light chain may be an initial response and if so may influence the sequent reactions in the activation of platelets with collagen.

It has been reported that Ca^{2+}-dependent phosphorylation of various platelet proteins plays a major role in the regulation of platelet function (1-9). Biological stimuli such as thrombin or collagen induce the incorporation of ^{32}P into two endogenous proteins having approximate molecular weights of 40 K and 20 K-dalton (1,2). These studies led to the idea that Ca^{2+}-dependent phosphorylation of these 40K and 20 K-dalton proteins may be required for the secretion of platelet granule constituents such as serotonin (1-5). The 20 K-dalton peptide has been identified as the 20K-dalton light chain of platelet myosin, and to be phosphorylated by both Ca^{2+}-calmodulin-dependent protein kinase (MLCK) (7-9) and Ca^{2+}-activated phospholipid dependent protein kinase (PK-C)(10). The

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The abbreviations used are: MLC, myosin light chain; MLCK, myosin light chain kinase; PK-A, cAMP dependent protein kinase; PK-C, Ca^{2+}-activated, phospholipid-dependent protein kinase, EGTA, ethylene glycol bis (β-aminoethyl ester)-N,N,N',N'-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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40K-Da peptide, phosphorylated by PK-C, was purified and characterized, but the crucial role of this peptide phosphorylation has remained obscure (11,12). On the other hand, "calmodulin antagonists" have been used as pharmacological tools to facilitate the function of Ca^{2+}-calmodulin mediated systems in vivo and in vitro (9,13). In many tissues including human platelets, calmodulin exerted a pleiotropic effect on many cellular function, therefore, in vivo study "calmodulin antagonists" were not adequate to elucidate the role of the MLCK mediated pathway. Therefore, we decided to synthesize a potent and direct inhibitor of MLCK. ML-9, a newly synthesized compound, exhibited a selective inhibition toward MLCK not involving calmodulin. In this communication, we report evidence that the Ca^{2+}-calmodulin mediated myosin 20K-Da light chain phosphorylation may be an initial response of a series of reactions in platelets induced by collagen, and also describe effects of ML-9 on several enzymes, in vitro.

MATERIALS AND METHODS

Enzyme assays and other determinations were performed under the conditions described by Hidaka et al. (14). MLCK was treated with trypsin as described by Tanaka et al. (15). MLCK, PK-A and PK-C were prepared from fresh human platelets. Human blood was collected from healthy volunteers not ingesting any drugs for at least 1 week. MLCK was purified by CaM affinity chromatography following ion exchange chromatography on DEAE cellulose described by Hathaway and Adelstein (8). PK-C was partially purified by modification of the method described by Inoue et al. (16). PK-A was purified by modification of the method described by Beavo et al. (17). Human platelets suspended in Hepes buffered saline (145 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 10 mM Hepes, 5 mM glucose, pH 7.4 at 37°C) were incubated for 60 min at 30°C with either 0.5 mCi ml^{-1} of [^{32}P] orthophosphate (New England Nuclear) or 0.5 μCi ml^{-1} of [^{14}C]serotonin (Amersham), spun at 600 xg for 5 min and the supernatant discarded. The pellets were resuspended in the same buffer to give a platelet count of 5-10 x 10^8/ml. Platelet aggregation was studied turbidimetrically using a Rikadenki 4 channel Aggregometer (RAM41). The standard platelet reaction mixtures consisted of 0.45 ml of labelled platelet suspension, 0.05 ml of saline (control) or ML-9 (10,20 and 50 μM) and collagen (1 μg ml^{-1}) or TPA (20, 50 ng ml^{-1}). The platelets were preincubated with saline or ML-9 in the aggregometer (Rikadenki) for 1 min before stimulation with collagen or TPA. Secretion and protein phosphorylation studies were carried out simultaneously at 37°C. Serotonin release was measured as described by Costa and Murphy (18), and protein phosphorylation was analyzed by SDS-PAGE and alkaline-urea-PAGE under the conditions described by Laemmli (19) and Perrie and Perry (20) respectively, followed by autoradiography and densitometric tracing on the autoradiogram. Tryptic peptide mapping was carried out as described (10,21). The phosphorylation of platelet endogenous proteins was assayed in the reaction mixture (final 0.2 ml) containing 25 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 2 mM EGTA), 10 ng phosphatidylserine (or absence), 10 μM [γ-^{32}P]ATP, 0.001% leupeptin,

Fig. 1. Chemical structure of 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-9).

150 μg of platelet soluble proteins and various concentrations of ML-9 at 30°C for 3 min. Phosphoproteins were analyzed, as described by Kawamoto and Hidaka (22).

RESULTS AND DISCUSSION

Phosphorylation of myosin 20K-Da light chain catalyzed by MLCK can play a central role in the major regulatory system of the contractile proteins in platelets (1-9). We have investigated the role of this myosin 20 K-Da light chain phosphorylation, using synthetic compounds, W-7 as a calmodulin antagonist (9) and H-7 as a selective and potent inhibitor of PK-C (23). A newly synthesized selective inhibitor of MLCK, ML-9, was examined the effect on MLCK activity in vitro and on activation of human platelets with collagen. The chemical structure of ML-9, 1-(5-chloro-naphthalene-1-sulfonyl)-1H-hexahydro-1, 4-diazepine, is shown in Fig. 1. The effects of ML-9 on several enzymes are shown in Table 1. ML-9 exhibited a selective inhibition toward MLCK. The apparent Ki values were 3.8, 32 and 54 μM for MLCK, PK-A and PK-C, respectively. ML-9 also inhibited Ca^{2+}-independent activity of MLCK (trypsin treated MLCK), at a similar concentration. Kinetic analysis of the double-reciprocal curves revealed that the inhibition of MLCK produced by ML-9 was competitive with respect to ATP and noncompetitive with respect to the phosphate acceptor

Table 1
Effect of ML-9 on various enzymes

Enzymes	Ki (μM)
MLC kinase + Ca^{2+}-CaM	3.8
trypsin treated + EGTA	3.8
cAMP-dependent protein kinase	32
protein kinase C	54

(data not shown). These data indicate that ML-9 is a direct and selective inhibitor of MLCK. To examine the effect of ML-9 on the release reaction and protein phosphorylation, washed human platelets were preincubated with ^{32}P-orthophosphate or ^{14}C-serotonin and treated with collagen (1 μg ml^{-1}). The suspension of platelets was stirred.

When human platelets were stimulated with collagen, myosin 20 K-Da light chain phosphorylation was rapid and appeared to precede the shape change. Secretion, aggregation and phosphorylation of the 40 K-Da peptide followed. Two-dimensional peptide mapping of this phosphorylated myosin 20 K-Da light chain induced by collagen, following tryptic digestion, revealed that MLCK phosphorylated this myosin 20 K-Da light chain and the site phosphorylated by protein kinase C was not seen in the early phase during collagen activation (Fig. 2). We also investigated the effect of ML-9 on collagen induced activation of human platelets. ML-9 delayed the time course of the myosin 20 K-Da light chain phosphorylation, in a dose-dependent manner. We also observed delays in platelet aggregation, serotonin secretion and phosphorylation of the 40 K-Da peptide (Fig. 3). There was also a good temporal correlation among shape change, myosin 20 K-Da light chain phosphorylation and secretion, as described by Daniel et al. (6). ML-9 did not affect protein phosphorylation and secretion in

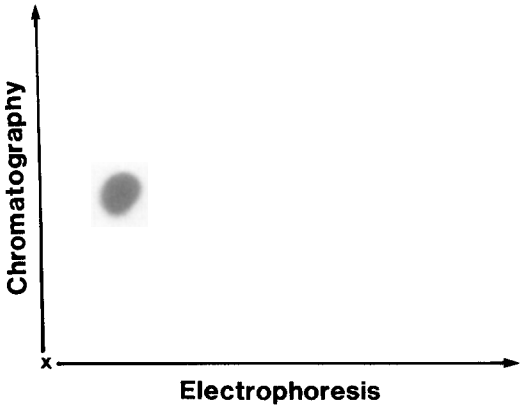

Fig. 2. The autoradiograph of two-dimensional peptide mapping of tryptic peptides of the ^{32}P labelled myosin 20 K-Da light chain in response to collagen (1 μg ml^{-1}) at 60s.

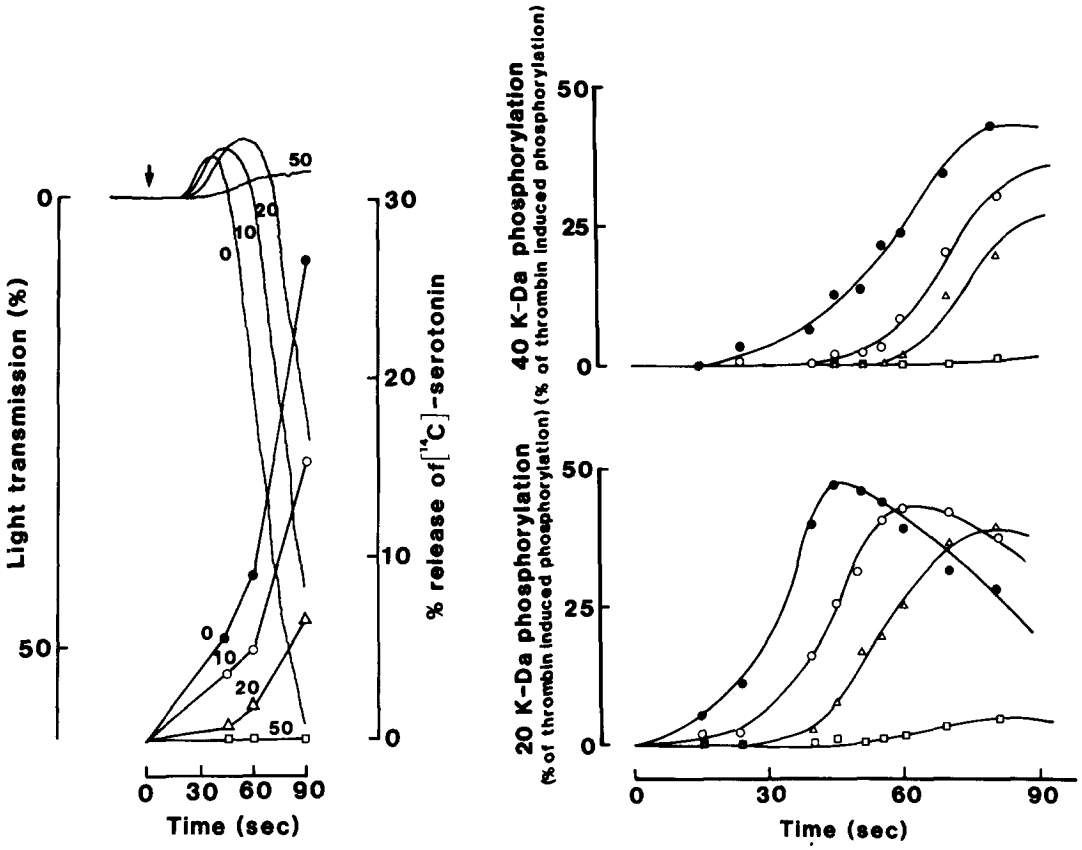

Fig. 3. Effect of ML-9 on platelet aggregation, secretion and protein phosphorylation in response to collagen.
Time course of platelet aggregation and [^{14}C]serotonin release (left), and the phosphorylation of 40 K-Da peptide and myosin 20 K-Da light chain (right) of washed human platelets prelabelled with [^{14}C]-serotonin or [^{32}P]orthophosphate in response to collagen (1 $\mu g\ ml^{-1}$) and the effect of ML-9 (the final concentration as indicated) preincubation as indicated under "Experimental Procedure". Closed circles, control; open circles, ML-9 10 μM; open triangle, ML-9 20 μM; open squares, ML-9 50 μM. Data for the phosphorylation of 40 K-Da peptide were represented relative to responses induced by 0.2 U ml^{-1} of thrombin at 40s. The phosphorylation of 20 K-Da light chain was analyzed for per cent phosphorylation on alkaline-urea PAGE as described in "Experimental Procedure".

unstimulated platelets (data not shown). We also examined the effect of ML-9 on the 40 K-Da peptide phosphorylation induced by the other agonist to clarify that the delay in the 40 K-Da peptide phosphorylation is not due to the direct effect of ML-9 on PK-C. As shown in Fig. 4, ML-9 did not inhibit the phosphorylation of either 40 K and 20 K-Da peptides induced by TPA, in the same dose range of ML-9. The effect of ML-9 was also investigated on calcium-dependent phosphorylation of myosin 20 K-Da light

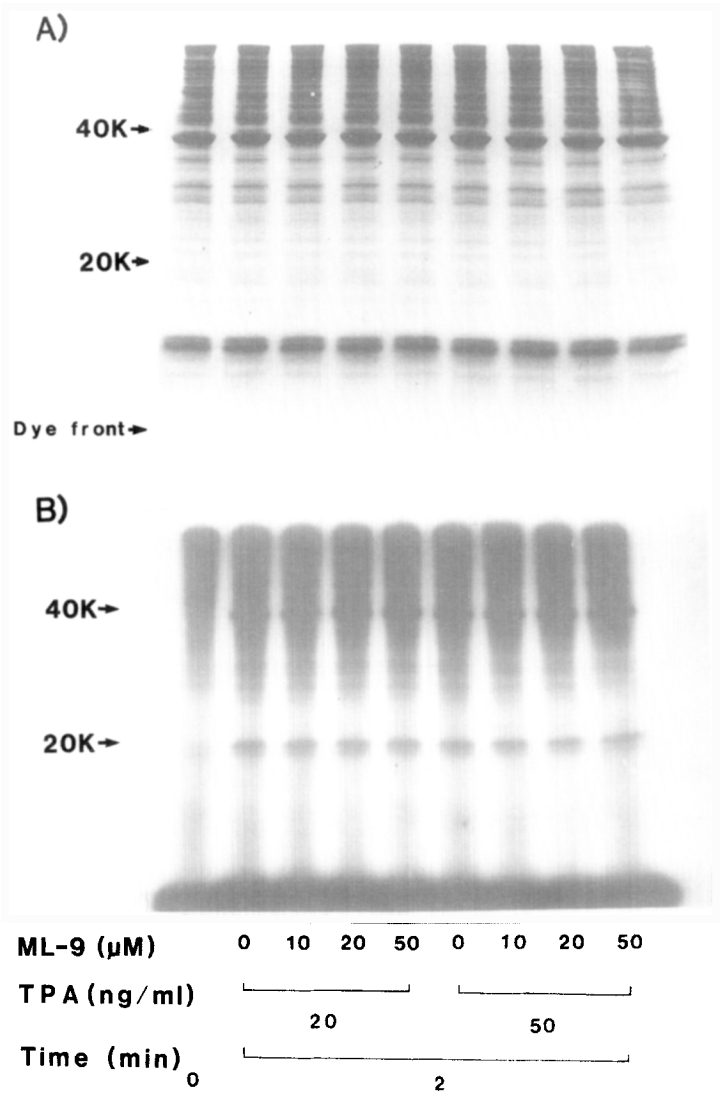

Fig. 4. Effect of ML-9 on platelet endogenous phosphorylation in response to TPA (20, 50 ng ml^{-1}) at 120S.
A) The Coomassie blue staining of the slab gels
B) The autoradiograph of the slab gels
Other conditions were as described in "Experimental Procedures"

chain and calcium-phospholipid-dependent phosphorylation of 40 K-Da peptide in a crude cell-free system (Fig. 5), using the 105,000 x g supernatant of the sonicated platelets, as enzyme and substrate sources. Phosphorylation of myosin 20 K-Da light chain occurred in the presence of calcium ion (100 μM), and the addition of EGTA to 2 mM inhibited this phosphorylation, thereby indicating that the myosin 20 K-Da light chain phosphorylation depends on calcium ion (8,9). Increasing the concentration of ML-9

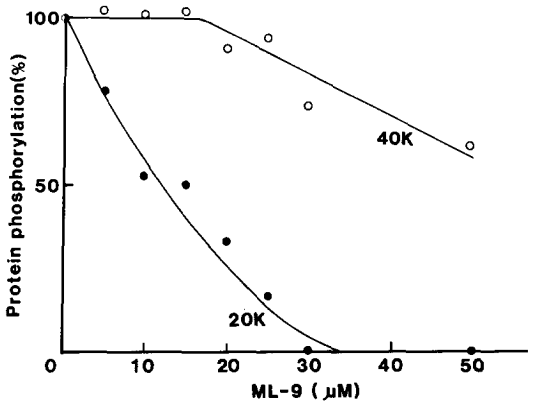

Fig. 5. Effect of ML-9 on the Ca^{2+}-dependent phosphorylation of myosin 20 K-Da light chain and Ca^{2+}-phospholipid-dependent phosphorylation of 40 K-Da peptide in vitro. Open symbols, 40 K-Da peptide phosphorylation, closed symbols, myosin 20 K-Da light chain phosphorylation. The data are expressed as a percentage of the control.

inhibited selectively the Ca^{2+}-dependent phosphorylation of this myosin 20 K-Da light chain with an IC_{50} value of 12 μM, as shown in Fig. 4. However, ML-9 produced little inhibition of Ca^{2+}-phospholipid-dependent phosphorylation of 40 K-Da peptide, in doses up to 50 μM. At higher concentrations of ML-9, ML-9 exhibited the pharmacological effect of a calmodulin antagonist, but at the concentrations used in the present work, ML-9 showed a potent and selective inhibition of MLCK. These findings suggest that myosin 20 K-Da light chain phosphorylation catalyzed by MLCK may be a key event in a series of reactions, such as aggregation, secretion and phosphorylation of the 40K-Da peptide, in case of activation of platelets with collagen. ML-9 is a novel inhibitor of MLCK and will aid in elucidating the function of MLCK mediated pathway in specific cellular processes.

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REFERENCES

1. Haslam, R.J. and Lynham, J.A. (1977) Biochem. Biophys. Res. Commun. 77, 714-722
2. Haslam, R.J., Lynham, J.A. and Fox, J.E.B. (1979) Biochem. J. 178, 397-406

3. Wallace, W.C. and Bensusan, H.B. (1980) J. Biol. Chem. 255, 1932-1937
4. Lyons, R.M. and Shaw, J.O. (1980) J. Clin. Invest. 65, 242-255
5. Bennet, W.F., Belville, J.S. and Lynch G. (1979) Cell 18, 1015-1023
6. Daniel, J.L., Molish, I.R., Rigmaiden, M. and Stewart, G. (1984) J. Biol. Chem. 259, 9826-9831
7. Daniel, J.L., Holmsen, H. and Adelstein, R.S. (1977) Thrombos Haemostas. 38, 984-989
8. Hathaway, D.R. and Adelstein, R.S. (1979) Proc. Natl. Acad. Sci. USA 76, 1653-1657
9. Nishikawa, M., Tanaka, T. and Hidaka, H. (1980) Nature 287, 863-865
10. Naka, M., Nishikawa, M., Adelstein, R.S. and Hidaka, H. (1983) Nature (Lond.) 306, 490-492
11. Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 97, 309-317
12. Imaoka, T., Lynham, J.A. and Haslam, R.J. (1983) J. Biol. Chem. 258, 11404-11414
13. Asano, M., Tanaka, T. and Hidaka, H. (1985) in Calmodulin Antagonists and Cellular Physiology (Hidaka, H. and Hartshorne, D.J., eds) pp 261-272, Academic Press, New York
14. Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036-5041
15. Tanaka, T., Naka, M. and Hidaka, H. (1980) Biochem. Biophys. Res. Commun. 92, 313-318
16. Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610-7616
17. Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Methods Enzymol. 38, 299-308
18. Costa, J.L. and Murphy, D.L. (1975) Nature 255, 407-408
19. Laemmli, U.K. (1970) Nature 227, 680-685
20. Perrie, W.T. and Perry, S.V. (1970) Biochem. J. 119, 31-38
21. Yamaguchi, M., Tanabe, K., Taguchi, Y.N., Nishizawa, M., Takahashi, T. and Matsukage, A. (1980) J. Biol. Chem. 255, 9942-9948
22. Kawamoto, S. and Hidaka, H. (1982) Biochem. Biophys. Res. Commun. 109, 1129-1133
23. Inagaki, M., Kawamoto, S. and Hidaka, H. (1984) J. Biol. Chem. 259, 14321-14323