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Selective Inhibition of Collagen-Induced Arachidonic Acid Liberation by 1-(5-Iodonaphthalene-1-Sulphonyl)-1H-Hexahydro-1,4-Diazepine Hydrochloride (ML-7), a Myosin Light Chain Kinase Inhibitor, in Washed Rabbit Platelets

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ABSTRACT. Effects of myosin light chain (MLC) kinase inhibitor ML-7 [1-(5-iodonaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine hydrochloride] and protein kinase C inhibitor H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydro-chloride] on collagen-induced platelet activation were investigated in washed rabbit platelets. Upon stimulation with collagen (1 μg/mL), H-7 decreased protein kinase C-mediated pleckstrin phosphorylation, but had no inhibitory effect on thromboxane (TX) A_2 formation or platelet aggregation. In contrast, ML-7 produced a concentration-dependent inhibition of the collagen-induced platelet aggregation and TXA_2 formation by preventing arachidonic acid (AA) liberation from membrane phospholipids. However, ML-7 had little effect on AA liberation induced by thrombin, Ca^{2+} ionophore A-23187 or melittin, suggesting that ML-7 may affect the signal transduction pathway specific for collagen-induced AA liberation, without direct inhibition of phospholipase A_2 activity. In indomethacin-treated platelets, collagen caused MLC phosphorylation and AA liberation in the absence of a significant increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or protein tyrosine phosphorylation. ML-7 inhibited both MLC phosphorylation and AA liberation induced by collagen in indomethacin-treated platelets. These results demonstrate that MLC phosphorylation and AA liberation are early events detectable in collagen-stimulated platelets, and suggest that ML-7 inhibits these early steps of collagen-induced signal transduction pathway in rabbit platelets. BIOCHEM PHARMACOL **54**;9: 1019–1026, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. collagen; rabbit platelet; phospholipase A_2; myosin light chain kinase; ML-7

The interaction of circulating platelets with collagen fibers exposed at injured vascular bed is a primary event triggering hemostasis. Several lines of evidence indicate that collagen-induced platelet activation involves the stimulation of tyrosine phosphorylation of the Fc-receptor γ-chain and the subsequent activation of $p72^{syk}$ and phospholipase C-γ2 in a tyrosine phosphorylation-dependent manner [1–4]. These signaling mechanisms have been shown to be direct effects of collagen and are usually observed with a high concentration of collagen (above 10 μg/mL) in a manner insensitive to cyclooxygenase inhibitors [1–4]. Furthermore, these effects of collagen are resistant to an increase in cyclic AMP [5, 6]. In contrast, it is also well known that platelet activation induced by a low concentration of collagen (1–5 μg/mL) is essentially dependent upon endogenously generated TX‡A_2 [7]. Studies from this and other laboratories demonstrated that AA liberation from membrane phospholipids, a key event for the effect of a low concentration of collagen, occurs without significant activation of phospholipase C [8–10], and is inhibited by an increase in cyclic AMP [8, 11]. It is therefore likely that collagen activates platelet function through different mechanisms depending upon the concentration tested. From the clinical point of view, it is important to understand the mechanism underlying the cyclooxygenase-dependent platelet activation elicited by a low concentration of collagen, since a cyclooxygenase inhibitor such as aspirin has been widely utilized as an antiplatelet agent to prevent thrombosis.

Recent studies have suggested that AA liberation from membrane phospholipids in response to several receptor

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‡ *Abbreviations:* AA, arachidonic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; ML-7, 1-(5-iodonaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine hydrochloride; ML-9, 1-(5-chlornaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine hydrochloride; MLC, myosin light chain; PKI, protein kinasae inhibitors; TX, thromboxane.

agonists is mediated by an 85 kDa cytosolic form of phospholipase A_2 [12, 13]. It has recently been demonstrated that cytosolic phospholipase A_2 is also involved in collagen-induced AA liberation in human platelets [14]. However, little is known concerning the signal transduction mechanism of collagen-induced cytosolic phospholipase A_2 activation. We have previously shown that a significant MLC phosphorylation preceded AA liberation, pleckstrin phosphorylation and platelet aggregation in response to a low concentration (1 μg/mL) of collagen in washed rabbit platelets [8]. Recent studies have suggested that in addition to the regulation of contractile proteins in muscle and nonmuscle cells, MLC phosphorylation affects several physiological responses, including catecholamine release [15], carcinoma cell attachment [16], and cell differentiation [17, 18].

To assess the role of MLC phosphorylation in collagen-induced phospholipase A_2 activation, we have examined the effects of ML-7, an MLC kinase inhibitor, on collagen-induced aggregation, AA liberation and protein phosphorylation in rabbit platelets compared to those of protein kinase C inhibitor H-7.

MATERIALS AND METHODS

Preparation of Washed Rabbit Platelets

Platelets were prepared from rabbit blood anticoagulated with acid-citrate dextrose. Platelets were washed twice with a HEPES buffer consisting of 130 mM NaCl, 4.7 mM KCl, 4.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.5 mM dextrose, 0.1% bovine serum albumin and 10 mM HEPES (pH 6.5). Platelets were finally suspended in HEPES buffer (pH 7.35) containing 2.0 mM $CaCl_2$.

Measurements of Platelet Aggregation

Platelet aggregation was monitored turbidometrically in a platelet aggregometer (Rikadenki Co. Ltd.) as described previously [19]. Platelet suspension (0.5 mL, 5×10^8 platelets/mL) was preincubated for 3 min at 37°C in the presence or absence of test agents and stimulated with collagen or other stimuli as indicated in the text.

Measurement of [^{3}H] AA Liberation

Platelets in plasma were incubated with [^{3}H]AA (5 μCi/mL) for 90 min at 37°, and then washed twice and suspended in HEPES buffer as described above. Reactions were performed in the aggregometer with monitoring platelet aggregation. At the end of the incubation periods, reactions were terminated by adding equal volumes of an ice-cold solution containing 20 mM EDTA, 20 μM indomethacin and 1.0% formaldehyde. The sample was centrifuged at 1200 × *g* for 5 min, and the [^{3}H]radioactivity in supernatant was measured by liquid scintillation counting. In a preliminary experiment with TLC analysis, [^{3}H]radioactivity released from [^{3}H]AA-labeled platelets to the extracellular medium was observed to consist mainly of AA, 12-hydroxyeicosatetraenoic acid and TXB_2.

Measurement of TXB_2 Formation

TXB_2 formation in agonist-stimulated platelets was assayed as described previously [8]. Briefly, platelet suspension (5×10^8 platelets/mL) was placed in the aggregometer and preincubated for 3 min with or without test agents. Platelets were then stimulated by collagen or other stimuli, and the reaction was terminated by adding an equal volume of an ice-cold solution containing 20 mM EDTA and 50 μM indomethacin. After centrifuging at 1200 × *g* for 5 min, supernatant was diluted with 10 mM Tris-HCl (pH 7.6), and the TXB_2 level was measured by radioimmunoassay.

Measurement of Protein Phosphorylation

Washed platelets were suspended in Ca^{2+}- and phosphate-free HEPES buffer (pH 7.4) and incubated with [^{32}P] orthophosphate (0.2 mCi/mL) for 60 min at 37°. The platelets were then washed twice and resuspended in the HEPES buffer as described above. Samples (0.25 mL) were placed in the aggregometer and stimulated with collagen in the presence or absence of ML-7 or H-7. The reaction was terminated by the addition of 0.25 mL of a 2 × sample buffer (6% sodium dodecyl sulfate, 4% 2-melcaptoethanol, 10% glycerin and 120 mM Tris-HCl, pH 6.7) and heated at 90° for 10 min. Aliquots containing 20 μg of protein were subjected to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The stacking and separating gels contained 3.5 and 11.5% acrylamide, respectively. Phosphorylated protein bands on the gel were detected by autoradiography.

Measurement of $[Ca^{2+}]_i$

Washed platelets were suspended in the Ca^{2+} free-HEPES buffer (pH 7.4) and incubated with 2 μM fura 2-acetoxymethyl ester at 37° for 20 min. The platelets were then washed twice and finally resuspended at 2×10^8 platelets/mL in the HEPES buffer (pH 7.4) containing 1.8 mM $CaCl_2$. The platelets were kept at room temperature during the experiments. Aliquot (2 mL) was transferred into a 10- × 10-mm quartz cuvette placed in the thermostat-regulated sample chamber of a dual excitation beam spectrophotometer (Hitachi, F-2000). The platelet suspension was continuously stirred with a circular stirring bar. The excitation wavelengths were 340 and 380 nm, and the fura-2 fluorescence emission was measured at 510 nm. At the end of the measurement, Triton X-100 was added to obtain maximal fluorescence, with excess EGTA then added to obtain minimal fluorescence. $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence at two excitation wavelengths as previously described.

Measurement of Protein Tyrosine Phosphorylation by Immunoblotting

Samples (0.25 mL) were placed in the aggregometer and preincubated for 3 min with or without ML-7 or indomethacin. The platelets were then stimulated by collagen. The reaction was terminated by addition of 0.25 mL of a 2 × sample buffer (6% sodium dodecyl sulfate, 4% 2-melcaptoethanol, 10% glycerin, 0.1 mM sodium vanadate and 120 mM Tris-HCl, pH 6.7) and heated at 90° for 10 min. Aliquots containing 20 μg of protein were subjected to a sodium dodecyl sulfate-polyacrylamide (11%) gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% BSA in TBS-T (100 mM NaCl, 0.1% Tween 20 and 10 mM Tris-HCl, pH 7.5) and incubated with the monoclonal anti-phosphotyrosine antibody (1:1000 in TBS-T containing 2% BSA) for 1 hr at room temperature. The blot was washed 4 times with TBS-T and incubated with horseradish peroxidase-conjugated sheep anti-mouse Ig (1:10000 in TBS-T) at 4° for 12–16 hr. After washing several times with TBS-T, the bolt was developed using the Amersham ECL System.

Materials

Collagen was purchased from Nycomed Arzneimittel (Munich, Germany). ML-7, ML-9 and H-7 were from Seikagaku Kogyo Co. Ltd. Thrombin was from Sigma Chemical Co. A-23167 and melittin were from Calbiochem. Fura 2-acetoxymethyl ester was from Wako Pure Chemicals. Monoclonal anti-phosphotyrosine antibody PY20 was from Transduction Laboratories. TXB_2 and anti-TXB_2 antiserum ere kindly provided by Ono Pharmaceutical Co. [^{3}H]AA (38 Ci/mmol), [^{32}P]orthophosphate (carrier-free), [^{3}H]TXB_2, horseradish peroxidase-conjugated sheep anti-mouse Ig (NA931), and ECL reagents were purchased from Amersham Japan. Other reagents used were of analytical grade available from commercial sources.

RESULTS

Effects of ML-7 and H-7 on Collagen-Induced Platelet Aggregation

Pretreatment of platelets with ML-7 (50 μM) markedly inhibited platelet aggregation induced by 1 μg/mL collagen. In contrast, H-7 (50 μM) had little effect on collagen-induced aggregation (Fig. 1A). Figure 1B shows the effects of different concentrations (1–100 μM) of ML-7 or H-7 on 1 μg/mL collagen-induced platelet aggregation. ML-7 produced a concentration-dependent inhibition of the aggregation with an IC_{50} value of *ca.* 10 μM, while H-7 did not show a significant effect. We observed that ML-9, another MLC kinase inhibitor, also had an inhibitory effect on collagen-induced platelet aggregation, with an IC_{50} value of *ca.* 30 μM (data not shown). Since platelet aggregation induced by 1 μg/mL of collagen was completely inhibited

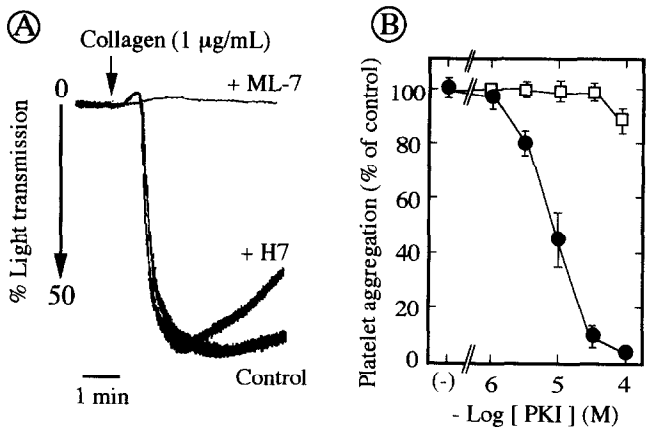

FIG. 1. Effects of ML-7 and H-7 on collagen-induced platelet aggregation. (A) Platelets were preincubated for 3 min with ML-7 (50 μM), H-7 (50 μM), or saline (control) and then stimulated with 1 μg/mL collagen. (B) Effects of different concentrations of the protein kinase inhibitors (PKI) ML-7 (●) or H-7 (□) on collagen (1 μg/mL)-induced aggregation. Values are means ± SEM from three experiments.

by indomethacin, collagen-induced aggregation at this concentration is totally dependent on endogenously generated TXA_2. ML-7 (50 μM) had no effect on the TXB_2 formation induced by AA (10 μM), but inhibited collagen-induced TXB_2 formation (data not shown). These results indicate that ML-7 prevented collagen-induced AA liberation from membrane phospholipids.

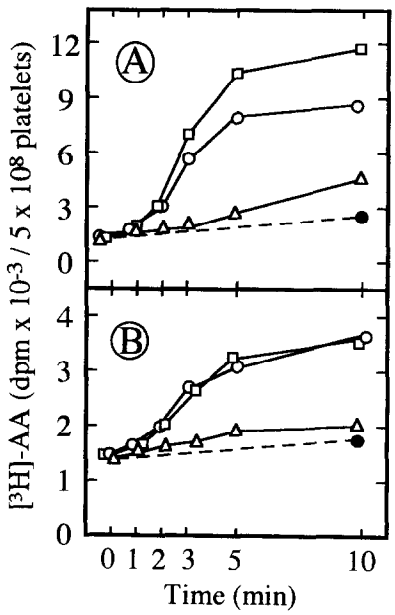

FIG. 2. Effects of ML-7 and H-7 on time-dependent AA liberation induced by collagen in the absence (A) or presence (B) of indomethacin (2.5 μM). Platelets prelabeled with [^{3}H] AA were stimulated with collagen (1 μg/mL) in the absence (○) or presence of ML-7 (50 μM, △) or H-7 (50 μM, □). ML-7 or H-7 was preincubated for 3 min. ●: basal release of [^{3}H] AA after a 10-min incubation. Values are means of two determinations. Two independent experiments yielded similar results.

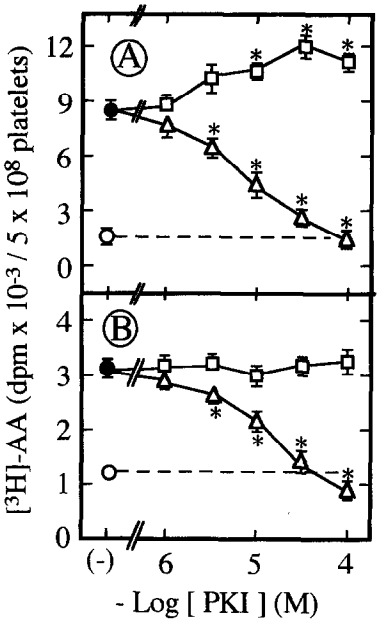

FIG. 3. Effects of different concentrations of protein kinase inhibitors (PKI) on collagen-induced AA liberation in the absence (A) or presence (B) of indomethacin (2.5 μM). Platelets prelabeled with [^{3}H] AA were stimulated with collagen (1 μg/mL) for 10 min in the absence (●) or presence of ML-7 (△) or H-7 (□) at the concentration indicated. ML-7 or H-7 was preincubated for 3 min before stimulation with collagen. ○: basal release of [^{3}H] AA after a 10-min incubation. Values are means ± SEM of three determinations. *$P < 0.05$ by Student's *t*-test for difference from control value.

Effects of ML-7 and H-7 on Collagen-Induced AA Liberation

We next examined the effects of ML-7 and H-7 on collagen-induced AA liberation from membrane phospholipids in [^{3}H]AA-labeled platelets (Fig. 2). AA liberation induced by collagen (1 μg/mL) was initiated after a time lag of approximately 1 min, reaching its maximum level at 5 min. ML-7 (50 μM) markedly inhibited collagen-induced AA liberation (Fig. 2A). On the contrary, H-7 (50 μM) apparently potentiated the maximum level of collagen-induced AA liberation, without affecting the duration of the time lag or the initial rate of AA liberation (Fig. 2A). In the presence of indomethacin (2.5 μM), collagen still elicited AA liberation without inducing aggregation (Fig. 2B). ML-7 (50 μM) almost abolished collagen-induced AA liberation from indomethacin-treated platelets. Interestingly, the potentiation of AA accumulation by H-7 was not observed in the presence of indomethacin (Fig. 2B). Figure 3 shows the effects of different concentrations of ML-7 and H-7 on collagen-induced AA liberation in [^{3}H]AA-labeled platelets. ML-7 produced a concentration-dependent inhibition of collagen-induced AA liberation either in the presence or absence of indomethacin. A significant inhibition by ML-7 was observed at a concentration above 3 μM, and 30 μM ML-7 totally inhibited the effects of collagen (Figs. 3A and B). The concentration-inhibition curve of ML-7 for collagen-induced AA liberation was similar to that for collagen-induced aggregation (Fig. 1). In contrast, H-7 potentiated collagen-induced AA liberation in a concentration-dependent manner only in the absence of indomethacin (Figs. 3A and B).

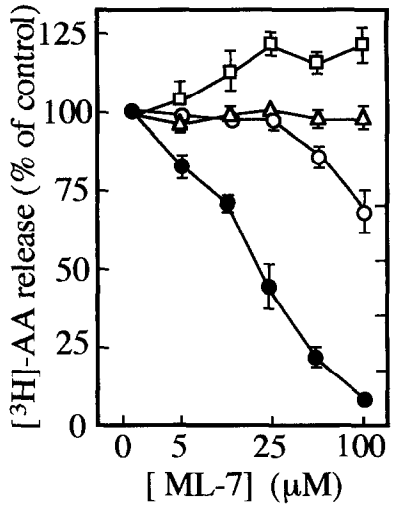

FIG. 4. Effects of ML-7 on AA liberation induced by different platelet stimuli. Platelets prelabeled with [^{3}H] AA were preincubated with ML-7 for 3 min at the concentrations indicated and then stimulated with collagen (1 μg/mL, ●), thrombin (0.1 U/mL, ○), A-23187(0.5 μM, □) or melittin (1 μg/mL, △) for 10 min. Results are presented as percent of the control response without ML-7. Values are means ± SEM of three determinations. The basal, collagen-, thrombin-, A-23187- and melittin-stimulated [^{3}H]AA liberation were 2314 ± 52, 9823 ± 182, 14241 ± 214, 11231 ± 291 and 8912 ± 113 dpm/5 × 10^8 platelets (N = 3), respectively.

Effects of ML-7 on AA Liberation Induced by Different Platelet Stimuli

To clarify whether ML-7 directly affects phospholipase A_2 activity, we examined the effects of ML-7 on AA liberation induced by several different platelet stimuli such as thrombin, Ca^{2+} ionophore A-23187, and melittin (Fig. 4). All these agents induced a marked AA liberation in [^{3}H]AA-labeled platelets. In contrast to its effects on the collagen-induced response, ML-7 had only a slight inhibitory effect on thrombin-induced AA liberation at a concentration higher than 50 μM. In addition, ML-7 did not inhibit A-23187- or melittin-induced AA liberation (Fig. 4). These results indicate that ML-7 did not directly inhibit platelet phospholipase A_2 activity and that the inhibition of AA liberation by ML-7 seemed to be selective for the collagen-induced response.

Effects of ML-7 and H-7 on Collagen-Induced Protein Phosphorylation

Stimulation of platelets prelabeled with [^{32}P]orthophosphate with collagen induced phosphorylation of 20-kDa MLC and 40-kDa pleckstrin (Fig. 5), mediated by MLC kinase and protein kinase C, respectively. Although H-7

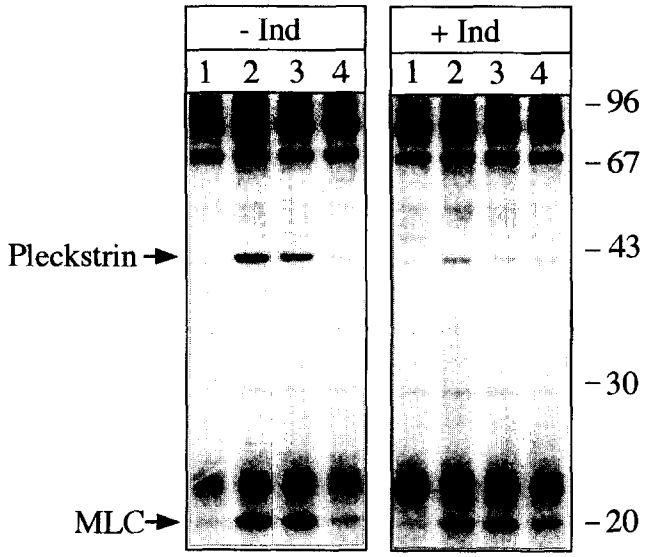

FIG. 5. Effects of ML-7 and H-7 on collagen-induced protein phosphorylation in the presence (+Ind) or absence (−Ind) of indomethacin (2.5 μM). [^{32}P]-labeled platelets were preincubated with ML-7 (50 μM) or H-7 (50 μM) for 3 min, and then stimulated with collagen (1 μg/mL) for 5 min. 1, control; 2, collagen; 3, H-7 plus collagen; 4, ML-7 plus collagen. The results shown are representative autradiograms from three independent experiments which yielded similar results.

decreased collagen-induced phosphorylation of 40 kDa pleckstrin without affecting that of 20 kDa MLC, ML-7 inhibited both pleckstrin and MLC phosphorylation (Fig. 5). In the presence of indomethacin (2.5 μM), collagen-induced 40-kDa pleckstrin phosphorylation was markedly inhibited, whereas 20-kDa MLC phosphorylation was still induced by collagen (Fig. 5). ML-7, but not H-7, inhibited this MLC phosphorylation (Fig. 5). Figure 6

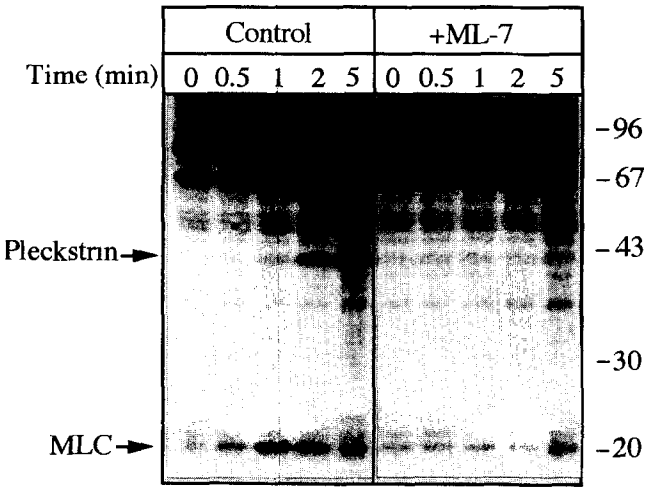

FIG. 6. Effects of ML-7 on collagen-induced time-dependent protein phosphorylation. [^{32}P]-labeled platelets were preincubated in the absence (control) or presence of 50 μM ML-7 (+ML-7) for 3 min and then stimulated with collagen (1 μg/mL). The results shown are representative autradiograms from three independent experiments which yielded similar results.

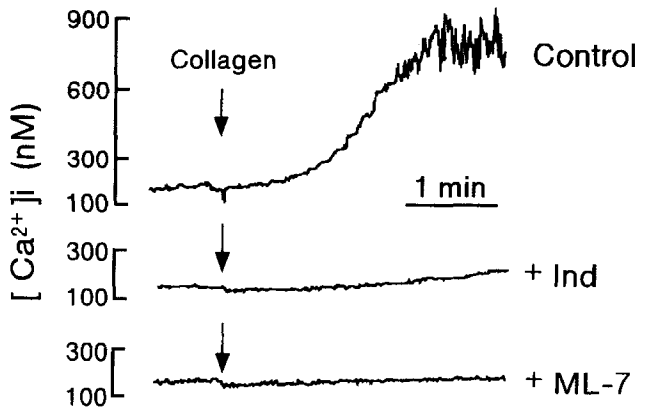

FIG. 7. Effects of ML-7 and indomethacin on the collagen-induced increase in $[Ca^{2+}]_i$. Fura2-loaded platelets were preincubated in the absence (control) or presence of 50 μM ML-7 (+ML-7) or 2.5 μM indomethacin (Ind) for 3 min, and then stimulated with collagen (1 μg/mL) as indicated by arrows. The results shown are representative traces from four independent experiments which yielded similar results.

shows the time-course of collagen-induced protein phosphorylation in the presence or absence of 50 μM ML-7. As we showed previously [8], collagen stimulated 20-kDa MLC phosphorylation within 30 sec, and maximally by 1 min. The MLC phosphorylation preceded the collagen-induced pleckstrin phosphorylation (Fig. 6), platelet aggregation (Fig. 1), and AA liberation (Fig. 2); the onset of these responses occurred after a 1-min lag following collagen stimulation. ML-7 totally inhibited collagen-induced phosphorylations of MLC and pleckstrin (Fig. 6).

Effects of ML-7 on Collagen-Induced $[Ca^{2+}]_i$ Increase

MLC kinase activity is known to be regulated by Ca^{2+}/calmodulin. We examined the effects of ML-7 on the collagen-induced increase in $[Ca^{2+}]_i$ (Fig. 7). Stimulation of fura2-loaded platelets with 1 μg/mL collagen did not change $[Ca^{2+}]_i$ during a 1-min lag phase, but then did gradually increase $[Ca^{2+}]_i$ from *ca.* 150 nM to 800 nM. The increase in $[Ca^{2+}]_i$ induced by collagen was abolished by ML-7 (50 μM) as well as indomethacin (2.5 μM). These results indicate that collagen induced a rapid MLC phosphorylation and AA liberation without a significant increase in $[Ca^{2+}]_i$.

Effects of ML-7 on Collagen-Induced Protein Tyrosine Phosphorylation

Recent studies have suggested that an activation of the protein tyrosine kinase cascade is involved in collagen-induced platelet activation [1–4, 20]. Under our experimental conditions, a few proteins having molecular masses of 64, 56, and 46 kDa were significantly phosphorylated on tyrosine in resting platelets (Fig. 8). These overall patterns of tyrosine phosphorylation did not change upon stirring for 3 min in the absence of collagen (Fig. 8). Following

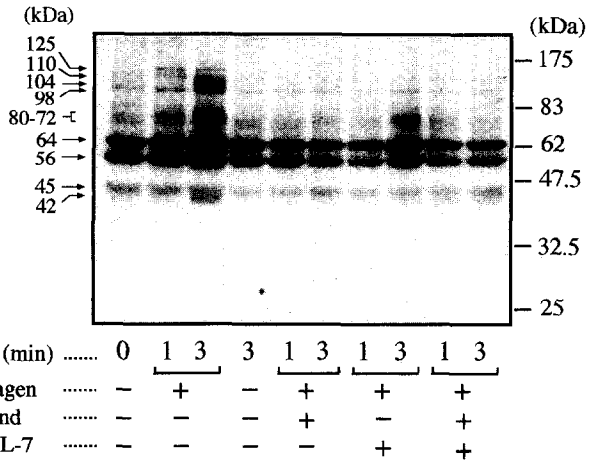

FIG. 8. Effects of ML-7 on collagen-induced protein tyrosine phosphorylation in the presence or absence of indomethacin. Platelets were preincubated with buffer, ML-7 (50 μM), indomethacin (2.5 μM) or ML-7 plus indomethacin for 3 min and then stimulated with collagen (1 μg/mL) for 1 min or 3 min as indicated. The results shown are representative immunoblottings from three independent experiments which yielded similar results.

stimulation with collagen (1 μg/mL) for 1 min, tyrosine phosphorylation increased in a number of proteins with molecular masses of 125, 110, 98 and 72–80 kDa (doublet or triplet). In addition to these proteins, tyrosine phosphorylation increased in 104 kDa and 42 kDa proteins after a 3-min stimulation with collagen (Fig. 8). Collagen-induced tyrosine phosphorylation was totally inhibited either by 50 μM ML-7 or 2.5 μM indomethacin (Fig. 8). These results suggest that tyrosine phosphorylation induced by 1 μg/mL collagen was largely mediated by cyclooxygenase metabolites of AA. We could therefore not evaluate the direct effect of ML-7 on collagen-induced tyrosine phosphorylation.

DISCUSSION

The present study demonstrates that ML-7, an MLC kinase inhibitor, is a potent inhibitor of collagen-induced platelet activation. The inhibition of collagen-induced platelet aggregation by ML-7 was accompanied by a parallel decrease in collagen-stimulated TXB_2 formation. ML-7, however, did not affect the conversion of AA to TXB_2, indicating that ML-7 has no effect on the cyclooxygenase-thromboxane synthetase pathway. In [^{3}H]AA-labeled platelets, we demonstrated that ML-7 markedly inhibited collagen-induced [^{3}H]AA liberation from membrane phospholipids. These results clearly indicate that ML-7 inhibits collagen-induced platelet activation by preventing AA liberation.

In contrast to ML-7, the protein kinase C inhibitor H-7 had little effect on collagen-induced platelet aggregation, although the inhibitory effect of H-7 on protein kinase C was confirmed by a decrease in 40 kDa pleckstrin phosphorylation induced by collagen. In [^{3}H]AA-labeled platelets, we observed that H-7 produced a potentiation, rather than inhibition, of collagen-induced AA liberation. These results are consistent with those reported by Börsch-Haubold et al. [14] in human platelets with another protein kinase C inhibitor, Ro31-8220. This effect of the protein kinase C inhibitor was explained by inhibition of negative feedback action of protein kinase C [21]. In agreement with this, the potentiation by H-7 of collagen-induced AA liberation was not observed in indomethacin-treated platelets in which collagen-induced protein kinase C activation was markedly impaired. These results indicate that collagen-induced AA liberation is not dependent on protein kinase C activation.

Recent studies have suggested that an 85 kDa cytosolic phospholipase A_2 is involved in the receptor agonist-induced AA liberation from membrane phospholipids in different types of cells, including platelets [12–14]. The present study demonstrates that ML-7 inhibited collagen-induced AA liberation, suggesting that ML-7 inhibits collagen-induced initial activation of the cytosolic phospholipase A_2. However, ML-7 does not appear to have a direct inhibitory effect on phospholipase A_2 activity, because it failed to inhibit AA liberation induced by Ca^{2+} ionophore A23187 or by the phospholipase A_2 activator melittin. In addition, AA liberation induced by thrombin, a physiological agonist acting via specific receptors, was only slightly inhibited by higher concentrations of ML-7. These results suggest that the signal transduction pathway involved in activation of cytosolic phospholipase A_2 induced by collagen is different from that induced by thrombin, and that ML-7 inhibits a signaling process specific for the collagen-induced response.

To understand the mechanism of action of ML-7, it is important to explore whether MLC phosphorylation is involved in the collagen-induced signal transduction mechanism. We have previously demonstrated that collagen induced a rapid MLC phosphorylation which apparently preceded AA liberation in rabbit platelets [8]. Results obtained in the present study confirm these previous observations and further demonstrate that inhibition of collagen-induced AA liberation by ML-7 was accompanied by a parallel decrease in collagen-induced rapid phosphorylation of MLC either in the presence or absence of indomethacin. Although there is little information in the literature concerning the relationship between MLC phosphorylation and phospholipase A_2 activation, a few reports from other laboratories have also demonstrated that collagen-induced platelet activation is highly sensitive to MLC kinase inhibitor. For example, ML-9, an MLC kinase inhibitor structurally related to ML-7, has been shown to inhibit collagen-induced platelet aggregation and MLC phosphorylation in human platelets [22, 23]. We also observed that ML-9 has similar effects in rabbit platelets to ML-7 on collagen-induced platelet aggregation and AA liberation. The inhibitory effects of ML-9 were slightly less than those of ML-7 on collagen-induced responses. This was consistent with the order of inhibitory potency of these agents on MLC kinase activity [23]. Furthermore, thyroid

hormones, such as thyroxin and triiodothyronine, have been reported to have a direct inhibitory effect on platelet MLC kinase activity [24], and were also found to selectively inhibit collagen-induced platelet aggregation without affecting thrombin- or phorbol ester-induced responses [25]. These findings strongly indicate that MLC phosphorylation plays an important role in collagen-induced platelet activation. Nakano et al. [26] have demonstrated that collagen-induced initial phospholipase A_2 activation was selectively inhibited by cytochalasin B, and suggested that rearrangement of platelet cytoskeleton would play an important role in collagen-induced initial phospholipase A_2 activation. MLC phosphorylation may be involved in such a collagen-induced rearrangement of cytoskeleton.

It is, however, also apparent that MLC phosphorylation alone is not sufficient to induce AA liberation, since several platelet agonists such as ADP and TXA_2 induce MLC phosphorylation without inducing AA liberation in rabbit platelets*. Collagen would induce an additional signal transduction mechanism to stimulate phospholipase A_2. Recent studies have provided accumulating evidence that collagen-induced platelet response is associated with activation of several protein-tyrosine kinase, such as $p72^{syk}$ [1, 2, 5, 27], $p60^{c\text{-}src}$ [5] and $p120^{FAK}$ [28], and involves tyrosine phosphorylation-dependent activation of phospholipase C-γ2 [3, 4], which leads to phosphoinositide hydrolysis, cytosolic Ca^{2+} mobilization, protein kinase C activation and eventually platelet aggregation. These responses are, however, insensitive to cyclooxygenase inhibitors and require high concentrations of collagen (above 10 μg/mL). In the present study, we observed that stimulation with 1 μg/mL of collagen also induced tyrosine phosphorylation of several proteins with molecular masses of 125, 110, 104, 98, 72–80, and 42 kDa. These data are in agreement with results reported by Bachelot et al. [19] with 7.5 μg/mL of collagen. However, the tyrosine phosphorylation as well as the phosphoinositide hydrolysis and protein kinase C activation induced by 1 μg/mL of collagen were totally inhibited by indomethacin. These results suggest that collagen-induced AA liberation in indomethacin-treated rabbit platelets occurs in the absence of significant tyrosine phosphorylation or phospholipase C-γ2 activation. However, since Yanaga et al. [1] have demonstrated that collagen, even at 1 μg/mL, induced tyrosine phosphorylation of Syk, a nonreceptor tyrosine kinase, after immunoprecipitation with an ant-Syk antibody in indomethacin-treated human platelets, we cannot exclude the possibility that our experimental condition could not detect a small collagen-induced increase in tyrosine phosphorylation. Further experiments are required to evaluate the effect of ML-7 on such a collagen-induced tyrosine phosphorylation of Syk.

The present study also demonstrated that collagen-induced MLC phosphorylation occurred in the absence of an apparent increase in $[Ca^{2+}]_i$. Since MLC kinase is activated by Ca^{2+} through a calmodulin-dependent mechanism, there is a possibility that collagen induces MLC phosphorylation without activating the MLC kinase. Recently, a small GTP-binding protein Rho has been found to modulate MLC phosphorylation either by inhibiting MLC phosphatase [29] or activating Rho kinase, which can phosphorylate MLC directly [30]. It would be interesting to evaluate whether collagen can activate the Rho-dependent pathway, and if so, whether ML-7 can affect Rho-dependent MLC phosphorylation. In any case, it is important to note that collagen-induced AA liberation and MLC phosphorylation were absolutely dependent on extracellular Ca^{2+} in rabbit platelets [8].

In conclusion, the present study demonstrates that the MLC kinase inhibitor ML-7 is a potent and selective inhibitor of collagen-induced AA liberation in washed rabbit platelets. Although further work is required to explore the causal relationships between phospholipase A_2 activation and MLC phosphorylation, the present results are an important step toward understanding the initial signal transduction mechanism of collagen-induced platelet activation.

* Matsuoka I, Nakahata N and Nakanishi H, unpublished observations.

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