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ML-7 and W-7 facilitate thromboxane A_2-mediated Ca^{2+} mobilization in rabbit platelets

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Abstract

The effects of 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-7), a myosin light chain kinase inhibitor, and (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin antagonist, on thromboxane A_2 receptor-mediated signal transduction were examined in rabbit washed platelets. ML-7 and W-7 at 10–30 μM slightly potentiated the aggregation induced by a thromboxane A_2 receptor agonist, 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin $F_2\alpha$ (U46619), in spite of their known inhibitory actions. ML-7 and W-7 concentration-dependently enhanced U46619-induced phosphoinositide hydrolysis and the increase in internal free Ca^{2+} concentration in the presence or absence of external Ca^{2+}. While ML-7 and W-7 inhibited basal GTPase activity, they augmented U46619-induced activation of GTPase in a concentration-dependent manner. The present results suggest that ML-7 and W-7 enhance thromboxane A_2 receptor-mediated signal transduction at the receptor/G protein coupling, leading to the enhancement of phosphoinositide hydrolysis and Ca^{2+} mobilization, independently of the inhibition of myosin light chain kinase or calmodulin.

Keywords: W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide); ML-7 (1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine); Thromboxane A_2; G-protein; Phosphoinositide hydrolysis; Ca^{2+}

1. Introduction

Thromboxane A_2 receptor stimulation causes phosphoinositide hydrolysis by phosphoinositide-specific phospholipase C through pertussis toxin-insensitive GTP binding protein (G protein) (Baldassare et al., 1993; Houslay et al., 1986). One of the G proteins which couples with thromboxane A_2 receptors has been identified as $G_{q/11}$ in human platelets from the experiments using $G_{q/11}\alpha$ antiserum, QL (Shenker et al., 1991). Activation of phosphoinositide-specific phospholipase C results in accumulations of two second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol. IP_3 mobilizes Ca^{2+} ions from internal storage sites (Berridge and Irvine, 1984), and 1,2-diacylglycerol activates protein kinase C in the presence of Ca^{2+} and acidic phospholipids (Nishizuka, 1984).

An increase in internal free Ca^{2+} concentration ($[Ca^{2+}]_i$) results in activation of a Ca^{2+}-dependent process. Calmodulin, a Ca^{2+}-binding protein, is involved in a wide variety of cellular processes including activation of myosin light chain kinase which causes phosphorylation of myosin light chain (Hathaway and Adelstein, 1979). Phosphorylated myosin by myosin light chain kinase increases actin-activated Mg^{2+}-ATPase activity (Adelstein and Conti, 1975) and is thought to be associated with platelet activation such as shape change, aggregation and secretion (Daniel et al., 1977, 1984). Myosin light chain of 20 kDa is phosphorylated in agonist-stimulated platelets such as thrombin or collagen (Daniel et al., 1981; Itoh et al., 1992; Lokeshwar and Bourguignon, 1992), suggesting that activation of myosin light chain kinase through calmodulin has a critical role in platelet activation.

To determine the role of Ca^{2+}-calmodulin systems, many synthetic drugs named 'calmodulin antagonists' such as W-7 and W-13 have been used (Kanamori et al., 1981). W-7 has been shown to bind only the Ca^{2+}-calmodulin complex at the hydrophobic sites of calmodulin (Tanaka and Hidaka, 1980) and to inhibit Ca^{2+}-calmodulin-dependent protein kinases, resulting in the inhibition of phosphodiesterase (Hidaka et al., 1978) or myosin light chain

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kinase (Nishikawa et al., 1980). Thrombin- or collagen-induced platelet aggregation is inhibited by preincubating with W-7 in human platelets (Nishikawa et al., 1980), suggesting that Ca^{2+}-calmodulin systems might be important for platelet activation. To elucidate the role of myosin light chain kinase, myosin light chain kinase inhibitors such as ML-7 and ML-9 have been synthesized and used. ML-9 has been reported to bind at or near the ATP-binding sites on the kinase molecule (Saitoh et al., 1987). ML-9 inhibits platelet aggregation and 20 kDa phosphorylation induced by collagen in human platelets (Saitoh et al., 1986). Therefore, myosin light chain kinase activation by the Ca^{2+}-calmodulin complex is thought to be indispensable for platelet activation including shape change and aggregation. In the present study, we used W-7 and ML-7 to examine thromboxane A_2 receptor-mediated platelet activation. Unexpectedly, we found that ML-7 and W-7 augmented the function of receptor/G protein coupling, independently of the inhibition of myosin light chain kinase or calmodulin.

2. Materials and methods

2.1. Materials

9,11-Dideoxy-9α,11α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) and bovine serum albumin (fraction V, fatty acid-free) were obtained from Sigma (St. Louis, MO, USA). Fura-2/AM was purchased from Dojindo (Kumamoto, Japan). 1-(5-Iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-7) and (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) were obtained from Seikagaku Co. Tokyo, Japan. [^{3}H]Myo-inositol (23.4 Ci/mmol) and [γ-^{32}P]GTP (30 Ci/mmol) were obtained from DuPont/New England Nuclear (Boston, MA, USA). Other chemicals or drugs were of reagent grade or of the highest quality available.

2.2. Platelet preparation

Whole blood was taken from male rabbits weighing 2.5–3.5 kg, and then mixed gently with one-seventh volume of ACD solution (65 mM citric acid, 85 mM trisodium citrate, 2% glucose) containing 5 μM indomethacin. Blood was centrifuged at $250 \times g$ for 10 min at 25°C and platelet-rich plasma was obtained by centrifugation of the supernatant at $180 \times g$ for 5 min to remove contaminated erythrocytes and leucocytes. Platelet-rich plasma was centrifuged at $650 \times g$ for 10 min and the pellet was resuspended in Tyrode-Hepes-albumin solution (138 mM NaCl, 2.68 mM KCl, 1.05 mM $MgCl_2 \cdot 6H_2O$, 4 mM $NaHCO_3$, 10 mM Hepes, 0.1% glucose, 0.35% bovine serum albumin, 5 μM indomethacin, pH 6.35). Platelets were washed twice, and finally resuspended in Tyrode-Hepes-albumin solution (pH 7.35) at the optimum concentration.

2.3. Measurement of platelet shape change and aggregation

Platelet shape change and aggregation were monitored by light transmission in a 6-channel aggregometer (PAM-6C, Erma Co., Tokyo, Japan). Washed platelets at $3–5 \times 10^8$/ml were preincubated in aggregometer cuvettes (total volume of 400 μl) with constant stirring at 37°C for 10 min. After 1 mM $CaCl_2$ was added, platelet suspension was incubated for 5 min. ML-7 and W-7 were preincubated for 5 min before the addition of U46619. In shape change studies, 1 mM EGTA was added instead of 1 mM $CaCl_2$.

2.4. Measurement of internal free calcium concentrations

Changes in internal free calcium concentration ($[Ca^{2+}]_i$) were measured by monitoring the intensity of fura-2 fluorescence. Washed platelets were incubated with 1 μM fura-2/AM at 37°C for 15 min. After washing the platelets twice, fura-2-loaded platelets were resuspended at about 1×10^8/ml. Fura-2 fluorescence at 510 nm was monitored with excitation at 340 nm and 380 nm in 1.5 ml of the platelet suspension in the quartz cell with constant stirring at 37°C by a fluorospectrophotometer (F-2000, Hitachi, Tokyo, Japan). Change in $[Ca^{2+}]_i$ was calculated from the 340/380 nm ratio as previously described (Nakahata et al., 1994).

2.5. Measurement of inositol phosphates

Washed platelets, suspended in albumin-free Tyrode-Hepes solution (pH 7.35), were labeled with 25 μCi/ml [^{3}H]myo-inositol at 37°C for 1 h. Platelets were washed with Tyrode-Hepes-albumin solution (pH 7.35), and resuspended at $3–6 \times 10^8$/ml. After platelet suspensions (360 μl) were preincubated for 10–12 min, they were incubated with drugs for 10–12 min in a final volume of 400 μl of the buffer containing 1 mM $CaCl_2$ or 1 mM EGTA. Reaction was terminated by the addition of 400 μl of ice-cold 10% trichloroacetic acid. The trichloroacetic acid extracts were washed three times with diethyl ether to remove trichloroacetic acid. Diethyl ether in the sample was removed by keeping the samples at 47°C for 30 min. Total [^{3}H]inositol phosphates and [^{3}H]IP_3 were separated by anion exchange column (AG 1X8, formate form, 100–200 mesh) as previously described (Nakahata et al., 1989, 1992).

2.6. Preparation of platelet membranes

Platelet pellets were suspended in ice-cold lysis buffer (5 mM EDTA-10 mM Hepes, pH 7.4) and sonicated five times for 15 s at intervals of 1 min on ice by a sonicator (Nihon Seiki Co., Tokyo, Japan). The homogenate was centrifuged at $40\,000 \times g$ for 10 min at 4°C to obtain

membranes. The membranes were washed twice with the above buffer. The membranes were dissolved in a small volume of 0.32 M sucrose-10 mM Hepes-5 mM $MgCl_2$ (pH 7.4), and stored at −80°C until use.

2.7. Measurement of GTPase activity

Platelet GTPase activity was assessed by a method previously described (Houslay et al., 1986; Nakahata et al., 1995), with minor modifications. In brief, the reaction mixture contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 0.5 mM EGTA, 1 mM ATP, 12 mM phosphocreatine, 0.1 μM [γ-^{32}P]GTP (30 Ci/mmol), 50 units/ml creatine phosphokinase and various drugs in a final volume of 100 μl. Platelet membranes were suspended in 10 mM Tris-HCl (pH 7.4) just before use. The reaction was initiated by the addition of membranes (final 5 μg/each tube) and was carried out for 5 min at 30°C. After 400 μl of ice-cold 5% Norit A in 20 mM phosphate buffer (pH 7.4) was added, the sample was vigorously mixed and centrifuged at 15000 rpm for 2.5 min by a microcentrifuge (Tomy Seiko. Co., Tokyo, Japan). Radioactivity of aliquots (200 μl) of the supernatant was counted by scintillation counting.

2.8. Determination of protein and data analysis

Protein was measured by dye-binding method using bovine serum albumin as a standard (Bradford, 1976). Data were represented as the mean ± S.E., and a statistically significant difference was determined by Student's *t*-test.

3. Results

3.1. Effects of ML-7 and W-7 on U46619-induced platelet aggregation and shape change

In the presence of 1 mM Ca^{2+}, U46619 at a concentration of 1 μM caused platelet aggregation after a transient shape change. ML-7, a myosin light chain kinase inhibitor, and W-7, a calmodulin antagonist, at 10–50 μM did not inhibit platelet aggregation induced by U46619 (Fig. 1), but slightly potentiated the aggregation in the presence of external Ca^{2+} in rabbit platelets. ML-7 and W-7 at 100 μM inhibited U46619-induced aggregation, probably by their specific actions. ML-7 and W-7 at high concentrations caused a slow shape change by themselves, suggesting that these drugs might have a non-specific action to induce shape change. The potentiating effects of ML-7 and W-7 on platelet aggregation differed in platelet preparations, and appeared prominently in the preparation which had a weaker ability of aggregation (data not shown). In contrast, ML-7 and W-7 at 50 μM significantly inhibited ADP (30 μM)- or ionomycin (10 μM)-induced aggregation (data not shown).

3.2. Effects of ML-7 and W-7 on U46619-induced increase in $[Ca^{2+}]_i$

U46619 increased $[Ca^{2+}]_i$ in fura-2-loaded platelets. The increase in $[Ca^{2+}]_i$ in the presence of 1 mM Ca^{2+} was much larger than that in the presence of 1 mM EGTA, reflecting Ca^{2+} influx from external medium (Fig. 2). Preincubation with ML-7 for 3 min resulted in a concentra-

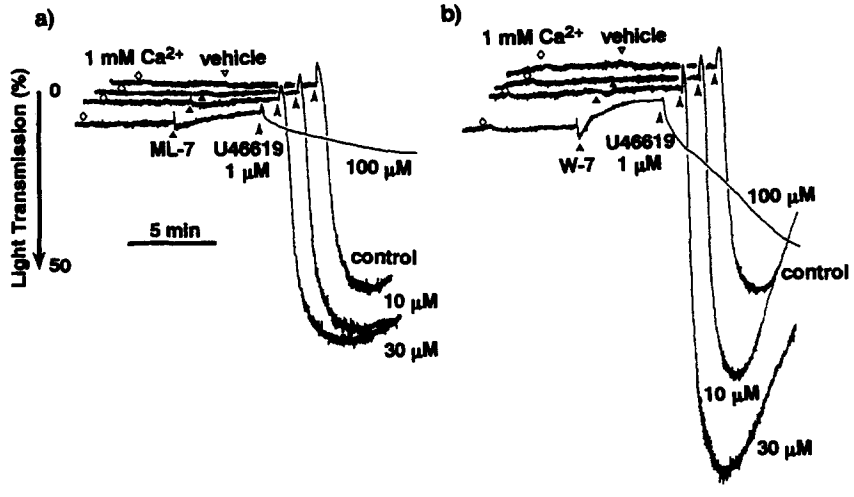

Fig. 1. Effect of ML-7 or W-7 on U46619-induced aggregation. Washed platelets (3–5 × 10^8/ml) were preincubated with the indicated concentrations of ML-7 (a) or W-7 (b) for 5 min at 37°C in the presence of 1 mM Ca^{2+}. It was then stimulated with 1 μM U46619. The aggregation was recorded as described under Materials and methods. The data are representative of several experiments with similar results.

tion-dependent potentiation of the U46619-induced rise in $[Ca^{2+}]_i$ in the presence of Ca^{2+} (Fig. 2a and Fig. 2c). In the presence of 1 mM Ca^{2+}, ML-7 augmented a transient phase of the rise in $[Ca^{2+}]_i$ more potently than a sustained phase (Fig. 2a). In the presence of 1 mM EGTA, U46619 caused a transient increase in $[Ca^{2+}]_i$ and returned to nearly basal level (Fig. 2b). ML-7 also concentration-dependently augmented the U46619-induced rise in $[Ca^{2+}]_i$ in the presence of EGTA (Fig. 2b and Fig. 2d). Similar results were obtained with W-7 in the presence or absence of 1 mM Ca^{2+} (Fig. 3). These results indicate that ML-7 and W-7 may have potentiating effects on U46619-induced Ca^{2+} mobilization, independently of the existence of external Ca^{2+}.

3.3. Phosphoinositide hydrolysis

It has been suggested that thromboxane A_2 receptor stimulation causes IP_3 formation, which mobilizes Ca^{2+} from the internal Ca^{2+} store. Therefore, we studied the effects of ML-7 and W-7 on U46619-induced phosphoinositide hydrolysis to determine whether the potentiating effect on Ca^{2+} mobilization induced by U46619 was related to the facilitation of phosphoinositide hydrolysis. U46619 caused phosphoinositide hydrolysis in the presence or absence of external Ca^{2+} (Fig. 4). In the presence of external Ca^{2+}, ML-7 potentiated U46619-induced phosphoinositide hydrolysis in a concentration-dependent manner (Fig. 4). Interestingly, the potentiation of phosphoinositide hydrolysis by ML-7 was much more apparent in the absence than presence of external Ca^{2+}. ML-7-induced potentiation of U46619-induced phosphoinositide hydrolysis showed the same concentration dependency observed in its potentiation of Ca^{2+} mobilization (Fig. 2). ML-7 at a concentration of 100 μM caused phosphoinositide hydrolysis by itself in the presence of 1 mM Ca^{2+} (156% compared to the basal level of phosphoinositide hydrolysis), suggesting a non-specific effect of this drug (Fig. 4).

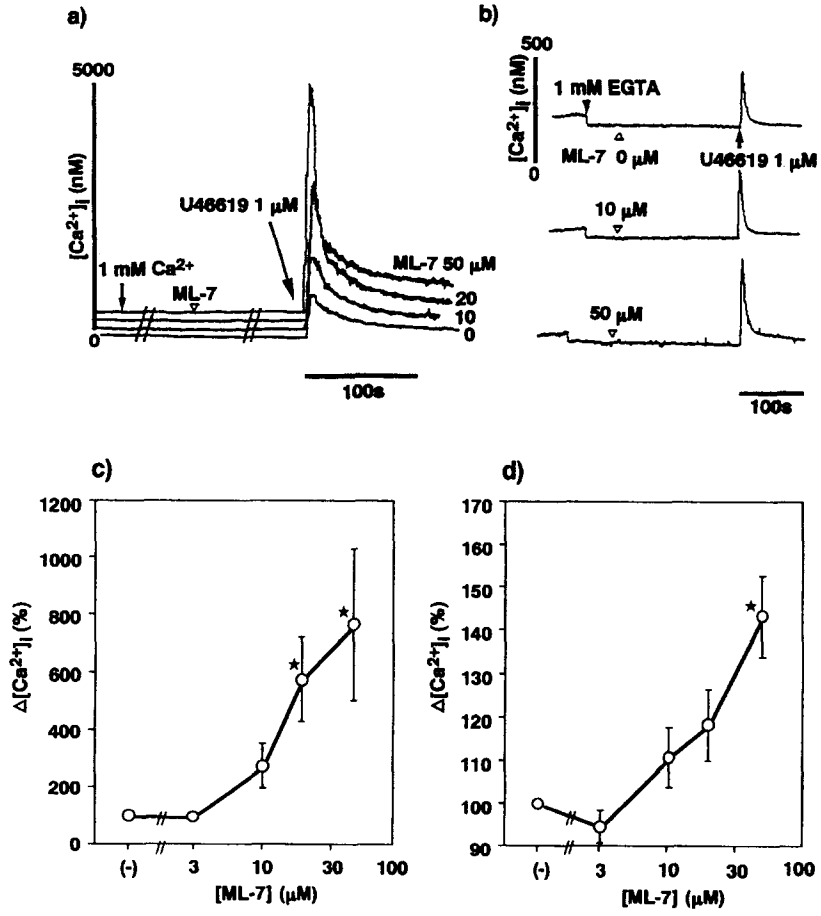

Fig. 2. Potentiating effects of ML-7 on U46619-elicited Ca^{2+} mobilization in the presence or absence of external Ca^{2+} ions. Washed platelets were loaded with 1 μM fura-2/AM for 15 min at 37°C. After washing, platelets (1×10^8/ml) were preincubated with each concentration of ML-7 for 3 min in the presence of 1 mM Ca^{2+} or 1 mM EGTA and stimulated with 1 μM U46619. The changes in $[Ca^{2+}]_i$ were calculated as described under Materials and methods. (a) Typical responses in the presence of 1 mM Ca^{2+}. (b) Typical responses in the presence of 1 mM EGTA. (c) Concentration-dependent augmentation of U46619-induced Ca^{2+} mobilization by ML-7 in the presence of 1 mM Ca^{2+}. (d) Concentration-dependent augmentation of U46619-induced Ca^{2+} mobilization by ML-7 in the presence of 1 mM EGTA. U46619 (1 μM)-induced increase in $[Ca^{2+}]_i$ in the presence of ML-7, expressed as the increase without ML-7 was 100%. Each point represents the mean ± S.E. from three experiments. * Significant difference from U46619 alone without ML-7 ($P < 0.05$).

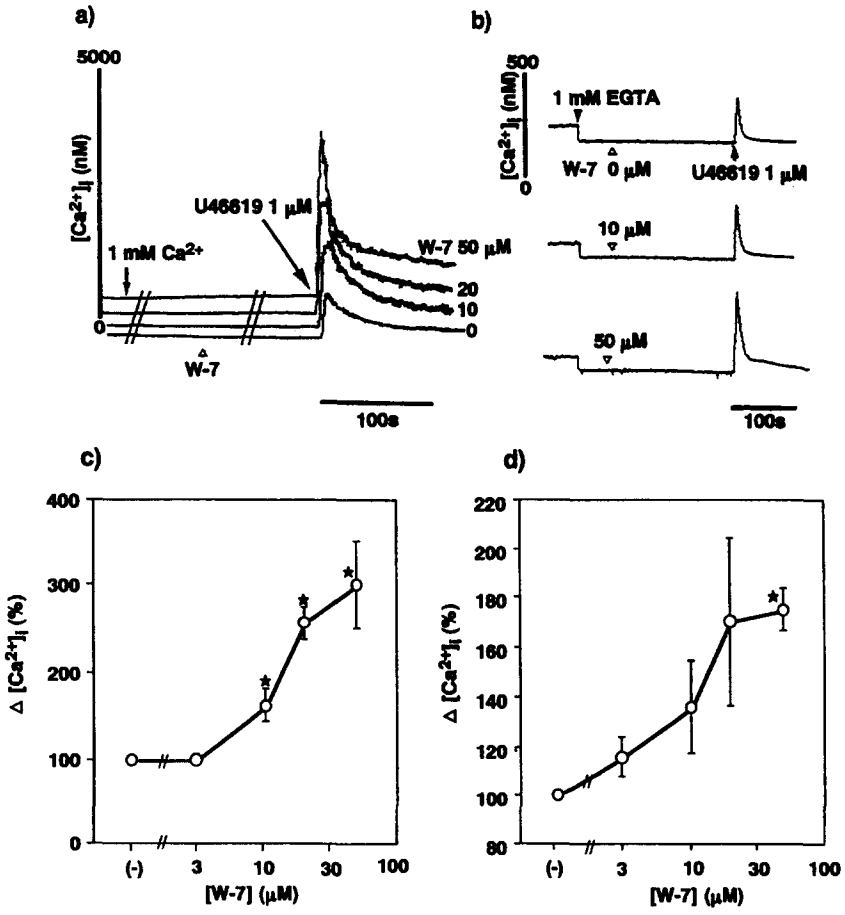

Fig. 3. Potentiating effects of W-7 on U46619-induced Ca^{2+} mobilization in the presence or absence of external Ca^{2+} ions. Washed platelets were loaded with 1 μM fura-2/AM for 15 min at 37°C. After washing, platelets (1×10^8/ml) were preincubated with each concentration of W-7 for 3 min in the presence of 1 mM Ca^{2+} or 1 mM EGTA and stimulated with 1 μM U46619. The changes in $[Ca^{2+}]_i$ were calculated as described under Materials and methods. (a) Typical responses in the presence of 1 mM Ca^{2+}. (b) Typical responses in the presence of 1 mM EGTA. (c) Concentration-dependent augmentation of U46619-induced Ca^{2+} mobilization by W-7 in the presence of 1 mM Ca^{2+}. (d) Concentration-dependent augmentation of U46619-induced Ca^{2+} mobilization by W-7 in the presence of 1 mM EGTA. U46619 (1 μM)-induced increase in $[Ca^{2+}]_i$ in the presence of W-7, expressed as the increase without W-7, was 100%. Each point represents the mean ± S.E. from three experiments. * Significant difference from U46619 alone without W-7 ($P < 0.05$).

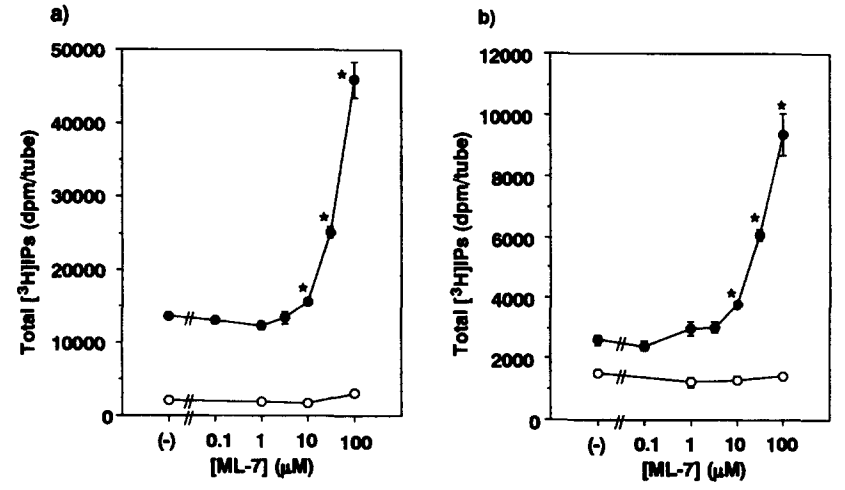

Fig. 4. Effect of ML-7 on U46619-induced phosphoinositide hydrolysis in rabbit platelets. (a) Washed platelets were labeled with 25 μCi/ml of [^{3}H]inositol for 1 h. After washing, platelets were incubated with each concentration of ML-7 in the presence (●) or absence (○) of 1 μM U46619 for 12 min in 10 mM Li^+-containing buffer in the presence of 1 mM Ca^{2+} (a) or 1 mM EGTA (b). Each point represents the mean ± S.E. from three determinations.

W-7 also concentration-dependently potentiated U46619-induced phosphoinositide hydrolysis in the presence or absence of external Ca^{2+} (Fig. 5), similar to ML-7. W-7 at high concentrations caused phosphoinositide hydrolysis by itself, independently of external Ca^{2+}.

To ensure the potentiating effects of ML-7 and W-7 on Ca^{2+} mobilization, IP_3 was determined instead of total inositol phosphates (Fig. 6). U46619 (1 μM) increased [^{3}H]IP_3 30 s after its addition. Preincubation of platelets with ML-7 (50 μM) or W-7 (50 μM) for 3 min resulted in a significant augmentation in U46619-induced [^{3}H]IP_3 accumulation. These results suggest that the potentiating effect of ML-7 or W-7 on Ca^{2+} mobilization induced by U46619 might be due to the facilitation of phosphoinositide hydrolysis.

3.4. Effects of ML-7 and W-7 on U46619-induced GTPase activation

To examine the possible site of action by ML-7 or W-7, GTPase activity was measured using [γ-^{32}P]GTP in platelet membranes. As shown in Fig. 7, ML-7 and W-7 inhibited the basal level of GTPase activity in a concentration-dependent manner. However, U46619-induced GTPase activation was augmented by ML-7 and W-7. For instance, ML-7 at 100 μM augmented U46619-elicited GTPase activation by 1.4- to 2.3-fold compared to that in the absence of ML-7 ($n = 8$). The potency of ML-7 was different in membrane preparations. Similar to ML-7, W-7 at 100 μM augmented U46619-induced GTPase activation by 1.2- to 2.6-fold ($n = 10$). The measurement of GTPase activity had been performed in the buffer containing 0.5 mM EGTA, where calmodulin could not form complexes with Ca^{2+} and not activate myosin light chain kinase. Therefore, ML-7 and W-7 augmented thromboxane A_2-mediated signal transduction at the receptor/G protein level, independently of inhibitions of myosin light chain kinase and calmodulin.

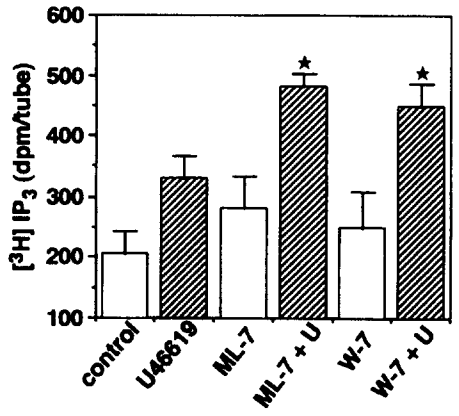

Fig. 6. Potentiating effects of ML-7 and W-7 on U46619-induced [^{3}H]IP_3 accumulation. Washed platelets were labeled with 25 μCi/ml of [^{3}H]inositol for 1 h. Platelets were preincubated with ML-7 (50 μM) or W-7 (50 μM) for 3 min, and they were then stimulated by U46619 (1 μM) for 30 s in the presence of 1 mM Ca^{2+} without LiCl. [^{3}H]IP_3 was separated by an anion exchange column (AG 1X8). The data represent the mean ± S.E. from three determinations. * Significant difference from U46619 alone ($P < 0.05$).

4. Discussion

It has been reported that myosin light chain kinase inhibitors and calmodulin antagonists have inhibitory ef-

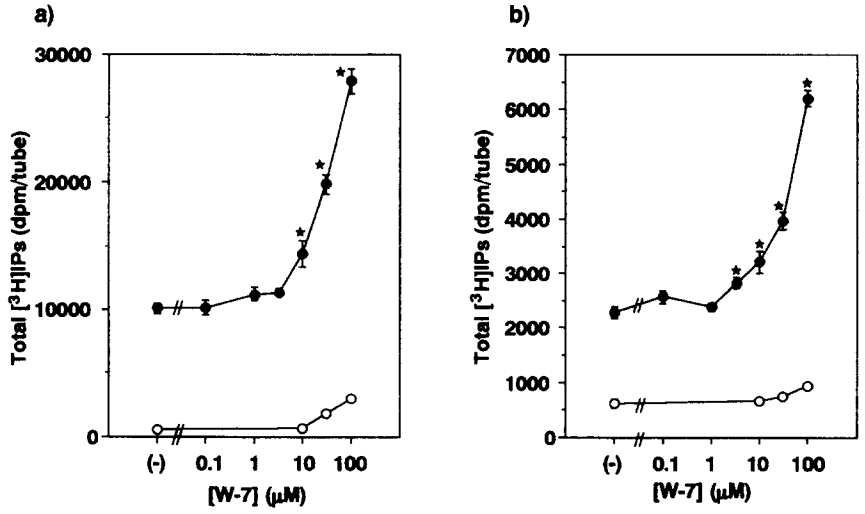

Fig. 5. Effect of W-7 on U46619-induced phosphoinositide hydrolysis in rabbit platelets. Washed platelets were labeled with 25 μCi/ml of [^{3}H]inositol for 1 h. After washing, platelets were incubated with each concentration of W-7 in the presence (●) or absence (○) of 1 μM U46619 for 10 min in 10 mM Li^+-containing buffer in the presence of 1 mM Ca^{2+} (a) or 1 mM EGTA (b). Each point represents the mean ± S.E. from three determinations. * Significant difference from U46619 alone without W-7 ($P < 0.05$).

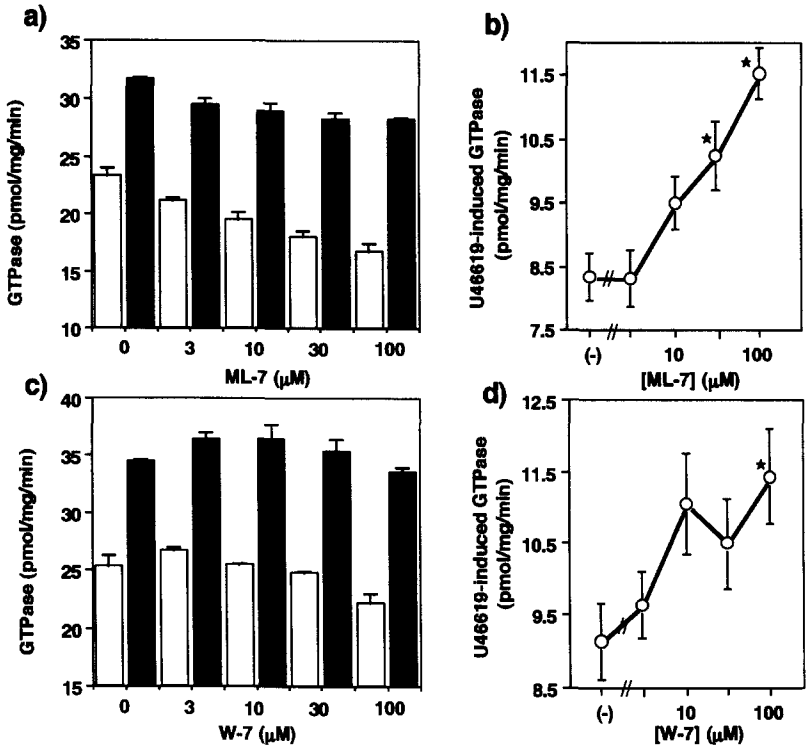

Fig. 7. Effects of ML-7 and W-7 on U46619-induced GTPase activation in platelet membranes. Platelet membranes (5 μg/tube) were incubated with the indicated concentrations of ML-7 (a) or W-7 (c) in the presence (hatched columns) or absence (open columns) of 1 μM U46619. Augmentations of U46619 (1 μM)-induced GTPase activation by ML-7 and W-7 were expressed in (b) and (d), respectively. Released ^{32}P was counted as described under Materials and methods. The data represent the mean ± S.E. from three determinations. * Significant difference from U46619 alone ($P < 0.05$).

fects on platelet activation including aggregation, secretion, and shape change (Nishikawa et al., 1980; Saitoh et al., 1986). ML-7 or W-7 inhibits phosphorylation of 20 kDa myosin light chain induced by thrombin or collagen in platelets.

In the present study, we examined the effects of ML-7 and W-7 on thromboxane A_2-stimulated platelet activation. Unexpectedly, these drugs at low concentrations did not inhibit, but rather, slightly potentiated, U46619-induced platelet aggregation. Trifluoperazine, a calmodulin antagonist, has been reported to potentiate thrombin-induced aggregation by facilitation of Ca^{2+} mobilization and secretion in human platelets (Rao, 1987). Similar results have also been reported using other calmodulin antagonists, fendiline and calmidazolium (Lückhoff et al., 1991). However, these two compounds could inhibit aggregation in spite of potentiating effects on secretion from human platelets. This discrepancy might be due to the balance between the inhibition of myosin light chain kinase and the potentiation of secretion.

ML-7 and W-7 potentiated significantly the U46619-induced increase in $[Ca^{2+}]_i$, independently of external Ca^{2+}. These phenomena are concomitant with the potentiating effects of ML-7 and W-7 on U46619-induced phosphoinositide hydrolysis. Since these drugs at 100 μM could inhibit aggregation in spite of significant potentiating effects on phosphoinositide hydrolysis and Ca^{2+} mobilization, the activation of myosin light chain kinase or calmodulin might be inhibited fully by these drugs at high concentrations. In additon to the potentiation of phosphoinositide hydrolysis and Ca^{2+} mobilization by ML-7 and W-7, U46619-induced activation of GTPase was also potentiated by these drugs. Thromboxane A_2 receptor is known to communicate with $G_{q/11}$, leading to an activation of phospholipase C-β(Baldassare et al., 1993; Nakahata et al., 1995). We can then assume that ML-7 and W-7 interact with $G_{q/11}$ resulting in an activation of phospholipase C and mobilization of Ca^{2+}, which in turn potentiate the aggregation induced by U46619.

Nishikawa et al. (1980) have shown that W-7 at a concentration of 100 μM completely inhibits the phosphorylation of myosin light chain in thrombin-stimulated human platelets. Furthermore, W-7 inhibits endogenous phosphorylation of myosin light chain from human platelets in vitro in a concentration-dependent manner (20–200 μM) with an IC_{50} of 80 μM. Since slightly lower concentrations of W-7 (10–100 μM) augmented thromboxane A_2-mediated Ca^{2+} signaling, the balance of myosin light chain kinase inhibition and augmenting Ca^{2+} signaling may result in the final response, i.e. potentiation or inhibition of aggregation.

The present study demonstrates that ML-7 and W-7 potentiate U46619-induced aggregation by activation of $G_{q/11}$. However, the activation of U46619-induced GT-

Pase by ML-7 or W-7 was smaller than those of phosphoinositide hydrolysis or Ca^{2+} mobilization. Therefore, we could not exclude other mechanisms of the augmentation of Ca^{2+} mobilization besides the activation of $G_{q/11}$. Recently, it has been reported that $p72^{syk}$, a non-receptor type tyrosine kinase, is rapidly activated by thromboxane A_2 mimetics, 9α,11β-epoxy-methanoprostaglandin H_2 (U44069) and 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2) in porcine platelets (Maeda et al., 1993). The activation of tyrosine kinase including $p72^{syk}$ might regulate platelet function such as aggregation (Asahi et al., 1992; Bachelot et al., 1992; Taniguchi et al., 1993). Then, it is possible that the preincubation with ML-7 or W-7 results in the modification of acitivity of tyrosine kinases including $p72^{syk}$ which in turn augments U46619-induced Ca^{2+} mobilization.

ML-7 and W-7 stimulate thromboxane A_2 receptor-mediated activation of GTPase in rabbit platelet membranes. As GTPase activity was measured in the presence of 0.5 mM EGTA, membrane-bound calmodulin could not be activated. It has been reported that W-7 only binds the Ca^{2+}-calmodulin complex at the hydrophobic site of the protein, but not bound in the absence of Ca^{2+} ions (Tanaka and Hidaka, 1980). Therefore, W-7 may have another site to modulate thromboxane A_2 receptor-coupled G protein activation than calmodulin. ML-9 has been suggested to bind at or near the ATP-binding site on the myosin light chain kinase (Saitoh et al., 1987). ML-9 also binds to both of Ca^{2+}-calmodulin-dependent and -independent myosin light chain kinase with a similar concentration dependency (Tanaka et al., 1980; Saitoh et al., 1987). ML-7 would also bind to myosin light chain kinase in the absence of Ca^{2+} ions. However, ML-7-induced potentiation of GTPase is not mediated via Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase in the present experiments, because the potentiation is observed in the presence of EGTA. Spence and Houslay (1989, , 1993 have shown that a membrane perturbing agent, benzyl alcohol, changes G protein-mediated signaling of adenylate cyclase. Therefore, one of the possible mechanisms of the potentiation of ML-7 and W-7 in thromboxane A_2 receptor-mediated Ca^{2+} mobilization is their membrane perturbing action that changes G protein function. To elucidate this possibility, further experiments are necessary.

ML-7 and W-7 are similar in their chemical structure by having a halogen molecule at position 5 of the naphthalene ring. W-7 and ML-7 have chloride and iodine at position 5 of the naphthalene ring, respectively. The similarity of the chemical structure between them might be related to the analogous effects of these drugs on thromboxane A_2 receptor-mediated signal transduction.

In the present study, we show that ML-7 and W-7 potentiate thromboxane A_2 receptor/G protein coupling in rabbit platelets besides the inhibition of myosin light chain kinase or calmodulin. Therefore, it is necessary to pay attention to the interpretation of the results derived from the experiments in which these drugs are used as a myosin light chain kinase inhibitor or a calmodulin antagonist. Further detailed analysis of the acting site of ML-7 or W-7 may be helpful to elucidate the regulation of thromboxane A_2 receptor-coupled G proteins including $G_{q/11}$.

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