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Selective Modulation of Calcium-Dependent Myosin Phosphorylation by Novel Protein Kinase Inhibitors, Isoquinolinesulfonamide Derivatives

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SUMMARY

Ca^{2+}-dependent myosin phosphorylation by Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLC-kinase) and protein kinase C were studied using selective inhibitors, isoquinolinesulfonamide derivatives. Both protein kinases were potently inhibited by 1-(8-chloro-5-isoquinolinesulfonyl)piperazine (HA-156) and its derivatives. Kinetic analysis indicated that HA-156 inhibited both enzymes competitively with respect to ATP, and K_i values of HA-156 for MLC-kinase and protein kinase C were 7.3 and 7.2 μM, respectively. To clarify molecular mechanisms of the isoquinolinesulfonamides to inhibit the Ca^{2+}-dependent protein kinases, we examined the structure-activity relationships of HA-156 and its derivatives. The dechlorinated analogues, HA-100 and HA-142, markedly decreased the affinity for MLC-kinase, suggesting that the inhibitory effect of isoquinolinesulfonamide derivatives depends upon hydrophobicity of the compounds. There is a good correlation between MLC-kinase inhibition and hydrophobicity determined by reverse phase chromatography. In contrast, HA-140 and HA-142 showed weak inhibition of protein kinase C, suggesting that the electron density of the nitrogen in the isoquinoline ring of the compounds correlates with the potency to inhibit protein kinase C activity. These pairs of isoquinolinesulfonamides will aid in elucidating the biological roles of Ca^{2+}-dependent myosin phosphorylation in intact cells. HA-156 and HA-140 inhibited myosin light chain phosphorylation in platelets exposed to collagen, whereas HA-142 and HA-100 did not, significantly. These isoquinolinesulfonamide derivatives should prove to be useful tools for distinguishing between the biological functions of Ca^{2+}-activated, phospholipid-dependent, and Ca^{2+}/calmodulin-dependent myosin light chain phosphorylation, *in vivo*.

Biological functions of vascular smooth muscle and blood platelets, including contraction and secretion, are expressed through cellular contractile activity. The Ca^{2+}/CaM-dependent phosphorylation of myosin light chain catalyzed by MLC-kinase may be the major regulatory system of contractile proteins in smooth muscle and nonmuscle cells (1–7). We obtained evidence that protein kinase C is responsible for phosphorylation of the myosin light chain, during platelet activation (8, 9). Studies *in vitro* indicated that the phosphorylation by protein kinase C modified the Ca^{2+}/CaM-dependent regulation of the actin-myosin interaction (10). Pharmacological antagonism of each myosin phosphorylation is expected to be a fruitful approach to manipulation of cellular responses and for elucidation of the biological role of myosin phosphorylation in various cells and tissues. A number of agents such as antipsychotic drugs, local anesthetics, and polypeptide cytotoxins inhibit protein kinase C (11). Most of these compounds interact with phospholipids and thereby block enzyme-activating processes. However, these drugs may also interfere with the metabolism of phospholipids or other functions related to phospholipids, and these phospholipid-interacting agents may interact with the Ca^{2+}/CaM complex, because the active region of CaM is hydrophobic, as is the case with phospholipids (12–16). There are striking similarities between protein kinase C and some CaM-dependent enzymes, events which may account for their susceptibility to inhibition by the same type of drugs. This is a serious disadvantage since natural stimuli which activate protein kinase C with phosphatidylinositol breakdown usually elevate the levels of cytosolic Ca^{2+} and lead to activation of CaM-dependent reactions.

In continuing efforts to elucidate the regulatory mechanism and functional roles of each Ca^{2+}-dependent myosin phosphorylation, a series of isoquinolinesulfonamide and naphthalenesulfonamide derivatives was synthesized in our laboratory, the

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ABBREVIATIONS: CaM, calmodulin; MLC-kinase, myosin light chain kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*^{1},*N*^{1}-tetraacetic acid; EDTA, ethylenediaminetetraacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P-light chain, phosphorylatable light chain; HA-156, 1-(8-chloro-5-isoquinolinesulfonyl)piperazine.

objective being to acquire compounds which would directly inhibit each Ca^{2+}-dependent protein kinase (17, 18). We report herein the structure-activity relationship for selective inhibition of MLC-kinase and protein kinase C, by analogues of isoquinolinesulfonamide derivatives.

Materials and Methods

Isoquinolinesulfonamide derivatives were synthesized by the method of Hidaka *et al.* (18). Phosphatidylserine (pig liver) was purchased from Serdary Research Laboratories, Inc. Chloroform was removed from this phospholipid by a stream of nitrogen and the phospholipid was sonicated in water for 1 min to produce a suspension of 0.5 mg/ml. [γ-^{32}P]ATP was obtained from Amersham, England. All other chemicals were reagent grade.

CaM was isolated from bovine brain and purified by the procedure reported by Yazawa *et al.* (19). Myosin light chain was purified from chicken gizzard by the method of Hathaway and Haeberle (20). Protein kinase C from rabbit brain was purified by the method of Inagaki *et al.* (21), and MLC-kinase was prepared from chicken gizzard by the method of Walsh *et al.* (22). Both were homogenous as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. cAMP-dependent protein kinase catalytic subunit was prepared from bovine heart by the method of Beavo *et al.* (23). cGMP-dependent protein kinase from pig lung was partially purified by the method of Kuo and Greengard (24). Protein was determined by the method of Lowry *et al.* (25) with bovine serum albumin as the standard.

Enzyme assay and determinations. Protein kinase activities were determined under the conditions described by Inagaki *et al.* (17), as follows. MLC-kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0) and 10 mM magnesium acetate, 0.1 mM calcium chloride or 1 mM EGTA, 100 ng of CaM, 5–100 μM [γ-^{32}P]ATP (4×10^5 cpm), 20 μM smooth muscle 20,000-Da myosin light chain, and 0.6 μg of MLC-kinase. Protein kinase C activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 0.5 mM calcium chloride or 1 mM EGTA, 10 μg of PS, 3.3–20 mM [γ-^{32}P]ATP (4×10^5 cpm), 20 μM smooth muscle myosin 20,000-Da light chain, and 0.3 μg of the enzyme. MLC-kinase and protein kinase C were treated with trypsin as described by Tanaka *et al.* (26) and Inoue *et al.* (27), respectively. cAMP-dependent protein kinase was measured in a reaction mixture (0.2 ml) containing 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 2 mM EGTA, 3.3–20 μM [γ-^{32}P] ATP (4×10^5 cpm), 0.5 μg of the enzyme, and 100 μg of histone H2B. cGMP-dependent protein kinase activity was assayed in a reaction mixture (0.2 ml) containing 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 2 mM EGTA, 1 μM cGMP, 3.3–20 μM [γ-^{32}P]ATP (4×10^5 cpm), 100 μg of histone H2B, and 2.4 μg of the enzyme. Assays were performed at 30° for 5 min and reaction was terminated by the addition of 1 ml of ice-cold 20% trichloroacetic acid following addition of 500 μg of bovine serum albumin as a carrier protein. The sample was centrifuged at 3,000 rpm for 15 min, the pellet was resuspended in ice-cold 10% trichloroacetic acid solution, and the centrifugation-resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 1 N NaOH, and radioactivity was measured by a liquid scintillation counter. Adenylate cyclase activity was assayed in platelet membrane fractions and guanylate cyclase activity was assayed in soluble fractions after centrifugation of platelet-sonicated homogenate at $105{,}000 \times g$ for 15 min. Activities of adenylate cyclase and guanylate cyclase were determined as described by Nakazawa *et al.* (28) and Hagiwara *et al.* (29). The myosin ATPase assay was carried out at 25° in a volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.0), 500 mM KCl, 5 mM EDTA, 0.01 mg/ml of myosin from rabbit skeletal muscle prepared by the method of Perry (30), and 0.2–1.0 mM ATP for myosin (K^+, EDTA)-ATPase; 50 mM Tris-HCl (pH 7.0), 500 mM KCl, 10 mM $CaCl_2$, 0.1 mg/ml myosin, and 0.2–1.0 mM ATP for myosin Ca^{2+}-ATPase. Assays were performed for 5 min at 25° and the reaction was terminated by the addition of 1 ml of 20% trichloroacetic acid. The preparations were filtered, and inorganic phosphate was measured according to the method of Martin and Dotty (31).

Phosphorylation of 20-k Da protein in platelets. Human blood was collected from healthy volunteers not ingesting any drugs for at least 1 week. 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 30°) were incubated for 60 min at 30° with 0.5 mCi/ml of [^{32}P] orthophosphate and spun at $600 \times g$ for 5 min; then, the supernatant was discarded (32). The pellets were resuspended in the same buffer to give a count of $5–10 \times 10^8$/ml. The platelets were preincubated with saline or drugs for 60 min before stimulation with collagen (1 μg/ml). The reaction was stopped by the addition of 0.5 ml of 0.6 N $HClO_4$ at indicated times directly into the stirred cuvette. Samples were analyzed for mol of phosphate/mol of P-light chain on alkaline-urea polyacrylamide gel electrophoresis as described by Daniel *et al.* (33). Tryptic peptide mapping was carried out as described (9, 10). In brief, the stained protein band was cut from a 15% polyacrylamide electrophoresis slab gel and transferred to a glass tube. The gel slice was washed and dried, and then 50 μg/ml of trypsin in 0.5 ml of NH_4HCO_3 (pH 8.4) were added to each tube. After incubation for 20 hr at 37°, the supernatant (which contained most of the tryptic peptide) was lyophilized and the residue was redissolved in 20 μl of TLE buffer (acetic acid/formic acid/H_2O = 15:5:8). A 3- to 6-μl portion of this solution was spotted on a silica gel-coated thin layer chromatography plate, and tryptic peptides were resolved by electrophoresis in the first dimension and ascending chromatography in the second dimension. Electrophoresis was carried out at 950 V for 80 min in TLE buffer using a Pharmacia flat bed apparatus FBE 3000 with cooling at 4°. Chromatography in thin layer chromatography buffer (butanol/pyridine/acetic acid/H_2O = 32.5:25:5:20) in a thin layer chromatography chamber was continued for about 4 hr until the front reached the top. The plate was then dried and examined by autoradiography using Kodak X-Omat R film.

Determination of retention index. The retention index of each compound was determined by the method of Backer *et al.* (34). High pressure liquid chromatography was carried out using a Nippon Bunko Inc. Tri-Rotor III system and C_{18} reverse phase column (Finepak SIL $C_{18}S$). The mobile phase was composed of a phosphate buffer (pH 7.0) and methanol (3:7), and the flow rate was 1.0 ml/min. The retention index of the drug studied was defined according to Eq. 1 using

$$\text{retention index} = 100 \times \frac{\log K_D - \log K_N}{\log K_{N+1} - \log K_N} + 100N \qquad (1)$$

where K_D, K_N, and K_{N+1} are the observed capacity factors for the test compound, for 2-ketoalkane standard eluting just before the test compound, and for higher homologue, respectively. The capacity factors (K) of drugs and standards were determined from the observed retention time (T_R) using Eq. 2, where T_O is the void volume, as detected by the solvent front:

$$K = \frac{T_R - T_O}{T_O} \qquad (2)$$

Results

Inhibition of MLC-kinase and protein kinase C by HA-156. An isoquinolinesulfonamide, 1-(8-chloro-5-isoquinolinesulfonyl)-piperazine (HA-156), was found to inhibit both Ca^{2+}-dependent myosin phosphorylation by MLC-kinase and protein kinase C (Fig. 1). This compound showed potent inhibition toward both cofactor-activated and trypsin-treated enzymes, suggesting the compound directly interacted with the catalytic site of the enzymes. Kinetic analysis using Lineweaver-Burk plots indicated that HA-156 inhibited both enzymes, competitively with respect to ATP (Fig. 2) and noncompetitively with

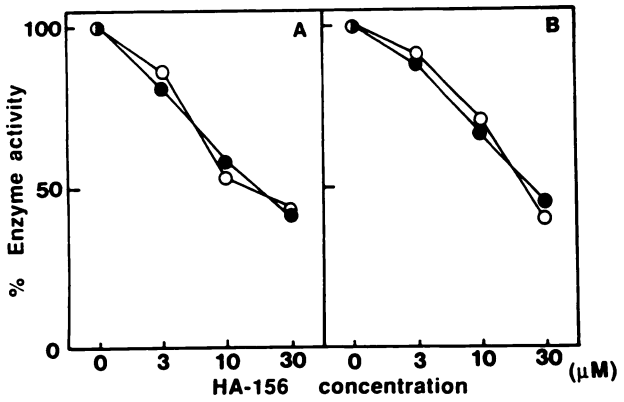


Fig. 1. Dose-dependent inhibition of MLC-kinase (A) and protein kinase C (B) by HA-156. Both kinase activities are determined using myosin light chain as a substrate protein. Trypsin digestion of both enzymes and myosin phosphorylation assay were performed under conditions as described under Materials and Methods. ○, cofactor-activated enzyme activity; ●, trypsin-treated enzyme activity. Phosphotransferase activities are expressed as a percentage of the control enzyme activity in the absence of HA-156.

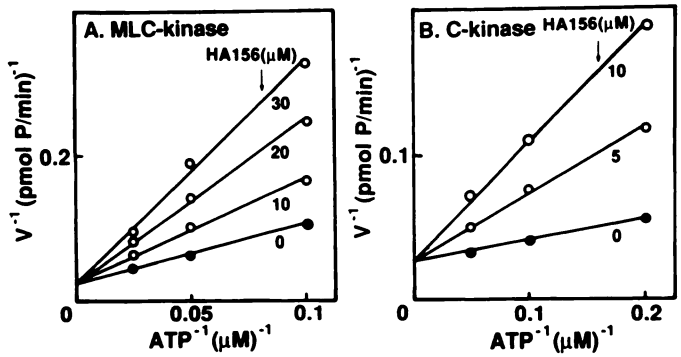


Fig. 2. Inhibition patterns of MLC-kinase (A) and protein kinase C (B) by HA-156. Reciprocal velocity was plotted vs. 1/[ATP] at 20 μM myosin light chain with varying HA-156 concentrations. All other conditions are as described under Materials and Methods. The lines are those fit to the data point by using the equations for simple linear competitive inhibition.

respect to myosin light chain and cofactors such as Ca^{2+}, phospholipids, and CaM (data not shown).

Activity-structure study of HA-156 and its derivatives. We investigated the structural requirements for MLC-kinase and protein kinase C inhibition of the compounds, as shown in Fig. 3. Kinetic analysis also showed that these derivatives inhibit MLC-kinase and protein kinase C competitively with respect to ATP (Fig. 4). Also, Fig. 3 clearly showed that chlorination of the aromatic ring increased the potency of these compounds in inhibition of MLC-kinase, but not of protein kinase C. Inhibition of MLC-kinase by chlorinated compounds (HA-156 and HA-140) was 8- to 10-fold more potent than that of dechlorinated compounds (HA-100 and HA-142), suggesting that the actions of isoquinolinesulfonamide derivatives, as inhibitors of MLC-kinase, depend on the hydrophobicity of compounds. To determine the hydrophobicity of the compounds, we measured the retention indices of the drugs on reverse phase high pressure chromatography, according to the method of Baker *et al.* (34). Retention indices of HA-156, HA-140, HA-100, and HA-142 were 890, 882, 773, and 738, respectively. These indices were found to correlate with the K_i values for MLC-kinase inhibition (Fig. 5). The correlation coefficient is $r = 0.99$, suggesting that the ability of isoquinolinesulfonamide derivatives to inhibit MLC-kinase activity is closely related to the hydrophobicity of these compounds.

Effect of isoquinolinesulfonamide derivatives on ATP- and/or GTP-utilizing enzymes. Since these isoquinolinesulfonamide derivatives compete with ATP in both Ca^{2+}-dependent protein kinase reactions, we investigated the effects of these compounds on a wider range of protein kinases and other ATP- and/or GTP-utilizing enzymes such as adenylate cyclase and guanylate cyclase from human platelets and myosin ATPases from rabbit skeletal muscle. Table 1 summarized the results obtained with HA-156 and its derivatives. HA-156 and HA-100 exhibited potent inhibition toward protein kinase C and cyclic nucleotide dependent protein kinases, whereas these enzymes were only slightly affected by HA-140 or HA-142. All these compounds at the concentration of 1 mM did not affect the activities of myosin ATPases, adenylate cyclase, or guanylate cyclase.

Inhibition of myosin light chain phosphorylation in platelets. We examined the inhibitory effects of isoquinolinesulfonamide derivatives on myosin light chain phosphorylation in the collagen-stimulated platelets. As shown in Fig. 6, the 20,000-Da phosphorylatable myosin light chain (P-light chain) phosphorylation reached a maximal value of 0.80 mol of phosphate per mol of P-light chain at 60 sec without any compounds. Two-dimensional phosphopeptide mapping of this phosphorylated 20-kDa light chain, following tryptic digestion, revealed that MLC-kinase phosphorylated this myosin 20-kDa light chain and the site phosphorylated by protein kinase C was not seen in the early phase during collagen activation (Fig. 6, *inset B*). Pretreatment of the platelets with 100 μM HA-156 or HA-140 followed by collagen activation resulted in a marked reduction of radioactive phosphate incorporation into the 20-kDa light chain, whereas HA-100 or HA-142 did not reveal any significant effect.

Discussion

There are at least two different protein kinases related to Ca^{2+}-dependent phosphorylation of smooth muscle and non-

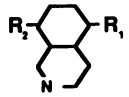
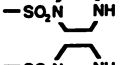
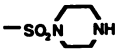
	R_1	R_2	chemical name	Ki(μM) MLC-kinase	Ki(μM) Protein kinase C
		-H	1-(5-isoquinolinesulfonyl)-piperazine(HA-100)	61	6.5
		-Cl	1-(8-chloro-5-isoquinoline-sulfonyl)-piperazine(HA-156)	7.3	7.2
	-H		1-(8-isoquinolinesulfonyl)-piperazine(HA-142)	70	96
	-Cl		1-(5-chloro-8-isoquinoline-sulfonyl)-piperazine(HA-140)	8.7	68

Fig. 3. Chemical structures and enzyme inhibition of isoquinolinesulfonamides. MLC-kinase and protein kinase C activities were determined as described under Materials and Methods.

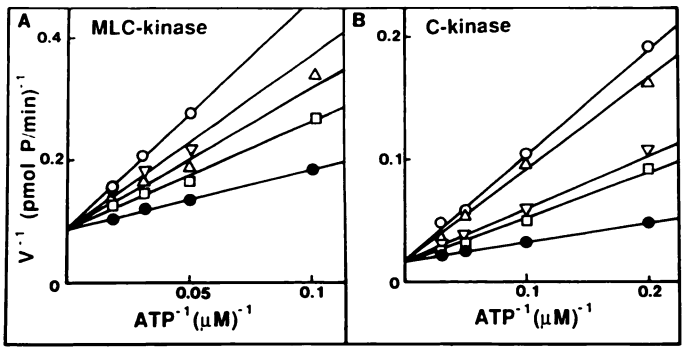


Fig. 4. Inhibition pattern of MLC-kinase (A) and protein kinase C (B) by isoquinolinesulfonamide derivatives. MLC-kinase activity was assayed in the absence (●) or presence of 20 μM HA-156 (○) or HA-140 (▽), and 200 μM HA-100 (△) or HA-142 (□). Protein kinase C activity was determined in the absence (●) or presence of 30 μM of HA-156 (○) or HA-100 (▽), and 200 μM HA-140 (△) or HA-142 (□). Reciprocal velocity is plotted versus 1/[ATP]. All other conditions are as described under Materials and Methods.

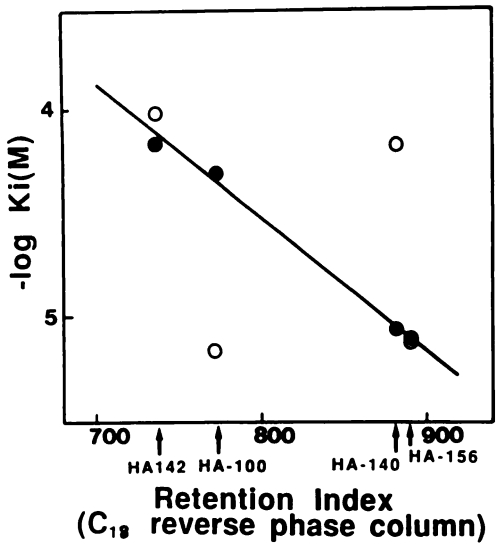


Fig. 5. Hydrophobicity and protein kinase inhibition of isoquinolinesulfonamides. The *ordinate* expresses the log (K_i^{-1}) determined in Fig. 3 and the *abscissa* shows the retention indices through C_{18} reverse phase column of isoquinolinesulfonamide derivatives tested under conditions described under Materials and Methods. ●, K_i for MLC-kinase; ○, K_i for protein kinase C.

TABLE 1

Effect of isoquinolinesulfonamide derivatives on ATP/GTP-utilizing enzymes

IC_{50} values of isoquinolinesulfonamide compounds are presented. Data are mean values of two independent experiments. Each protein kinase activity was assayed with 10 μM ATP. See Materials and Methods for details.

	IC_{50}			
	HA-156	HA-100	HA-140	HA-142
	μM			
MLC-kinase	22	240	19	270
Protein kinase C	11	12	180	>500
cAMP-dependent protein kinase	8	8	450	>500
cGMP-dependent protein kinase	6	4	300	>500
Myosin ATPase				
Ca^{2+}-ATPase	>1000	>1000	>1000	>1000
K^+, EDTA-ATPase	>1000	>1000	>1000	>1000
Guanylate cyclase	>1000	>1000	>1000	>1000
Adenylate cyclase	>1000	>1000	>1000	>1000

muscle myosin. One of the protein kinases is MLC-kinase, which is activated by the Ca^{2+}/CaM complex (35), and the other is protein kinase C, which requires phospholipids and diglyceride as cofactors (36). Therefore, isoquinolinesulfonamide derivatives which selectively inhibit protein kinase C and/or MLC-kinase may be useful tools for differentiating Ca^{2+}-dependent phosphorylation of myosin *in vitro* and *in vivo*. Kinetic data indicated that the isoquinolinesulfonamides, HA-156, HA-100, HA-140, and HA-142 showed competitive inhibition with ATP toward both enzymes, suggesting that these compounds bind to the ATP-binding site of the protein kinases. The structure-activity study revealed that chlorination of the isoquinoline ring increased the hydrophobicity and the potency of these compounds in inhibition of MLC-kinase. We previously showed that the potency of various compounds to antagonize CaM is closely related to their extent of lipid solubility (37). Furthermore, the hydrophobic regions of CaM, which can be exposed after alteration of conformation of CaM induced by Ca^{2+} binding, serve as an interface for the binding of CaM to the regulatory sites on target enzymes, including MLC-kinase, and bind to lipophylic compounds (13). In this report the importance of hydrophobic regions in the catalytic site of MLC-kinase as the ATP- or isoquinolinesulfonamide-binding site was suggested. The chlorinated and dechlorinated isoquinolinesulfonamide derivatives were also examined with regard to inhibition of protein kinase C. The K_i values of the chlorinated derivatives (HA-156 and HA-140) and the dechlorinated ones (HA-100 and HA-142) for protein kinase C were 7.2, 68, 6.5, and 96 μM, respectively. The affinity of isoquinolinesulfonamides for protein kinase C cannot be explained solely by nonspecific hydrophobic events. As shown in Fig. 3, the 5-isoquinolinesulfonamide derivatives such as HA-156 and HA-100 were more potent inhibitors of protein kinase C than the 8-isoquinolinesulfonamide derivatives such as HA-140 and HA-142. A similar relation was seen with other pairs of isoquinolinesulfonamides: *N*-(2-aminoethyl)-8-chloro-5-isoquinolinesulfonamide and *N*-(2-aminoethyl)-5-chloro-8-isoquinolinesulfonamide, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide and *N*-(2-aminoethyl)-8-isoquinolinesulfonamide (data not shown). It has been reported that the electron density of nitrogen in the isoquinoline ring of 5-isoquinolinesulfonamide derivatives is significantly higher than that of 8-isoquinolinesulfonamides (38, 39). Therefore, the affinity of isoquinolinesulfonamide derivatives for protein kinase C may depend on the extent of electron density and the position of nitrogen in the isoquinoline ring. HA-156 and HA-100 also potently inhibited cyclic nucleotide-dependent protein kinases. Recently, our group (40) and Parker *et al.* (41) revealed the amino acid sequence of protein kinase C and suggested that the catalytic part of protein kinase C is highly homologous to that of cyclic nucleotide-dependent protein kinases. In addition, we previously showed that isoquinolinesulfonamides such as H-7 and H-9, which interact with the ATP-binding site of enzymes, potently inhibited protein kinase C and cyclic nucleotide-dependent protein kinases but revealed comparatively weak inhibition for casein kinase I and II, and MLC-kinase. Our results may support evidence showing the homology of the ATP-binding site of protein kinase C and cyclic nucleotide-dependent protein kinases.

Fig. 6 indicates that a series of compounds tested can be used for *in vivo* study, and the data given in Fig. 6 showed that the MLC-kinase inhibitors, HA-156 and HA-140, decreased the

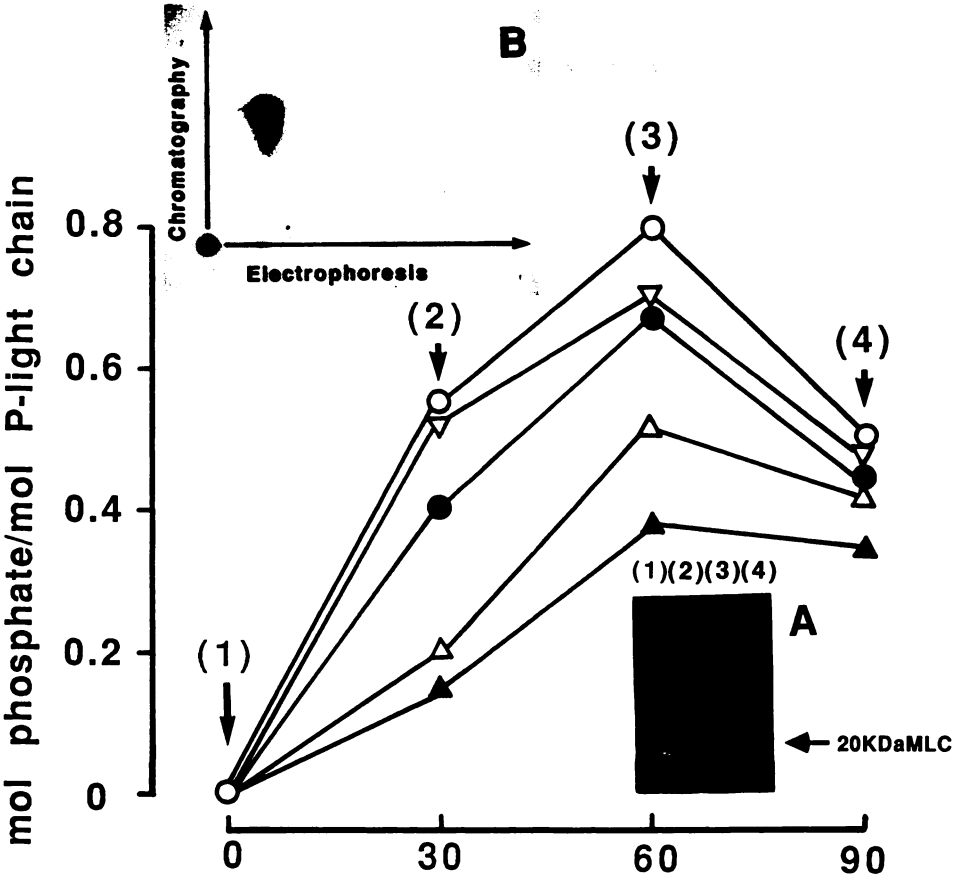


Fig. 6. Time course of 20-kDa peptide phosphorylation in platelets induced by collagen and the effect of isoquinolinesulfonamide preincubation. Samples of washed platelets (0.5 ml) were stirred at 30° in Hepes-buffered saline. Collagen (5 μl of 0.1 mg/ml) was added at time zero. The reaction was stopped by the addition of 0.6 N $HClO_4$ at indicated times directly into the stirred cuvette. Samples were analyzed for percentage of phosphorylation on alkaline-urea polyacrylamide gel electrophoresis as described by Daniel *et al.* (33). The platelets were incubated in the absence of any drugs (○: control) and in the presence of 100 μM HA-142 (▽), HA-140 (△), HA-100 (●), and HA-156 (▲), respectively. *Inset A*, Autoradiograph of platelet endogenous phosphorylation stimulated by 1 μg/ml collagen without any drugs. *Inset B*, Two-dimensional peptide mapping of ^{32}P-labeled 20-kDa peptide presented in the autoradiograph (40 sec).

phosphorylation of myosin light chain in collagen-induced platelets, in accordance with the results of Saitoh *et al.* (42), whereas the protein kinase C inhibitor, HA-100, did not. The data presented in this report are pertinent for designing new inhibitors with a potentially greater specificity for either MLC-kinase or protein kinase C. Highly specific derivatives will be most useful for studies on the function of Ca^{2+}-dependent myosin phosphorylation.

Acknowledgments

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