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Articles

N-(6-Phenylhexyl)-5-chloro-1-naphthalenesulfonamide, a Novel Activator of Protein Kinase C†

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ABSTRACT: Naphthalenesulfonamide derivatives were used to study the mechanism of regulation of Ca^{2+}-dependent smooth muscle myosin light chain phosphorylation catalyzed by Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) and myosin light chain kinase. Derivatives such as *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9), with a hydrophobic residue at the end of a hydrocarbon chain, stimulated Ca^{2+}-activated, phospholipid-dependent myosin light chain phosphorylation in a Ca^{2+}-dependent fashion. There was no significant effect of these compounds on Ca^{2+}–calmodulin (CaM) dependent myosin light chain phosphorylation. On the other hand, derivatives with the guanidino or amino residue at the same position had an inhibitory effect on both Ca^{2+}–phospholipid- and Ca^{2+}–CaM-dependent myosin light chain phosphorylation. These observations suggest that activation of Ca^{2+}-activated, phospholipid-dependent myosin light chain phosphorylation by naphthalenesulfonamide derivatives depends on the chemical structure at the end of hydrocarbon chain of each compound. SC-9 was similar to phosphatidylserine with regard to activation, and the apparent K_m values for Ca^{2+} of the enzyme with this compound and phosphatidylserine were 40 μM and 80 μM, respectively. Kinetic analysis indicated that 12-*O*-tetradecanoylphorbol 13-acetate increased the affinity of the enzyme with SC-9 for calcium ion. However, kinetic constants revealed that the K_m value of protein kinase C activated by SC-9 for substrate myosin light chain was 5.8 μM, that is, about 10 times lower than that of the enzyme with phosphatidylserine, and that the V_{max} value with SC-9 was 0.13 nmol·min^{-1}, that is, 3-fold smaller than that seen with phosphatidylserine. These findings suggest that SC-9 is a novel and potent synthetic activator of protein kinase C. These naphthalenesulfonamide derivatives should prove to be useful tools for elucidating the mechanism of activation of protein kinase C and for distinguishing between Ca^{2+}-activated, phospholipid-dependent and Ca^{2+}–CaM-dependent myosin light chain phosphorylation of smooth muscle, in vitro.

The contractile activity in smooth muscle and nonmuscle cells appears to be regulated by a mechanism that involves the phosphorylation and dephosphorylation of the 20000-dalton (Da) light chain of myosin (Adelstein & Eisenberg, 1980; Hartshorne & Siemankowski, 1981). There are at least two types of Ca^{2+}-dependent phosphorylation of smooth muscle and nonmuscle myosin light chain. One enzyme catalyzing the phosphorylation of the 20000-Da myosin light chain is myosin light chain kinase (MLC[1] kinase), which is activated by the Ca^{2+}–calmodulin (CaM) complex (Sobieszek & Small, 1977; Sherry et al., 1978). Recently, it was demonstrated that

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[1] Abbreviations: MLC, myosin light chain; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; SC-9, *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CaM, calmodulin; HMM, heavy meromyosin; SC-10, *N*-(*n*-heptyl)-5-chloro-1-naphthalenesulfonamide; W-109, *N*-(6-guanidinohexyl)-5-chloro-1-naphthalenesulfonamide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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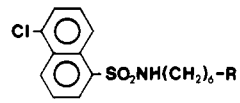

	R	hydrophobic fragmental constant of R
SC-9		1.90
SC-10	$-CH_3$	0.89
W-109	$-NHC(=NH)NH_2$	-5.66
W-7	$-NH_2$	-1.54

FIGURE 1: Chemical structures of naphthalenesulfonamide derivatives and hydrophobic fragmental constants. Abbreviations: SC-9, *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide; SC-10, *N*-(*n*-heptyl)-5-chloro-1-naphthalenesulfonamide; W-109, *N*-(6-guanidinohexyl)-5-chloro-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide. Hydrophobic fragmental constants are those of Leo et al. (1975).

isolated myosin light chain and intact myosin could serve as a substrate for another species of Ca^{2+}-dependent protein kinase (protein kinase C), requiring phosphatidylserine and diglyceride as cofactors (Takai et al., 1979; Endo et al., 1982; Naka et al., 1983). We also found that protein kinase C and MLC kinase catalyze the phosphorylation of different sites within the 20000-Da light chain of smooth muscle heavy meromyosin (HMM) and that sequential phosphorylation of HMM by MLC kinase and protein kinase C decreases the actin-activated MgATPase activity, as compared to that following phosphorylation by MLC kinase alone (Nishikawa et al., 1983, 1984). Most recently, we reported that protein kinase C incorporated phosphate into two sites of myosin light chain kinase and that this phosphorylation resulted in a reduced affinity for calmodulin (Ikebe et al., 1985). This dual regulation of the Ca^{2+}-dependent myosin light chain phosphorylation system by Ca^{2+}–phospholipid and Ca^{2+}–CaM is complex. Several derivatives of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) such as *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9) activate myosin light chain phosphorylation by protein kinase C, without any effect on Ca^{2+}–CaM-dependent myosin light chain phosphorylation. We now report newly synthesized activators of Ca^{2+}-activated, phospholipid-dependent myosin light chain phosphorylation, the structure–activity relationship of the activators, and the mechanism of activation of these synthetic compounds, in comparison with findings of phospholipids and other naturally occurring activators of protein kinase C.

Experimental Procedures

Materials. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and its derivatives were synthesized by the method of Hidaka et al. (1978). Chemical structures of these compounds are shown in Figure 1. W-7 and W-109 were dissolved in water. SC-9 and SC-10 were dissolved in dimethyl sulfoxide at a final concentration of 10 mM, and then the solution was diluted with water for enzyme assay. Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) from rabbit brain was prepared by the procedure of Inagaki et al. (1985). This preparation was confirmed to be free of other interfering enzymes, endogenous phosphate acceptor proteins, cAMP, cGMP, and Ca^{2+}–calmodulin. MLC kinase was purified from chicken gizzard by the method of Adelstein and Klee (1981). Myosin light chain from chicken gizzard myosin was prepared essentially by the method of Perrie and Perry (1970). The light chain was separated from calmodulin by DEAE-cellulose chromatography. The partially purified holoenzyme of cAMP-dependent protein kinase II (second DE-52 step) from bovine heart and cGMP-dependent protein kinase from pig lung were prepared by modification of the methods of beavo et al. (1974) and Kuo and Greengard (1974), respectively. Phosphatidylserine (beef brain) was from Serdary Research Laboratory, 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. Calmodulin (CaM) was isolated from bovine brain and purified as described (Hidaka et al., 1979). [γ-^{32}P]ATP was obtained from Amersham (England), and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was from P-L Biochemicals Inc. Trypsin was from Worthington Biochemical Corp., and soybean trypsin inhibitor was from Sigma Chemical Co. All other chemicals were of reagent grade.

Assay Procedure. Protein kinases were assayed by measuring the incorporation of ^{32}P into myosin light chain from [γ-^{32}P]ATP. Protein kinase C activity was assayed in a reaction mixture containing, in a final volume of 0.2 mL, 25 mM Tris-HCl (pH 7.0), 10 mM magnesium chloride, 30 μg of smooth muscle 20000-dalton myosin light chain, 10 μM [γ-^{32}P]ATP (4×10^5 cpm), and 0.5 μg of the enzyme. The concentrations of Ca^{2+}, phosphatidylserine, and TPA employed are indicated in each experiment. The concentration of Ca^{2+} was calculated by Ca^{2+}–EGTA buffer. MLC kinase activity was determined under the condition described earlier (Tanaka et al., 1980) in a reaction mixture containing, in a final volume of 0.2 mL, 40 mM Tris-HCl (pH 7.0), 5 mM magnesium chloride, 0.25 mM calcium chloride or 1 mM EGTA, 200 ng of calmodulin, 10 μM [γ-^{32}P]ATP (4×10^5 cpm), 30 μg of smooth muscle 20000-dalton myosin light chain, and 0.6 μg of MLC kinase. The incubation was carried out for 12 min in the assay of protein kinase C and for 5 min in the assay of MLC kinase. The activities of cAMP-dependent protein kinase and cGMP-dependent protein kinase were assay under the conditions described earlier (Hidaka et al., 1984). The reaction was terminated by the addition of 1 mL of 20% trichloroacetic acid following addition of 1 mg of bovine serum albumin as a carrier protein. The sample was centrifuged at 3000 rpm for 15 min, the pellet was resuspended in 5% trichloroacetic acid solution, and the centrifugation–resuspension cycle was repeated 3 times. The final pellet was dissolved in 2 mL of 1 N NaOH. Protein kinase C was treated with trypsin, as described by Inoue et al. (1977). The binding between $^{45}CaCl_2$ or [^{3}H]W-7 and phosphatidylserine in the absence or presence of SC-9 was studied by the method of Feinstein (1964).

Determination. Radioactivity of ^{32}P was determined by using a Beckman liquid scintillator counter, Model LS-7000. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard protein.

Results

New Activators of Protein Kinase C. Chemical structures of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and its derivatives are shown in Figure 1. *N*-(6-Phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9), *N*-(*n*-heptyl)-5-chloro-1-naphthalenesulfonamide (SC-10), and *N*-(6-guanidinohexyl)-5-chloro-1-naphthalenesulfonamide (W-109) are the derivatives of W-7 substituted with phenyl, methyl, and guanidino residues, respectively, for the amino residue of W-7. The hydrophobic fragmental constants (Leo et al., 1975) of phenyl, methyl, guanidino, and amino residues are 1.90, 0.89, −5.66, and −1.54, respectively.

In the experiment shown in Figure 2A, SC-9 and SC-10 further enhanced the myosin light chain phosphorylation by protein kinase C, dose dependently, in the presence of Ca^{2+}

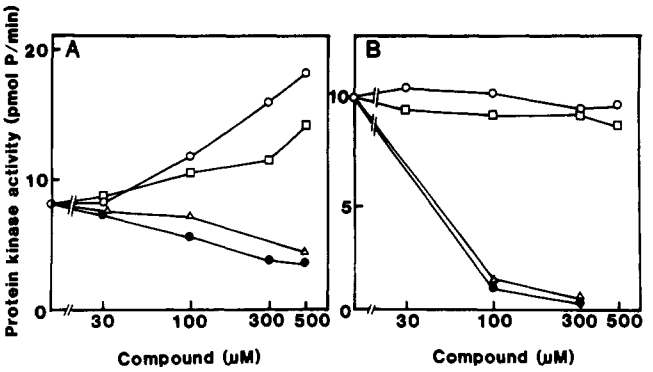

FIGURE 2: Effect of naphthalenesulfonamide derivatives on myosin light chain phosphorylation by protein kinase C (A) or myosin light chain kinase (B). Protein kinase C was assayed as described under Experimental Procedures, in the presence of Ca^{2+} (1×10^{-6} M), phosphatidylserine (50 μg/mL), and TPA (100 ng/mL). Myosin light chain kinase was assayed as described under Experimental Procedures, in the presence of Ca^{2+} (2.5×10^{-4} M) and calmodulin (200 ng/tube). Assay was done (○) with SC-9, (□) with SC-10, (△) with W-109, or (●) with W-7.

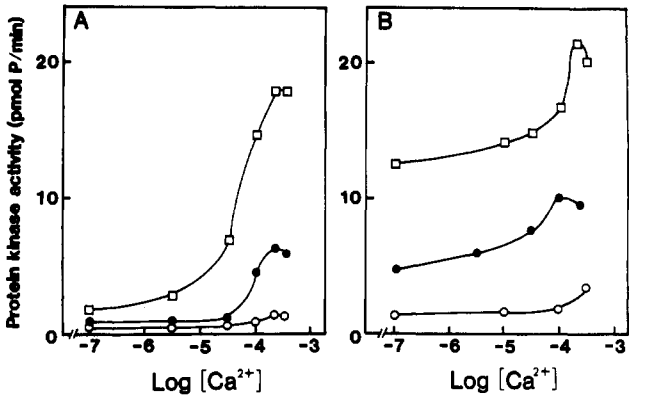

FIGURE 3: Effect of SC-9 on the reaction velocity of protein kinase C at various concentrations of Ca^{2+}. Protein kinase C was assayed as described under Experimental Procedure, at various concentrations of Ca^{2+}, in the absence (A) or presence (B) of a fixed amount of TPA (100 ng/mL). Assay was done (□) with SC-9 (200 μM), (●) with phosphatidylserine (50 μg/mL), or (○) with neither SC-9 nor phosphatidylserine.

(1×10^{-6} M), phosphatidylserine (50 μg/mL), and TPA (100 ng/mL). Moreover, SC-9 and SC-10 activated the histone phosphorylation catalyzed by protein kinase C. However, these compounds could not activate myosin light chain phosphorylation by MLC kinase in the presence or absence of the Ca^{2+}–calmodulin complex (Figure 2B). SC-9 could neither stimulate nor inhibit cAMP-dependent protein kinase II and cGMP-dependent protein kinase, in a concentration of up to 300 μM (data not shown).

On the other hand, both Ca^{2+}-dependent myosin light chain phosphorylations were inhibited by W-7 and by W-109 (Tanaka et al., 1982). The findings that SC-9 or SC-10 but not W-109 nor W-7 enhanced the protein kinase C activity suggest that the stimulation of Ca^{2+}-activated, phospholipid-dependent myosin light chain phosphorylation by these derivatives may be dependent on the phenyl or methyl residue, of which the hydrophobic fragmental constant is positive, and presumably on the extent of hydrophobicity of each compound.

Mechanism of the Activation. The concentration dependence on Ca^{2+} of protein kinase C catalyzed phosphorylation of myosin light chain in the presence of SC-9 or phosphatidylserine is illustrated in Figure 3. As shown in Figure 3A, SC-9 stimulated myosin light chain phosphorylation by protein kinase C, in a Ca^{2+}-dependent fashion in the absence of phosphatidylserine and TPA. The apparent K_m values for Ca^{2+} of protein kinase C assayed with SC-9 and with phosphatidylserine were 40 μM and 80 μM, respectively. However, the relative reaction velocity of this phosphorylation with SC-9 was much greater than that with phosphatidylserine (50 μg/mL), which was used in almost saturated quantities. It has been reported that when a small amount of either TPA or diolein was supplemented to phospholipid, the K_m value for Ca^{2+} of protein kinase C was dramatically decreased to the 10^{-6} M range in histone phosphorylation (Castagna et al., 1982). As shown in Figure 3B, the SC-9-induced activation of myosin light chain phosphorylation was greatly enhanced with the concomitant decrease in the Ca^{2+} concentration, when a small amount of TPA (100 ng/mL) was added. The apparent K_m value for TPA of protein kinase C activated by SC-9 was similar to that of the enzyme activity with phosphatidylserine (data not shown).

The initial rates of phosphorylation of MLC by protein kinase C were studied over a wide range of substrate concentrations and analyzed by double-reciprocal plots (Figure 4). The K_m values for myosin light chain of protein kinase C with SC-9 or phosphatidylserine, as an activator, were 5.8 μM and 56 μM, respectively. As the K_m value of protein kinase C with SC-9 for myosin light chain was about 10 times smaller than that of the enzyme with phosphatidylserine, the reaction velocity of protein kinase C with SC-9 was much higher than that with phosphatidylserine, in the presence of a low concentration of myosin light chain. However, the V_{max} values of protein kinase C activated by SC-9 or phosphatidylserine were 0.13 nmol·min^{-1} and 0.35 nmol·min^{-1}. The V_{max} value of protein kinase C activated by SC-9 for myosin light chain

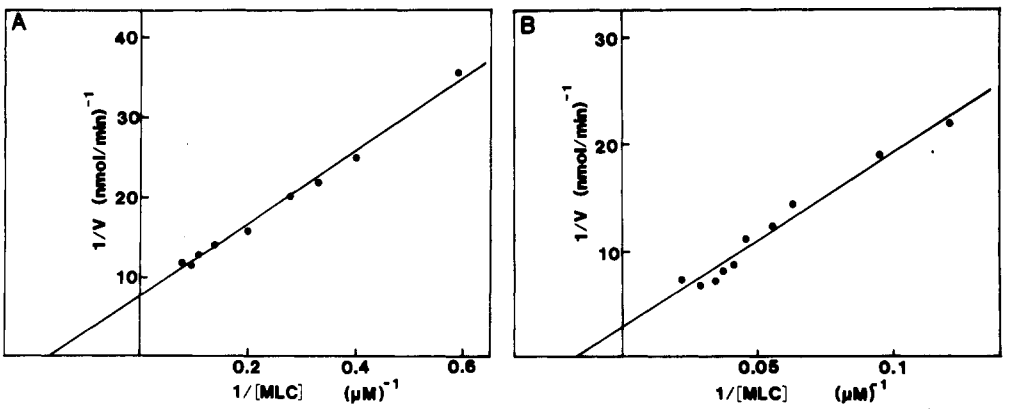

FIGURE 4: Double-reciprocal plots of phosphorylation of myosin light chain by SC-9- (A) and phosphatidylserine- (B) activated protein kinase C. The enzyme was assayed as described under Experimental Procedures with 500 μM ATP and 2×10^{-4} M Ca^{2+}, except for various amounts of myosin light chain in the presence of SC-9 (500 μM) or phosphatidylserine (50 μg/mL).

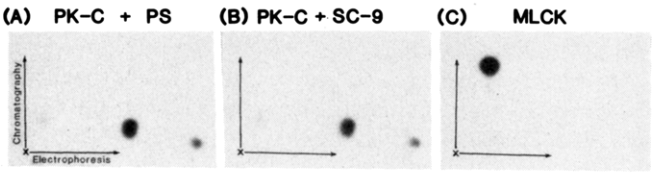

FIGURE 5: Two-dimensional chromatography of tryptic phosphopeptides following phosphorylation of myosin light chain by protein kinase C with phosphatidylserine (A), protein kinase C plus SC-9 (B), or myosin light chain kinase with Ca^{2+}–calmodulin complex (C), as described (Naka et al., 1983).

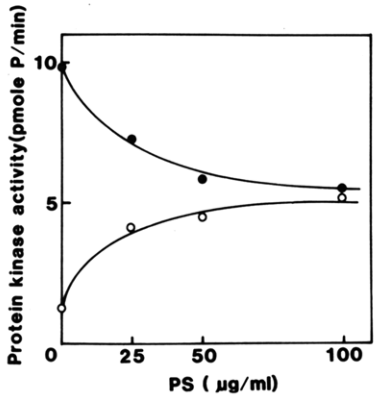

FIGURE 6: Effect of SC-9 on the reaction velocity of protein kinase C with various concentrations of phosphatidylserine. Protein kinase C was assayed as described under Experimental Procedures with 2×10^{-4} M Ca^{2+} at various concentrations of phosphatidylserine in the presence of 200 μM SC-9 (●) or absence of SC-9 (○).

was about 3-fold smaller than that of the enzyme activity with phosphatidylserine. Autoradiograms of the two-dimensional peptide maps of the tryptic fragments of myosin light chain phosphorylated by protein kinase C with SC-9 or phosphatidylserine as activators yielded identical major radioactive spots (Figure 5), which differed from evidence obtained in the case of catalysis by myosin light chain kinase (Nishikawa et al., 1983). These results indicate that the sites on myosin light chain phosphorylated by protein kinase C with SC-9 are similar to those in the case of phosphatidylserine, except for the myosin light chain requirement.

The effect of SC-9 on the reaction velocity of Ca^{2+}-activated, phospholipid-dependent myosin light chain phosphorylation with various concentrations of phosphatidylserine is shown in Figure 6. Although SC-9 produced further stimulation of myosin light chain phosphorylation of protein kinase C in the presence of a low concentration of phosphatidylserine, the SC-9-induced activation of protein kinase C was progressively inhibited by the addition of increasing amounts of phosphatidylserine, reaching the reaction velocity obtained with phosphatidylserine alone, used in saturated quantities. The apparent K_i value of phosphatidylserine for protein kinase C with SC-9 seemed to be similar to the apparent K_m value of phosphatidylserine for protein kinase C in the absence of SC-9. These results suggest that the SC-9 binding sites on protein kinase C are also responsible for binding of phosphatidylserine. Protein kinase C was reported to be alternatively activated, in an irreversible manner, by limited proteolysis with trypsin, and the catalytically active fragment produced was entirely independent of Ca^{2+} and phospholipids (Inoue et al., 1977). Trypsin-treated protein kinase C was no longer sensitive to activation or inhibition by SC-9 or W-7 in the absence of Ca^{2+} and phosphatidylserine (data not shown). Thus, SC-9 and W-7 apparently do not interact with the catalytical fragment of the enzyme. As the chemical structure of SC-9 is similar to that of W-7 (Figure 1) and we wanted to study the relationship between the action sites of SC-9 and W-7, we investigated the effect of W-7 on SC-9-activated myosin light chain phosphorylation by protein kinase C. W-7 inhibited the SC-9- (200 μM) induced activation of protein kinase C, in a dose-dependent manner, with an IC_{50} value of 110 μM. Kinetic analysis revealed that inhibition of Ca^{2+}-activated, SC-9-dependent myosin light chain phosphorylation by W-7 was the mixed type, with respect to SC-9, and that the K_i value was 60 μM. The effects of SC-9 on the binding of W-7 or calcium ion to phosphatidylserine were studied in order to examine the relationship among the binding sites of SC-9, W-7, and calcium ion. There was no significant inhibition of [^{3}H]W-7 or $^{45}Ca^{2+}$ binding to phosphatidylserine by SC-9 up to 1 mM, thereby suggesting that SC-9 might interact with the protein kinase C system at sites different from calcium ion or W-7 binding sites on phosphatidylserine.

Discussion

We found that newly synthesized compounds such as *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide are calcium-dependent activators of protein kinase C. These synthetic activators and phosphatidylserine have similar effects on protein kinase C activity, as illustrated by the following points: (1) These compounds can also activate protein kinase C in a Ca^{2+}-dependent manner with a similar K_m value for Ca^{2+}. (2) TPA also reduces the requirement for Ca^{2+} of the SC-9 activated protein kinase C, and the apparent K_m value for TPA of SC-9-activated protein kinase C was similar to those of phosphatidylserine-activated enzyme. (3) Although SC-9 produced further stimulation of myosin light chain phosphorylation by protein kinase C with a low concentration of phosphatidylserine, no further enhancement of the enzymatic activity was observed in the presence of a saturated amount of phosphatidylserine. (4) The calmodulin antagonist W-7, which inhibits the phosphatidylserine-induced protein kinase C activity competitively with respect to phosphatidylserine, also inhibited myosin light chain phosphorylation catalyzed by SC-9-activated protein kinase C.

However, the K_m value for myosin light chain and the V_{max} value of protein kinase C activated by SC-9 are about 10 and 3 times smaller, respectively, than that activated by phosphatidylserine. It was reported that further enhancement of protein kinase C activity was observed when phosphatidylserine was supplemented with phosphatidylethanolamine (Kaibuchi et al., 1981), which was similar to the effect of SC-9 on protein kinase C in the presence of phosphatidylserine. However, phosphatidylethanolamine enhances the enzyme activation by a marked increase in the affinity of the enzyme for Ca^{2+} as well as for phosphatidylserine, diglyceride, and TPA, which was not observed with SC-9. These observations suggest that SC-9 is a novel and unique activator of Ca^{2+}-activated, phospholipid-dependent myosin light chain phosphorylation. Although the exact binding sites of SC-9 on protein kinase C remain to elucidated, the possibility that SC-9 has pharmacological actions on myosin light chain may be excluded, for the following reasons: (1) This stimulatory effect of SC-9 on protein kinase C was also observed when histone was used as the substrate. (2) Activation of myosin light chain phosphorylation by protein kinase C was not observed after treatment of the enzyme with trypsin.

Naturally occurring substances, including retinoic acid (Ohkubo et al., 1984), lipid A (Wightman & Paetz, 1984), and unsaturated fatty acids (McPhail et al., 1984) other than phosphatidylserine, have been found to activate protein kinase C. The activation of protein kinase C by these substances may relate to structural similarities with phosphatidylserine.

However, these naphthalenesulfonamide derivatives have no fatty acid side chain moiety, which is a component of the naturally occurring activators of the enzyme. Moreover, it should be noted that some naphthalenesulfonamide derivatives are activators and others are inhibitors of protein kinase C, depending on the chemical structure.

These naphthalenesulfonamide derivatives, which are selective activators of protein kinase C, without significant effects on MLC kinase activity, and with the phenyl or methyl residue at the end of hydrocarbon chain of the naphthalenesulfonamides, have a positive hydrophobic fragmental constant. On the other hand, agents that inhibited both protein kinase C and MLC kinase activity had amino and guanidino residues, which have a negative hydrophobic fragmental constant and are positively charged moieties. Therefore, these studies on the structural requirements for the enzyme activation clearly suggest the importance of hydrophobic residues at the hydrocarbon chain of the naphthalenesulfonamides. These residues may be responsible for increases in the hydrophobicity of the compounds, and stimulation of the enzyme would follow. However, as not all the amphiphilic compounds could activate protein kinase C, it can be predicted that this effect of SC-9 is brought about not only by virtue of its amphiphilic property but also by virtue of other physiochemical factors yet to be elucidated. We reported that hydrophobic quinazolinesulfonamide derivatives activated calcium ion dependent cyclic nucleotide phosphodiesterase instead of the calcium–calmodulin complex (Tanaka et al., 1983). However, SC-9 and SC-10 proved to have no significant effects on Ca^{2+}–CaM-dependent myosin light chain kinase.

There are at least two different protein kinases related to Ca^{2+}-dependent phosphorylation of smooth muscle and nonmuscle myosin. One protein kinase is MLC kinase, which is activated by the Ca^{2+}-CaM complex (Adelstein & Eisenberg, 1980; Hartshorne & Siemankowski, 1981), and the other is protein kinase C, which requires phospholipids and diglyceride as cofactors (Endo et al., 1982; Naka et al., 1983). Therefore, SC-9 and SC-10, which stimulated protein kinase C mediated myosin light chain phosphorylation without significant effects on MLC kinase activity, may be useful tools for differentiating the type of kinase responsible for in vitro phosphorylation of myosin light chain. Furthermore, investigation undertaken to examine the effects of SC-9 on the other two types of protein kinases, cAMP-dependent protein kinase and cGMP-dependent protein kinase, indicated that SC-9 is a selective activator of protein kinase C but not of cAMP-dependent protein kinase or cGMP-dependent protein kinase. These results suggest that SC-9 will be generally useful to identify the activity of protein kinase C in crude cell extracts.

Pharmacological activators of protein kinase C, such as SC-9, will aid in elucidating the biological role of this protein phosphorylation in intact cells. Exposure of Swiss 3T3 cells to SC-9 led to a dose-related increase in hexose uptake, an event also observed when the cells were treated with TPA. This activation by SC-9 was inhibited by the specific protein kinase C inhibitor H-7 (Nishino et al., unpublished observations). This phenomenon suggests that SC-9 may activate protein kinase C in intact cells and can be used for in vivo studies.

Ca^{2+} binds directly to calmodulin and not to the enzyme, it induces a conformational change that exposes hydrophobic groups, and the Ca^{2+}–calmodulin complex is then capable of activating MLC kinase (Laporte et al., 1980; Tanaka & Hidaka, 1980). The mechanism of activation of protein kinase C, at the molecular level, remains the subject of ongoing study. Certain interactions of the phospholipids or of these derivatives with hydrophobic regions on the enzyme also seem to be involved.

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Registry No. TPA, 16561-29-8; W-7, 65595-90-6; SC-9, 102649-78-5; SC-10, 102649-79-6; W-109, 102649-80-9; Ca, 7440-70-2; protein kinase, 9026-43-1.

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Medium-Chain Acyl Coenzyme A Dehydrogenase from Pig Kidney Has Intrinsic Enoyl Coenzyme A Hydratase Activity†

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ABSTRACT: The flavoprotein medium-chain acyl coenzyme A (acyl-CoA) dehydrogenase from pig kidney exhibits an intrinsic hydratase activity toward crotonyl-CoA yielding L-3-hydroxybutyryl-CoA. The maximal turnover number of about 0.5 min^{-1} is 500–1000-fold slower than the dehydrogenation of butyryl-CoA using electron-transferring flavoprotein as terminal acceptor. *trans*-2-Octenoyl- and *trans*-2-hexadecenoyl-CoA are not hydrated significantly. Hydration is not due to contamination with the short-chain enoyl-CoA hydratase crotonase. Several lines of evidence suggest that hydration and dehydrogenation reactions probably utilize the same active site. These two activities are coordinately inhibited by 2-octynoyl-CoA and (methylenecyclopropyl)acetyl-CoA [whose targets are the protein and flavin adenine dinucleotide (FAD) moieties of the dehydrogenase, respectively]. The hydration of crotonyl-CoA is severely inhibited by octanoyl-CoA, a good substrate of the dehydrogenase. The apoenzyme is inactive as a hydratase but recovers activity on the addition of FAD. Compared with the hydratase activity of the native enzyme, the 8-fluoro-FAD enzyme exhibits a roughly 2-fold increased activity, whereas the 5-deaza-FAD dehydrogenase is only 20% as active. A mechanism for this unanticipated secondary activity of the acyl-CoA dehydrogenase is suggested.

The medium-chain (or general) acyl-CoA[1] dehydrogenase catalyzes the α,β-unsaturation of acyl-CoA thio esters with concomitant reduction of enzyme-bound FAD (Beinert, 1963). With preferred substrates, such as octanoyl-CoA, the product *trans*-2-octenoyl-CoA is bound very tightly to the reduced enzyme (Steyn-Parve & Beinert, 1958; Thorpe et al., 1981):

$$E\cdot FAD\cdot octanoyl\text{-}CoA \rightleftharpoons E\cdot FAD_{2e}\cdot octenoyl\text{-}CoA$$

This complex is then reoxidized in two one-electron steps by electron-transferring flavoprotein (Hall & Lambeth, 1980; Reinsch et al., 1980; Gorelick et al., 1985) in an ordered mechanism in which enoyl-CoA product dissociates from the oxidized enzyme (McKean et al., 1979). In the second step of mitochondrial β-oxidation, these thio esters are hydrated by enoyl-CoA hydratases to their L-3-(OH)-acyl-CoA derivatives (Stern et al., 1956; Wakil & Mahler, 1954; Wakil, 1956). They are then oxidized to their corresponding 3-keto derivatives by 3-(OH)-acyl-CoA dehydrogenases and finally cleaved to yield acetyl-CoA and a shortened acyl-CoA by 3-ketoacyl-CoA thiolases.

This paper demonstrates that pig kidney medium-chain acyl-CoA dehydrogenase, when freed of significant contaminating hydratase activities, retains an intrinsic short-chain enoyl-CoA hydratase (crotonase) activity:

$$crotonyl\text{-}CoA + H_2O \rightleftharpoons L\text{-}3\text{-}(OH)\text{-}butyryl\text{-}CoA$$

A characterization of this unanticipated secondary activity of the oxidized dehydrogenase is presented.

Experimental Procedures

Materials

Crotonyl-CoA, octanoyl-CoA (lithium salt), and CoASH were obtained from P-L Biochemicals. *trans*-2-Octenoic acid and 2-octynoic acid were from Pfaltz & Bauer, and *trans*-2-hexadecenoic acid was purchased from ICN Pharmaceuticals Inc. FAD, NAD^+, bovine serum albumin (fatty acid free), bovine liver crotonase, and 3-hydroxyacyl-CoA dehydrogenase were from Sigma. Octyl-Sepharose CL-4B, protein A–Sepharose CL-4B, and Sephacryl S-200 were from Pharmacia. FAD analogues were prepared as described previously (Thorpe & Massey, 1983). 8-Chlororiboflavin was a generous gift of Dr. John Lambooy, University of Maryland. The remaining riboflavin derivatives were generous gifts from Dr. Vincent Massey, University of Michigan.

Methods

General. Visible and UV spectra were recorded on Varian 219 and Perkin-Elmer 552 spectrophotometers. NMR spectra were recorded on a Bruker Aspect 3000 250-MHz spectrometer. Unless otherwise stated, all buffers contained 0.3 mM

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[1] Abbreviations: acyl-CoA, acyl coenzyme A; HPLC, high-pressure liquid chromatography; FAD, flavin adenine dinucleotide; NAD^+, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PCMB, *p*-(chloromercuri)benzoate.

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