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Purification of a Protein C Activator from the Venom of the Southern Copperhead Snake (*Agkistrodon contortrix contortrix*)†

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ABSTRACT: A protease has been purified by ion-exchange chromatography from the venom of *Agkistrodon contortrix contortrix* (Southern copperhead snake) that can activate the vitamin K dependent protein, protein C. The apparent molecular weight of this protease, determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, was 20000 under nonreducing conditions. Incubation of this protease with plasma resulted in a prolongation of the clotting time and a time-dependent increase in amidolytic activity. Incubation of the protease with purified protein C resulted in an increase in both amidolytic and anticoagulant activity. The protease had no inhibitory effect on thrombin, factor V, fibrinogen, or factor X. It had slight clotting activity toward fibrinogen. The apparent K_m of the protease for protein C was 0.28 μM. Calcium ions were observed to inhibit protein C activation with an apparent K_i of 0.2 mM. Ethylenediaminetetraacetic acid, diisopropyl fluorophosphate, and soybean trypsin inhibitor were observed to inhibit the venom protease. These results suggest that the venom of the Southern copperhead snake contains a protease that is a specific activator of protein C.

A variety of proteases that can either promote or inhibit blood coagulation have been isolated from snake venoms. Protease activators of factors V (Hanahan et al., 1972) and X (Kisiel et al., 1976b) have been obtained from Russell's viper venom. Prothrombin activators have been found in a number of venoms including *Crotalus adamanteus* (Markland & Damus, 1971), *Echis carinatus* (Morita et al., 1976), *Echis coloratus*, *Notechis scutatus scutatus* (Kornalik & Blomback, 1975), and *Oxyuranus scutellatus scutellatus* (Owen & Jackson, 1973). Thrombin-like enzymes have been isolated from the venom of a number of snakes of the *Bothrops* genus (Holleman & Weiss, 1976; Stocker et al., 1974) as well as from the venoms of *Crotalus adamanteus* (Markland & Damus, 1971), the *Crotalidae* genus (Denson et al., 1972), *Agkistrodon bilineatus*, *Agkistrodon contortrix mokeson*, and *Trimeresurus flavoviridis* (Denson et al., 1972), among others.

Several snake venoms have been reported to contain proteins that can inhibit blood coagulation. Inhibitors of prothrombin activation have been observed in the venoms of *Trimeresurus gramineus* (Ouyang & Yang, 1975) and *Agkistrodon acutus* (Ouyang & Teng, 1972). A protein isolated from *A. acutus* inhibited the participation of factor Xa in the prothrombinase complex (Teng & Seegers, 1981). However, none of the venoms that contain anticoagulant activities have been reported to have activators of protein C.

Protein C, a vitamin K dependent plasma protein, can be converted to a serine protease (activated protein C) by a number of enzymes. Protein C has been activated by trypsin (Esmon et al., 1976), the factor X activator from Russell's viper venom (Kisiel et al., 1976b), and thrombin (Kisiel et al., 1977). The activation of protein C by thrombin is slow and can be inhibited by calcium ions (Kisiel et al., 1976a). It is thought that in vivo activation of protein C requires the interaction between thrombin and thrombomodulin, an endothelial cell surface protein (Esmon & Owen, 1981). The thrombin–thrombomodulin complex can activate protein C approximately 10^4 times as fast as thrombin alone (Esmon et al., 1982). The thrombin–thrombomodulin complex requires the presence of calcium ions or other divalent cations for activity.

Activated protein C is a potent inhibitor of blood coagulation. Its anticoagulant action appears to be through the

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proteolytic inactivation of factors V (Kisiel et al., 1977; Walker et al., 1979; Suzuki et al., 1983; Marlar et al., 1982) and VIII (Vehar & Davie, 1980; Vehar et al., 1984; Fay et al., 1982; Marlar et al., 1982; Fulcher et al., 1984). In addition to its anticoagulant activity, activated protein C has amidolytic activity against synthetic peptide substrates (Kisiel et al., 1976a).

Experimental Procedures

Materials. Agkistrodon contortrix contortrix snake venom, sulfopropyl-Sephadex (SP-C50–120), and soybean trypsin inhibitor were from Sigma Chemical Co. The chromogenic substrates Bz-Ile-Pro-Arg-*p*-nitroanilide (S2288), Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S2222), and Bz-Pro-Phe-Arg-*p*-nitroanilide (S2302) were purchased from Helena Laboratories. All other reagents were of the highest grade available.

Proteins. Protein C, prothrombin, factor X, thrombin, and fibrinogen were of bovine origin. The purification of prothrombin and thrombin has been reported previously (Owen et al., 1974). Protein C and factor X were isolated as described elsewhere (Walker et al., 1979). Protein C and factor X were activated with the factor X activator from Russell's viper venom and purified as previously described (Walker et al., 1979). Barium-absorbed bovine plasma was the source of fibrinogen (Straughn & Wagner, 1966) and antithrombin III (Walker & Esmon, 1979). Protein concentrations were determined by absorbance at 280 nm. The molecular weights and extinction coefficients used to determine protein concentrations were as follows: activated protein C, 56000, $E^{1\%}_{1cm}$ 13.7 (Kisiel et al., 1977); factor Xa, 45000, $E^{1\%}_{1cm}$ 14.4; prothrombin, 72000, $E^{1\%}_{1cm}$ 15.5; thrombin, 37000, $E^{1\%}_{1cm}$ 21.4 (Owen et al., 1974); antithrombin III, 56000, $E^{1\%}_{1cm}$ 6.0 (Downing et al., 1978); protein C, 62000, $E^{1\%}_{1cm}$ 13.7 (Kisiel et al., 1976a); factor X, 55000, $E^{1\%}_{1cm}$ 12.4 (Jackson et al., 1968). For the purpose of this study we assumed an $E^{1\%}_{1cm}$ for the venom protease of 10.0.

Purification of the Venom Protease. Crude, lyophilized *A. contortrix contortrix* venom (100 mg) was reconstituted in 5 mL of 0.02 M imidazole buffer, pH 6.5, and dialyzed overnight in 0.1 M NaCl and 0.02 M imidazole, pH 6.5. The venom sample was applied to a column (0.9 cm × 30 cm) of sulfopropyl-Sephadex equilibrated in 0.1 M NaCl and 0.02 M imidazole, pH 6.5. The column was developed with a linear gradient from 0.1 to 0.6 M NaCl in the imidazole buffer (200 mL). Fractions (3 mL) were collected and assayed for anticoagulant activity with bovine plasma and for the ability to activate purified protein C.

Assays. Thrombin was assayed by a fibrinogen clotting assay. Factor Xa (Walker, 1980) and factor V (Kappeler, 1955) were assayed by one-stage clotting assays. Protein C activator was assayed by two methods that were based on either anticoagulant activity or amidolytic activity toward the synthetic substrate S2288. For both assays protein C was extensively dialyzed to remove any calcium ions that might be present. Anticoagulant activity in the sulfopropyl-Sephadex column fractions was assayed by incubating 100 μL of bovine plasma with 20 μL of the fraction at 37 °C. After 5 min, 100 μL of thromboplastin and 100 μL of buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mg/mL bovine serum albumin) were added to the incubation mixture. Clotting was initiated by the addition of 100 μL of 0.025 M $CaCl_2$. Prolongation of clotting time was determined relative to a blank that contained plasma incubated in the absence of venom. Those fractions that showed anticoagulant activity (clotting times greater than twice the clotting time of the blank) were further tested for protein C activator activity. Protein C (100 μL, 0.32 μM) was incubated for 15 min at 37 °C with 5 μL of the column fractions that contained anticoagulant activity. At the end of the incubation period, 10 μL of the protein C/venom mixture was added to an assay mixture containing 100 μL of bovine plasma, 100 μL of thromboplastin, and 90 μL of buffer (0.1 M NaCl, 0.02 M Tris-HCl,[1] pH 7.5, 1 mg/mL bovine serum albumin). Clotting was initiated by the addition of 100 μL of 0.025 M $CaCl_2$. Protein C activation was determined relative to a standard curve prepared by dilution of a stock solution of activated protein C. Protein C activation was also determined by an amidolytic assay. The amidolytic reaction mixture containing 0.17 μM protein C, 0.5 μg of venom protein in 0.1 M NaCl and 0.02 M Tris-HCl, pH 7.5, and 0.08 mM S2288 was incubated at 37 °C. The total volume of the reaction mixture was 1.0 mL. The formation of activated protein C was followed by measuring the appearance of *p*-nitroanilide absorbance at 405 nm. The amount of activated protein C present at a given time was proportional to the slope of the absorbance vs. time plot. Absorbance data were obtained with a computer on-line with a Beckman Model DU-8B spectrophotometer.

K_m Determination. The apparent K_m of the venom protease for activated protein C was determined by varying the concentration of protein C in the amidolytic reaction mixture from 10 to 150 nM and monitoring the change in absorbance at 405 nm over a 5-min period. A plot of the change in absorbance vs. time yielded a line, the slope of which was equivalent to the rate of activated protein C formation. Data were analyzed by the nonlinear regression analysis described by Cleland (1967).

Inhibitor Studies. Inhibitors of the venom-catalyzed protein C activation were tested by preincubating various concentrations with venom protease for 2 min at 37 °C, then adding this venom/inhibitor mixture to the amidolytic reaction mixture (described above), and monitoring the rate of activated protein C appearance. Concentrations of the inhibitors studied were as follows: soybean trypsin inhibitor (STI), 1.5–10 nM; diisopropyl fluorophosphate (DFP), 25–100 μM; EDTA, 0.1–2 mM; calcium, 12.5–100 μM; antithrombin III, 0.1–1 mM. To determine whether synthetic chromogenic substrates could be cleaved by the venom, 1.3 μg of the venom protease sample was incubated at 37 °C with 0.08 mM synthetic substrate in a final reaction volume of 1 mL. The change in absorbance at 405 nm was observed over a 5-min period. The synthetic substrates tested by this method were S2288, S2302, and S2222.

Results

When 20 μg of crude *A. contortrix contortrix* venom was assayed for anticoagulant activity in bovine plasma, it was observed to prolong the clotting time from 20 to >90 s. It also appeared that the venom could catalyze the activation of protein C. In order to determine the cause of prolongation and to investigate the possibility that activated protein C might be involved in this phenomenon, purification of the anticoagulant from crude venom was attempted.

A venom protease was purified in a single step from crude venom by ion-exchange chromatography on sulfopropyl-Sephadex (Figure 1). Fractions from this column were initially screened for anticoagulant activity and subsequently for protein C activator activity by using either the formation of an anticoagulant or development of amidolytic activity as an

[1] Abbreviations: SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate.

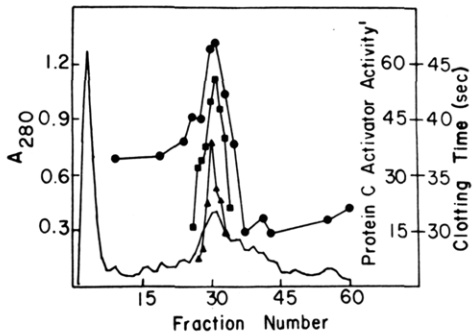

FIGURE 1: Chromatograph of the crude copperhead venom on sulfopropyl-Sephadex. Crude venom (100 mg) was dissolved in 5 mL of 0.02 M imidazole, pH 6.5, and dialyzed against 0.1 M NaCl and 0.02 M imidazole, pH 6.5. The venom was chromatographed at 22 °C on a column (0.9 cm × 30 cm) of sulfopropyl-Sephadex. The column was equilibrated in 0.1 M NaCl and 0.02 M imidazole, pH 6.5, and developed with a linear gradient from 0.1 to 0.6 M NaCl in the imidazole buffer (100 mL/chamber). Fractions (3 mL) were collected and monitored for protein (•), anticoagulant activity (●), and protein C activator activity by clotting (■) and amidolytic (▲) assay. Fractions 27–33 showed one protein band upon SDS–polyacrylamide gel electrophoresis.

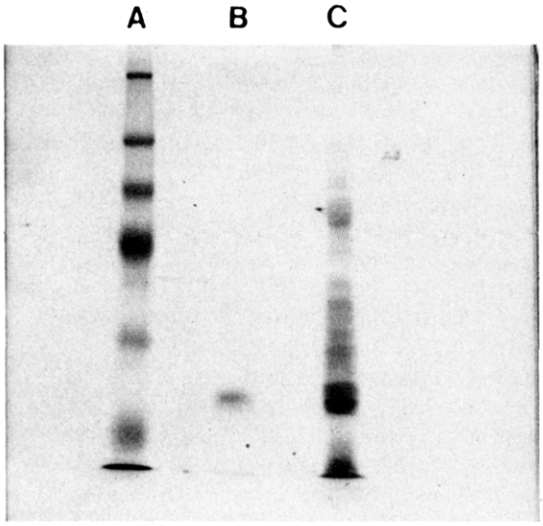

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the protein C activator from *A. contortrix contortrix*. Lane A: molecular weight standards containing myosin (M_r 200 000), phosphorylase B (M_r 97 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), chymotrypsinogen (M_r 25 700), and β-lactoglobulin (M_r 18 400). Lane B: purified protein C activator (18 μg). Lane C: crude venom (20 μg). The gel (12% polyacrylamide) was stained with Coomassie brilliant blue R.2.

end point. Anticoagulant as well as protein C activator activity was found near the middle of the salt gradient. The peak protein fractions showed one protein band upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2). Changes in either the pH or the salt gradient reduced the resolution of the column.

The venom activator appears to be a major protein constituent of the venom. The purified protein represented approximately 8% of the total venom protein. Electrophoresis of the crude venom revealed a major band that coelectrophoresed with the purified protein (Figure 2). It was difficult to determine the *x*-fold purification or yield because the crude venom apparently contains a number of activities that interfere with the assays used. The venom protease was stable when stored in solution at 4 °C for as long as 4 months.

The apparent molecular weight of this protein as determined by polyacrylamide gel electrophoresis was 20 000 under nonreducing conditions (Figure 2). A sample of the purified protein was chromatographed on a Pharmacia Superose 6 gel filtration column (0.6 cm × 65 cm). All fractions were assayed for protein and protein C activator activity. The position at which the major protein eluted confirmed a molecular weight of approximately 20 000. Protein C activator activity was found only in those fractions that contained the major protein peak.

The purified protease displayed anticoagulant activity when incubated with bovine plasma. Incubation of the protease with purified protein C resulted in the formation of activated protein C as measured by the appearance of anticoagulant activity, amidolytic activity, or the cleavage of the heavy chain of protein C monitored by SDS–polyacrylamide gel electrophoresis (data not shown). The venom protease did not cleave the synthetic substrate S2288. The apparent K_m of the protease for protein C, determined by the amidolytic assay, was 0.28 μM. The V_{max} for activation of protein C by the venom protease was 7.4 mol of activated protein C formed min^{-1} (mol of venom activator)$^{-1}$. If high concentrations of venom protease were incubated with protein C (i.e., protein C/venom protease = 10), cleavage of the light chain and inactivation of protein C could be detected.

No activation of prothrombin was detected when 25 μM prothrombin was incubated with 2.2 μM venom at 37 °C and the activity monitored by a clotting assay over a 30-min incubation period. SDS–polyacrylamide gel electrophoresis analysis of samples removed at various times during the 30-min incubation indicated that prothrombin was cleaved by the venom. An effort was made to determine if the venom cleavage of prothrombin contributed to the anticoagulant effect observed with bovine plasma. Protein C deficient plasma, containing 1.5 μM prothrombin, was incubated with and without venom. The plasma to which venom protease had been added exhibited the same clotting time as the plasma that was incubated in the absence of venom protease (18 s). When protein C was added back to the protein C deficient plasma and incubated with venom protease, the clotting time increased to 32 s. With an amidolytic assay, prothrombin was found to inhibit the venom-catalyzed activation of protein C, K_i = 1.7 μM. To determine if prothrombin inhibited the activation of protein C in plasma, excess prothrombin was added to the protein C deficient plasma that had been supplemented with protein C. The final concentrations of prothrombin in the protein C supplemented plasma were 2.25, 3.0, and 3.75 μM (1.5, 2.0, and 2.5 times the original concentration of prothrombin in the deficient plasma). These plasma samples were incubated with the venom protease and the clotting times determined. While the additional prothrombin caused a small decrease in the clotting time (32 s with protein C and no additional prothrombin to 27 s with protein C and 3.75 μM prothrombin), it failed to restore the clotting time observed in plasma in the absence of protein C. The incubation of bovine plasma with and without the venom resulted in a prolongation of clotting time from 23 (no venom) to 90 s (venom present in the incubation).

The incubation of 4.58 μM factor X with 2.2 μM venom protease resulted in no observable formation of factor Xa as determined by the clotting assay. Factor Xa (4.58 μM) was incubated with the same concentration of the venom protease assayed over 30 min. No loss of factor Xa activity was detected. There was also no apparent loss of thrombin activity when 0.5 μM thrombin was incubated with 0.33 μM venom protease. The protein C activator had no effect on plasma factor V activity. The protease had slight clotting activity toward fibrinogen.

Inhibition of protein C activation was determined by observing changes in S2288 hydrolysis following the incubation of the venom sample with potential inhibitors. Calcium ions were observed to inhibit protein C activation with an apparent K_i of 200 μM. The activation of protein C by the venom protease was also inhibited by soybean trypsin inhibitor (K_i, 3.8 μM), DFP (K_i, 95 μM), and EDTA (K_i, 8.5 mM). Antithrombin III, S2302, and S2222 had no effect on the venom activation of protein C.

Discussion

A protease from the venom of the Southern copperhead snake that catalyzed the activation of protein C was purified by ion-exchange chromatography. The enzyme, which appeared to be homogeneous following electrophoresis on SDS–polyacrylamide gels, was a major component of the venom. When protein C was treated with this protease, an increase in anticoagulant and amidolytic activity was observed. Cleavage of the heavy chain of protein C was observed on SDS–polyacrylamide gel electrophoresis of protein C samples that had been incubated with the venom protease. At very high levels of the enzyme, a second cleavage was observed in the light chain of protein C, which resulted in the loss of activity. In addition to activating protein C, this protease was able to cleave prothrombin and had weak activity toward fibrinogen. This specificity suggested that the protease was thrombin-like. However, it did not interact with several thrombin substrates including factor V and antithrombin III.

Prothrombin, while cleaved, was not activated by the venom protease. Neither did the cleavage of prothrombin appear to contribute to the anticoagulant effect of the venom protease in bovine plasma. In the absence of protein C the venom had little anticoagulant effect on plasma. Prothrombin, the other protein that we have observed to be a substrate, was a poor inhibitor of protein C activation. The addition actually tended to slightly decrease the anticoagulant effect of the venom protease. These results tend to suggest that the anticoagulant effect was not due to prothrombin inactivation.

Kinetic analysis of the activation of protein C by the venom protease suggested that its catalytic efficiency was similar to the thrombin–thrombomodulin complex. The K_m and V_{max} for the activation of protein C by the venom protease were 0.28 μM and 7.4 mol min^{-1} mol^{-1}, respectively. Esmon et al. (1983) have reported the K_m and V_{max} for the activation of protein C by thrombin, in the absence of calcium ion, to be 1.2 μM and 2.6 mol min^{-1} mol^{-1}. The activation of protein C by the thrombin–thrombomodulin complex in the presence of 3 mM calcium resulted in a K_m and V_{max} of 8.0 μM and 250 mol min^{-1} mol^{-1}, respectively (Esmon et al., 1983). The resulting activation reaction has a catalytic efficiency of 0.43×10^{-6} M^{-1} s for the venom activator compared to 0.003×10^{-6} for thrombin and 0.51×10^{-6} for the thrombin–thrombomodulin complex (Lollar et al., 1985). The venom protease, therefore, has a comparable efficiency to the thrombin–thrombomodulin complex and appears more efficient at catalyzing the activation of protein C than thrombin.

One difference between the venom protease and the thrombin–thrombomodulin complex was the effect of calcium ions. Thrombin–thrombomodulin activation was enhanced by calcium ions (Esmon et al., 1982) while calcium ions inhibited activation by both the venom protease and thrombin (Kisiel et al., 1977).

Earlier reports on venom anticoagulant activities have stressed that the mechanism by which these proteins exert their effect is through inhibition of prothrombin activation. The inhibition by the venom protein from *T. gramineus* was due to irreversible binding of the venom protein to the activating factors of prothrombin (Ouyang & Yang, 1975). The activation of prothrombin was inhibited by the *A. acutus* venom protein by the binding of this protein to factor Xa. The venom proteins from *T. gramineus* and *A. acutus*, then, produce their anticoagulant effect by a similar if not identical mechanism of action. Unlike these venom proteins, our results suggest that the purified anticoagulant protein isolated from *A. contortrix contortrix* is a protease that is specific for protein C. Assay of crude venoms obtained from the broad-banded copperhead and Northern copperhead indicated the presence of an anticoagulant activity and a protein C activator (data not shown). Protein C activators were not found in several other members of the *Agkistrodon* genus including *A. acutus*, *A. rhodostoma*, and *A. piscivorus piscivorus*.

The observations that a protein C activator can be easily purified from the venom of the Southern copperhead snake and that it is active in the absence of calcium suggest that the enzyme may be useful for the development of functional assays of protein C that do not require the separation of protein C from other plasma proteins.

Registry No. Ca, 7440-70-2; protein C, 60202-16-6; protein C activator, 102919-92-6.

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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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