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Two Types of Calcium-Dependent Protein Phosphorylations Modulated by Calmodulin Antagonists

Naphthalenesulfonamide Derivatives

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SUMMARY

Ca^{2+}-dependent protein phosphorylations activated by calmodulin or phospholipid were studied using selective inhibitors. Both protein phosphorylations were inhibited by *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and its derivatives. Kinetic analysis indicated that the primary effect of these agents was mediated through a competitive inhibition of enzyme activation by interaction with calmodulin or phospholipid, and K_i values of W-7 for calmodulin-dependent phosphorylation and phospholipid-dependent protein kinase were 12 μM and 110 μM, respectively. The addition of Ca^{2+} inhibited the binding of [^{3}H]W-7 to phosphatidylserine but not the binding to calmodulin. The potencies of naphthalenesulfonamide derivatives as inhibitors of Ca^{2+}, calmodulin-dependent protein kinase were dependent on the length of the alkyl chain (C_2–C_{10}) but not on Ca^{2+}-activated, phospholipid-dependent protein kinase. These results suggest that naphthalenesulfonamide derivatives may be more selective inhibitors of Ca^{2+}, calmodulin-dependent protein phosphorylation than is Ca^{2+}-activated, phospholipid-dependent protein kinase and that the mechanism of interaction between W-7 and phosphatidylserine differs from the interaction between W-7 and calmodulin. These agents are useful tools for elucidating the physiological role of Ca^{2+}-dependent protein phosphorylation.

INTRODUCTION

Protein phosphorylation is an established major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli (1). Calcium ion is an important intracellular messenger and is involved in the regulation of a variety of cell functions (2). There are at least two types of Ca^{2+}-dependent phosphorylations of protein in tissues. Some protein kinases are activated by the Ca^{2+}-calmodulin complex (3, 4), and another species of Ca^{2+}-dependent protein kinase, which requires phospholipid and diglyceride as cofactors, was described by Takai and co-workers (5). However, the precise relationship between these two types of Ca^{2+}-dependent protein phosphorylations has not been determined. Levin and Weiss (6) reported that neuroleptics bound to the Ca^{2+}-calmodulin complex and inhibited selectively Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. Recently, Mori *et al.* (7) and Schatzman *et al.* (8) demonstrated that certain antipsychotic drugs could also suppress Ca^{2+}-activated, phospholipid-dependent protein kinase. However, distinct differences in inhibition of these Ca^{2+}-dependent protein kinases by neuroleptics have not been reported (8).

We have now compared the effect of the naphthalenesulfonamide derivatives on these two kinds of Ca^{2+}-dependent protein phosphorylations, including myosin light-chain kinase from smooth muscle and Ca^{2+}-activated, phospholipid-dependent protein kinase in an attempt to elucidate the differences.

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylserine and diolein were purchased from Serdary Research Laboratories Inc. and Nakarai Chemicals, respectively. Lysine-rich histone (Type III-S) was obtained from Sigma Chemical Company. Adenosine 5′-[γ-^{32}P]triphosphate and $^{45}CaCl_2$ were obtained from Amersham International Limited. W-7,[1] [^{3}H]W-7, and its derivatives were synthesized by the method of Hidaka *et al.* (9). No. 233 and prenylamine were donated by Mitsubishi Chemical Industries Limited and Hoechst Japan Limited, respectively. Calmodulin was isolated from bovine brain and purified by the pro-

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[1] The abbreviations used are: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; No. 233, N^2-dansyl-L-arginine-4-*t*-butylpiperidine amide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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cedure reported by Yazawa *et al.* (10). The preparation was assessed to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Myosin light-chain kinase was purified from chicken gizzard by the method of Adelstein and Klee (11). Myosin light-chain of chicken gizzard, used as substrate for the kinase assay, was prepared by the method of Perrie and Perry (12). The light chain was separated from calmodulin by DEAE-cellulose chromatography (13). Ca^{2+}-activated phospholipid-dependent protein kinase was prepared from rabbit brain as described by Inoue *et al.* (14). This preparation was determined to be free of other interfering enzymes, endogenous phosphate acceptor proteins, and calmodulin, and its specific activity was 31 nmoles of ^{32}P per minute per milligram in the presence of calcium, phosphatidylserine, and diolein.

Methods. Myosin light-chain kinase activity was assayed as previously described (15). Unless otherwise noted, 0.1 μM calmodulin was used. Ca^{2+}-activated, phospholipid-dependent protein kinase was assayed as reported by Schatzman *et al.* (8). The assay system (0.2 ml) contained the following: Tris-HCl (pH 7.5), 5 μmoles; $MgCl_2$, 2 μmoles; lysine-rich histone, 50 μg; phosphatidylserine, 3 μg; diolein, 0.16 μg; [γ-^{32}P]ATP, 2 nmoles (5.0 × 10^5 cpm); EGTA, 0.05 μmole; 0.5 μg of Ca^{2+}-activated, phospholipid-dependent protein kinase in the presence or absence of $CaCl_2$, 0.1 μmole. The reaction was carried out for 5 min at 30°.

The binding of [^3H]W-7 to calmodulin and phosphatidylserine was investigated by the equilibrium binding technique of Hummel and Dreyer (16) on a Sephadex G-50 gel filtration column, as described previously (17). Interaction between [^3H]W-7 or $^{45}CaCl_2$ and phospholipids was studied by the method of Feinstein (18). Phospholipid phosphorus was determined by the method of Bartlett (19) and protein according to the method of Lowry *et al.* (20), with purified calmodulin or bovine serum albumin as a standard.

RESULTS

Inhibition of Ca^{2+}-dependent phosphorylation. We have reported that W-7, No. 233, and prenylamine inhibited myosin light-chain kinase activity of smooth muscle through an interaction with the Ca^{2+}-calmodulin complex (17). Thus, we investigated the effect of these calmodulin antagonists on Ca^{2+}-activated, phospholipid-dependent protein kinase activity. This enzyme activity was inhibited by these agents in a dose-dependent manner (Fig. 1). The IC_{50} values for W-7, No. 233, prenylamine, and a chlorine-deficient analogue of W-7 inhibiting Ca^{2+}-activated, phospholipid-dependent protein kinase were 131 μM, 58 μM, 255 μM, and 710 μM, respectively. These concentrations were higher than their respective IC_{50} values for the inhibition of the calcium, calmodulin-dependent myosin light-chain phosphorylation reported previously (17). This enzyme preparation did not contain calmodulin, as estimated by Ca^{2+}-dependent cyclic nucleotide phosphodiesterase activation, and was not activated by the addition of calmodulin up to 10 μM. Moreover, this protein kinase was activated, calcium-dependently, by phosphatidylserine and diolein, such being consistent with the results reported by other workers (5, 8).

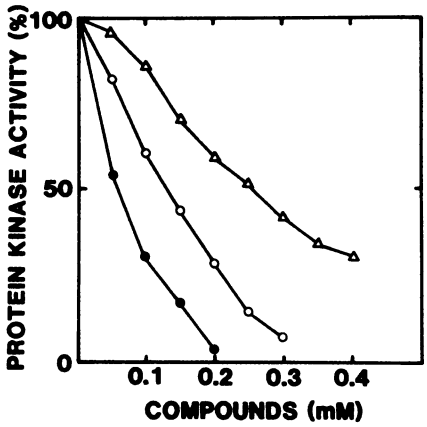

FIG. 1. *Inhibition of Ca^{2+}-activated, phospholipid-dependent protein kinase by various calmodulin antagonists*

Protein kinase activity was assayed as described under Experimental Procedures, with various concentrations of compounds added as indicated: ●——●, with No. 233; ○——○, with W-7; △——△, with prenylamine.

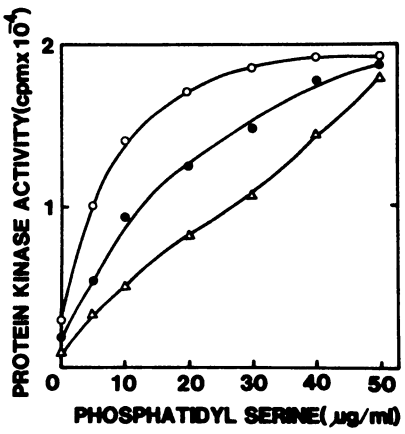

FIG. 2. *Effect of W-7 on Ca^{2+}-activated, phospholipid-dependent protein kinase as a function of phosphatidylserine concentration*

The protein kinase, from rabbit brain, was assayed as described under Experimental Procedures, except for the varying concentrations of phosphatidylserine and in the absence (○) or presence of W-7, 100 μM (●) or 200 μM (△).

These findings confirm the proposal that exogenously added calmodulin is not involved in Ca^{2+}-activated, phospholipid-dependent protein phosphorylation. They also suggest that calmodulin antagonists inhibit two types of Ca^{2+}-dependent protein kinases through different mechanisms (8) and may be less potent against Ca^{2+}-activated, phospholipid-dependent protein kinase than Ca^{2+}-calmodulin dependent protein kinase. As shown in Fig. 2, this inhibitory effect of W-7 on Ca^{2+}-activated, phospholipid-dependent protein kinase could be overcome by adding more phosphatidylserine. This was accompanied by an increased K_a for phosphatidylserine. The addition of W-7 not only increased the K_a of the protein kinase for Ca^{2+} but also decreased the extent of maximal enzyme activity (data not shown). The data shown in Fig. 2 are in accord with the data of Mori *et al.* (7) and Schatzman *et al.* (8) for neuroleptics. Kinetic analysis of W-7-induced inhibition of activation of Ca^{2+}-activated phospholipid-

dependent protein kinase revealed that this calmodulin antagonist inhibits this activity in a competitive fashion with phosphatidylserine. Its K_i value was 110 μM (Fig. 3A).

Differences in the mechanism of interaction W-7 with phosphatidylserine and calmodulin. We have already reported that W-7 inhibits Ca^{2+}, calmodulin-dependent myosin light-chain phosphorylation (17). To investigate further the mechanism of this inhibition and to compare the findings with those for Ca^{2+}-activated, phospholipid-dependent protein kinase, the activity of myosin light-chain kinase was determined under conditions of various concentrations of calmodulin and W-7. The data (Fig. 3B) were plotted as $1/v$ versus W-7 concentration, according to Dixon (21). As can be seen in Fig. 3B, W-7 inhibited myosin light-chain kinase in a competitive fashion with calmodulin. Its K_i value was 12 μM, which was significantly lower than that for inhibition of Ca^{2+}-activated, phospholipid-dependent protein kinase. These results are in agreement with data obtained in the phosphodiesterase inhibition (22) and [^{3}H]W-7 binding experiments (17). We then investigated [^{3}H]W-7 binding to purified calmodulin or to phosphatidylserine to determine whether W-7 inhibits these Ca^{2+}-dependent phosphorylations through interaction with calmodulin or phosphatidylserine. Here W-7 was found to bind to both phosphatidylserine and calmodulin. However, the effect of calcium ion on [^{3}H]W-7 binding to phosphatidylserine differed from that on binding to calmodulin (Fig. 4). In the absence of calcium ion (1 mM EGTA), the binding of W-7 to phosphatidylserine was maximal, and calcium ion inhibited this W-7 binding in a concentration-dependent manner, as shown in Fig. 4A. As shown in Table 1, similar results were also obtained and by using $^{45}CaCl_2$ according to the method of Feinstein (18). On the other hand, W-7 binding to calmodulin, which did not occur in the absence of calcium, was observed in the presence of 0.1 mM $CaCl_2$ (22). There was no significant inhibitory effect of calcium ion on this binding to 2 mM $CaCl_2$. These results suggest that W-7 interacts with these activators of Ca^{2+}-dependent protein kinases in a different fashion with calcium ion and with different affinities for calmodulin or phosphatidylserine.

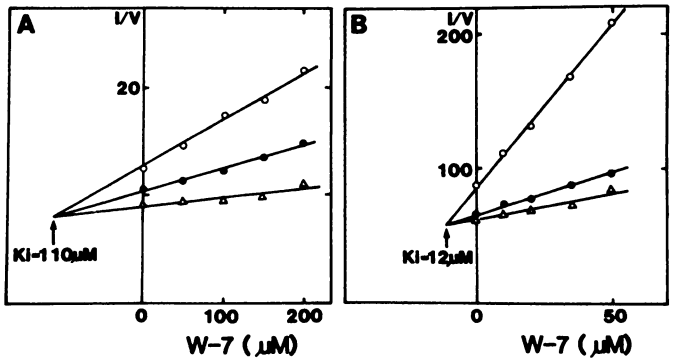

FIG. 3. *Kinetic analysis of W-7-induced inhibition of activation of Ca^{2+}-activated, phospholipid-dependent protein kinase (A) and myosin light-chain kinase (B) was carried out by using Dixon plots (21)*

Ca^{2+}-activated, phospholipid-dependent protein kinase activity (A) was measured as described under Experimental Procedures with phosphatidylserine, 10 μg/ml (○), 30 μg/ml (●), or 150 μg/ml (△). Myosin light-chain kinase (B) was assayed as described previously (15), with calmodulin, 30 ng/ml (○), 4 μg/ml (●), or 40 μg/ml (△).

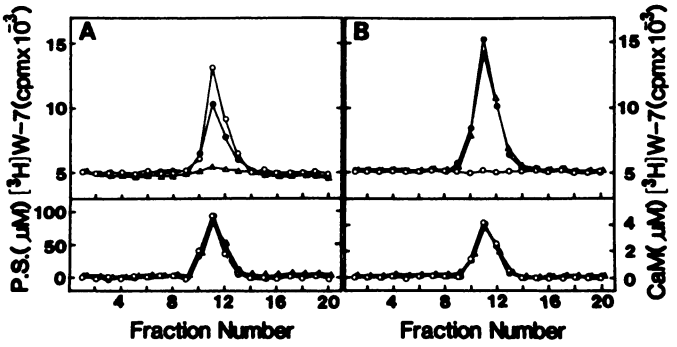

FIG. 4. *Elution profile for the measurement of W-7 binding to phosphatidylserine (A) or calmodulin (B)*

Sephadex G-50 (0.9 × 28.0 cm) was pre-equilibrated with buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM imidazole, 3 mM magnesium acetate, and 0.2 μM [^{3}H]W-7, plus 1 mM EGTA (○), 0.1 mM $CaCl_2$ (●), or 2 mM $CaCl_2$ (△) at 25°. Purified phosphatidylserine (*P.S.*) (180 μg) (A) or calmodulin (*CaM*) (180 μg) (B) was used for each experiment. Chloroform was removed from phosphatidylserine by a stream of nitrogen; the lipid was sonicated in 20 mM Tris-HCl (pH 7.5) and then used for the experiment. The gel filtration was carried out at 25° at a flow rate of 8.6 ml/hr, and 0.86-ml fractions were collected. Samples (0.5 ml) of each fraction were analyzed for radioactivity. Phospholipid phosphorus and protein were assayed as described under Experimental Procedures.

TABLE 1

Effect of W-7 on the binding of calcium by phosphatidylserine

Our methods were essentially those used by Feinstein for measurement of calcium binding by phospholipids (18). One milliliter of a solution containing 116 μmoles of NaCl, 2.5 μmoles of KCl, 10 nmoles of $^{45}CaCl_2$, 2.0 μmoles of Tris buffer (pH 7.4), and various concentrations of W-7 was combined with 2.0 ml of a mixture of two volumes of chloroform to one of methanol; the chloroform-methanol mixture contained 0.1 mg of phosphatidylserine per milliliter.

W-7	Bound calcium	Inhibition
mM	*nmoles*	%
None	8.75	0
0.3	8.23	6
0.5	7.09	19
1.0	1.23	86

Hydrocarbon chain length of naphthalenesulfonamides and inhibition. In recent work, we found that the actions of naphthalenesulfonamide derivatives, as selective inhibitors of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase, depended on the length of the hydrocarbon chain (C_5–C_{10}) (22). In the present work, we synthesized naphthalenesulfonamides with a shorter alkyl chain (C_2, C_3, and C_4) and investigated the effect of these naphthalenesulfonamides with various hydrocarbon chain lengths (C_2–C_{10}) on these two kinds of Ca^{2+}-dependent protein phosphorylations. As shown in Fig. 5, the inhibition of myosin light-chain phosphorylation clearly depended on the length of the alkyl chain (C_2–C_{10}), but the Ca^{2+}-activated, phospholipid-dependent protein kinase did not. IC_{50} values of these compounds of the series under study for Ca^{2+}-activated, phospholipid-dependent protein kinase were not related to the hydro-

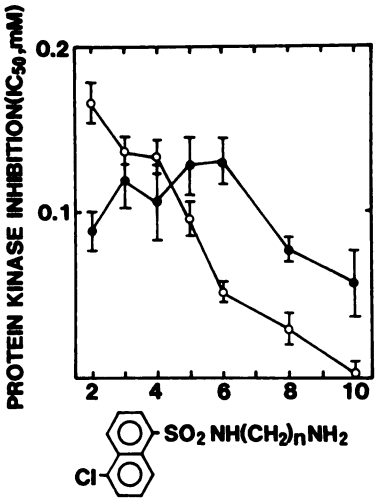

FIG. 5. *Effect of naphthalenesulfonamides of various hydrocarbon chain lengths on Ca^{2+}-activated, phospholipid-dependent protein kinase and Ca^{2+}, calmodulin-dependent myosin light-chain kinase*

Ca^{2+}-activated, phospholipid-dependent protein kinase (●) and myosin light-chain kinase (○) were assayed as described under Experimental Procedures with various concentrations of naphthalenesulfonamides, as indicated. The IC_{50} values were calculated as the concentration necessary to produce 50% inhibition of Ca^{2+}-activated, phospholipid-dependent protein kinase and myosin light-chain kinase. Data points represent the means and standard error of the IC_{50} values determined in four experiments.

carbon chain length of naphthalenesulfonamides. There was a significant difference in IC_{50} values for *N*-(10-aminodecyl)-5-chloro-1-naphthalenesulfonamide (C_{10}) or *N*-(2-aminoethyl)-5-chloro-1-naphthalenesulfonamide (C_2) between Ca^{2+}, calmodulin-dependent protein phosphorylation and Ca^{2+}-activated, phospholipid-dependent protein kinase. The small difference for the IC_{50} values of the two protein kinases for W-7 as compared with the difference in the K_i values (Fig. 3) may be due to the use of different amounts of each activator in the study.

DISCUSSION

Many diverse biological processes are regulated by the concentration of intracellular calcium (2). The mechanisms of the effects of calcium ion on cell function remain obscure; however, there is evidence suggesting that Ca^{2+}-dependent protein phosphorylation may serve as a final pathway for this second messenger (1). Ca^{2+}-dependent phosphorylation of endogenous substrate proteins are apparently regulated by the Ca^{2+}-calmodulin complex (3, 4). The small, acidic protein, calmodulin, appears to be ubiquitous throughout much of the plant and animal kingdoms. Recently, we demonstrated that W-7 and its derivatives bind to calmodulin in a Ca^{2+}-dependent manner and with high affinity (17), and that calmodulin may play an important role in platelet function (23), cell proliferation (24), vascular contraction (17), and insulin secretion (25) as determined by using the calmodulin antagonists, the naphthalenesulfonamides.

Another type of Ca^{2+}-dependent protein kinase, requiring phosphatidylserine and diglyceride as cofactors, was reported by Nishizuka and co-workers (26). The widespread occurrence of this protein kinase was demonstrated in various tissues and phyla of the animal kingdom (27, 28). The enzyme activity levels, as compared with those of cyclic AMP-dependent and cyclic GMP-dependent protein kinase, were exceedingly high in certain tissues (27, 28). This widespread and profound Ca^{2+}-activated, phospholipid-dependent protein kinase activity suggests potential roles for the calcium-mediated cellular processes. However, it is difficult to evaluate which Ca^{2+}-dependent protein phosphorylation system is predominantly involved in Ca^{2+}-dependent regulation of cell function in each tissue and species, although both of the protein kinases appear to differ with regard to physical and kinetic properties (11, 26). It was reported that neuroleptics such as trifluoperazine (8) and chlorpromazine (7) had an inhibitory effect on both Ca^{2+}-activated, phospholipid-dependent phosphorylation and Ca^{2+}, calmodulin-dependent protein phosphorylation. We found in our present work that structurally *unrelated* calmodulin antagonists such as W-7, No. 233, and prenylamine inhibited Ca^{2+}-activated, phospholipid-dependent protein kinase. These findings can be explained on the basis of the hydrophobic interaction between the hydrophobic regions of the Ca^{2+}-calmodulin complex and lipophilic calmodulin antagonists (29, 30) which may bind to phosphatidylserine.

The present studies clearly demonstrated definite differences between the two Ca^{2+}-dependent protein kinases in inhibitory potency, inhibitory interaction with calcium ion, and the activity-structure relationship of naphthalenesulfonamides. The IC_{50} values related to inhibition of the Ca^{2+}, calmodulin-dependent protein phosphorylation decreased with increase in the length of the alkyl chain. We have also noted that the potencies in human platelet aggregation inhibition and human platelet myosin light-chain kinase inhibition and affinity for Ca^{2+}-calmodulin were directly related to the hydrocarbon chain length of naphthalenesulfonamide derivatives (data not shown). Therefore, these naphthalenesulfonamide derivatives with various chain lengths can probably be used as potent and useful tools with which to differentiate the physiological role of these two Ca^{2+}-dependent protein phosphorylations in various cells and tissues.

As there are definite differences in the K_i values against each protein kinase, experimental data obtained with the use of these naphthalenesulfonamides should complement data obtained in biochemical studies on the relationship between the two protein kinases. It is of interest that, with a decrease in the affinity of Ca^{2+}-activated, phospholipid-dependent protein kinase for calcium ion in the presence of W-7, W-7 inhibited Ca^{2+} binding to phosphatidylserine and vice versa.

There are no ideal specific inhibitors of each Ca^{2+}-dependent protein kinase applicable for clarification of the functional significance of, and the interrelationship between, the two putative Ca^{2+}-dependent protein phosphorylation systems. Even if new compounds are synthesized, application may not be feasible because calmodulin antagonists are considered to bind to calmodulin hydrophobic regions (29, 30) and may to some extent interact with lipids.

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REFERENCES

1. Greengard, P. Phosphorylated proteins as physiological effectors: protein phosphorylation may be a final common pathway for many biological regulatory agents. *Science (Wash. D. C.)* **199**:146–152 (1978).
2. Rasmussen, H., and D. B. P. Goodman. Relationships between calcium and cyclic nucleotide in cell activation. *Physiol. Rev.* **57**:421–509, (1977).
3. Yamauchi, T., and H. Fujisawa. Most of the Ca^{2+}-dependent endogenous phosphorylation of rat brain cytosol proteins requires Ca^{2+}-dependent regulator protein. *Biochem. Biophys. Res. Commun.* **90**:1172–1178 (1979).
4. Dabrowska, R., D. Aromatorio, J. M. F. Sherry, and D. J. Hartshorne. Composition of the myosin light chain kinase from chicken gizzard. *Biochem. Biophys. Res. Commun.* **78**:1263–1272 (1977).
5. Takai, Y., A. Kishimoto, Y. Iwasa, Y. Kawahara, T. Mori, and Y. Nishizuka. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* **254**:3692–3695 (1979).
6. Levin, R. M., and B. Weiss. Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *Mol. Pharmacol.* **13**: 690–697 (1977).
7. Mori, T., Y. Takai, R. Minakuchi, B. Yu, and Y. Nishizuka. Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **255**:8378–8380 (1980).
8. Schatzman, R. C., B. C. Wise, and J. F. Kuo. Phospholipid-sensitive calcium-dependent protein kinase: inhibition by antipsychotic drugs. *Biochem. Biophys. Res. Commun.* **98**:669–676 (1981).
9. Hidaka, H., M. Asano, S. Iwadare, I. Matsumoto, T. Totsuka, and N. Aoki. A novel vascular relaxing agent, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide which affects vascular smooth muscle actomyosin. *J. Pharmacol. Exp. Ther.* **207**:8–15 (1978).
10. Yazawa, M., M. Sakuma, and K. Yagi. Calmodulin from muscle of marine invertebrates, scallop and sea anemone. *J. Biochem.* **87**:1313–1320 (1980).
11. Adelstein, R. S., and C. B. Klee. Purification and characterization of smooth muscle myosin light chain kinase. *J. Biol. Chem.* **256**:7501–7509 (1981).
12. Perrie, W. T., and S. V. Perry. An electrophoretic study of the low-molecular-weight components of myosin. *Biochem. J.* **119**:31–38 (1970).
13. Adelstein, R. S., M. A. Conti, D. R. Hathaway, and C. B. Klee. Phosphorylation of smooth muscle moysin light chain kinase by the catalytic subunit of adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **253**:8347–8350 (1978).
14. Inoue, M., A. Kishimoto, Y. Takai, and Y. Nishizuka. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J. Biol. Chem.* **252**:7610–7616 (1977).
15. Tanaka, T., M. Naka, and H. Hidaka. Activation of myosin light chain kinase by trypsin. *Biochem. Biophys. Res. Commun.* **92**:313–318 (1980).
16. Hummel, J. P., and W. J. Dreyer. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta* **63**:530–532 (1962).
17. Hidaka, H., T. Yamaki, M. Naka, T. Tanaka, H. Hayashi, and R. Kobayashi. Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. *Mol. Pharmacol.* **17**:66–72 (1980).
18. Feinstein, M. B. Reaction of local anesthetics with phospholipids, a possible chemical basis for anesthesia. *J. Gen. Physiol.* **48**:357–374 (1964).
19. Bartlett, G. R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466–468 (1959).
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
21. Dixon, M. The determination of enzyme inhibitor constants. *Biochem. J.* **55**:170–171 (1953).
22. Hidaka, H., M. Asano, and T. Tanaka. Activity-structure relationship of calmodulin antagonists: naphthalenesulfonamide derivatives. *Mol. Pharmacol.* **20**:571–578 (1981).
23. Nishikawa, M., T. Tanaka, and H. Hidaka. Ca^{2+}-calmodulin-dependent phosphorylation and platelet secretion. *Nature (Lond.)* **287**:863–865 (1980).
24. Hidaka, H., Y. Sasaki, T. Tanaka, T. Endo, S. Ohno, Y. Fujii, and T. Nagata. *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc. Natl. Acad. Sci. U. S. A.* **78**:4354–4357 (1981).
25. Niki, H., A. Niki, and H. Hidaka. Effect of a new calmodulin interacting agent (W-7) on insulin secretion. *Biomed. Res.* **2**:413–417 (1981).
26. Nishizuka, Y., Y. Takai, E. Hashimoto, A. Kishimoto, Y. Kuroda, K. Sakai, and H. Yamamura. Regulatory and functional compartment of three multifunctional protein kinase systems. *Mol. Cell. Biochem.* **23**:153–165 (1979).
27. Minakuchi, R., Y. Takai, B. Yu, and Y. Nishizuka. Widespread occurrence of calcium-activated, phospholipid-dependent protein kinase in mammalian tissues. *J. Biochem.* **89**:1651–1654 (1981).
28. Kuo, J. F., R. G. G. Andersson, B. C. Wise, L. Mackerlova, I. Salomonsson, N. L. Brackett, N. Katoh, M. Shoji, and R. W. Wrenn. Calcium-dependent protein kinase: widespread occurrence in various tissues and phyla of animal kingdom and comparison of effects of phospholipid, calmodulin, and trifluoperazine. *Proc. Natl. Acad. Sci. U. S. A.* **77**:7039–7043 (1980).
29. Tanaka, T., and H. Hidaka. Hydrophobic regions function in calmodulin-enzyme(s) interactions. *J. Biol. Chem.* **255**:11078–11080 (1980).
30. LaPorte, D. C., B. M. Wierman, and D. R. Storm. Calcium-induced exposure of hydrophobic surface on calmodulin. *Biochemistry* **19**:3814–3819 (1980).

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