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Naphthalenesulfonamides as Calmodulin Antagonists and Protein Kinase Inhibitors

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SUMMARY

N-(6-Aminoethyl)-5-chloro-1-naphthalenesulfonamide (A-3), which is a shorter alkyl chain derivative of the calmodulin (CaM) antagonist, W-7, was found to inhibit smooth muscle myosin light chain kinase (MLC-kinase) through a mechanism different from that related to W-7. Both the holoenzyme and the catalytic fragment, which is active without CaM, were susceptible to A-3 with a similar concentration dependency, thereby indicating that the inhibitory effect is due to the direct interaction of the compound with the enzyme molecule and not with the enzyme activator. Naphthalenesulfonamides are both CaM antagonists and direct inhibitors of MLC-kinase, and these actions depend on the length of the alkyl chain (C_2–C_8). Although the potencies in inhibiting CaM functions increased, the direct effects on MLC-kinase decreased with extension of the carbon chain of the derivatives. Kinetic studies indicated that A-3 inhibited MLC-kinase competitively with respect to ATP and that the K_i value was 7.4 μM. A-3 was also a competitive inhibitor of cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, casein kinase I, and casein kinase II, with respect to ATP. The K_i values of naphthalenesulfonamides for these enzymes also increased with extension of the carbon chain of the derivatives. These results suggest that naphthalenesulfonamides inhibit protein phosphorylation not only by inhibition of the enzyme-activating process but also by inhibition of the catalytic process. The mode of interaction between the derivatives and protein kinases differs from the interaction between the derivatives and CaM.

Diverse biological processes are regulated by the concentration of intracellular calcium (1). 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 (2–4). Ca^{2+}-dependent phosphorylation of endogenous substrate proteins seems to be regulated by the Ca^{2+}-CaM complex (5, 6). The small, acidic protein, CaM, is ubiquitous throughout much of the plant and animal kingdoms. Recently, we demonstrated that W-7 and its derivatives bind to CaM in a Ca^{2+}-dependent manner and with high affinity, and that CaM may play an important role in platelet function through Ca^{2+}-CaM-dependent myosin light chain phosphorylation (7). CaM, however, regulates various enzyme activities such as cyclic nucleotide phosphodiesterase, adenylate cyclase, (Ca^{2+} + Mg^{2+}) ATPase from human erythrocyte, and MLC-kinase. The CaM activation of these enzymes by CaM is inhibited by W-7 (7).

In the present work, we found that the shorter alkyl chain derivatives of W-7 preferentially inhibit MLC-kinase, as compared to other Ca^{2+}-CaM-dependent enzymes such as cyclic nucleotide phosphodiesterase, and that naphthalenesulfonamides not only interact with CaM but also directly inhibit MLC-kinase by binding competitively with respect to ATP. We also studied the effects of naphthalenesulfonamides on other protein kinases, including cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, MLC-kinase, casein kinase I, and casein kinase II.

Materials and Methods

W-7 and derivatives were synthesized by the method of Hidaka *et al.* (8). Cyclic [^{3}H]guanosine 3′,5′-monophosphate, guanosine 5′-[γ-^{32}P]triphosphate, and adenosine 5′-[γ-^{32}P]triphosphate were from Amersham International Limited. Trypsin (bovine pancreas), trypsin inhibitor (soybean), snake venom (*Crotalus atrox*), and histone type III-S were purchased from Sigma Chemical Company. Histone H2B was purchased from Boehringer-Mannheim Biochemicals. PS (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. CaM isolated from bovine brain was purified by the procedure described by Yazawa *et al.* (9). CaM-deficient Ca^{2+}-CaM cyclic nucleotide phosphodiesterase was partially purified from bovine brain by a modification of the method of Kincaid *et al.* (10).

Myosin light chain was prepared from chicken gizzard by the method

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ABBREVIATIONS: CaM, calmodulin; MLC-kinase, myosin light chain kinase; PS, phosphatidylserine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetate; A-3, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide.

of Hathaway and Haeberle (11). The partially purified holoenzyme of cAMP-dependent protein kinase I (second DE-52 step) and its purified catalytic subunits were prepared from rabbit skeletal muscle by the method of Beavo *et al.* (12). cGMP-dependent protein kinase from pig lung was partially purified by the method of Kuo and Greengard (13). Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) was prepared from rabbit brain, as described by Inagaki *et al.* (14). MLC-kinase was purified from chicken gizard by the method of Walsh *et al.* (15). Casein kinase I from rat liver was prepared by the method of Meggio *et al.* (16). Casein kinase II from rabbit skeletal muscle was prepared according to the method of Huang *et al.* (17). Myosin was prepared from rabbit skeletal white muscle by the method of Perry (18).

Enzyme assay and determinations. Cyclic nucleotide phosphodiesterase was measured according to the method of Hidaka and Asano (19). cAMP-dependent protein kinase 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, 2 mM EGTA, 1 μM cAMP or no cAMP, 3.3–20 μM [γ-^{32}P]ATP (4×10^5 cpm), 0.5 μg of the enzyme, 100 μg of histone H2B, and each compound as indicated. cGMP-dependent protein kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 50 mM magnesium acetate, 2 mM EGTA, 1 μM cGMP or no cGMP, 3.3–20 μM [γ-^{32}P]ATP (4×10^5 cpm), 100 μg of histone H2B, 2.4 μg of the enzyme, and each compound as indicated. 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 μM [γ-^{32}P]ATP (4×10^5 cpm), 100 μg of histone H1, and 0.3 μg of the enzyme. 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), 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. Casein kinase I 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 EGTA, 1 mM dithiothreitol, 3.3–20 μM [γ-^{32}P]ATP (4×10^5 cpm), 3.0 μg of the enzyme, and 800 μg of casein (Hammerstein quality). Casein kinase II activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 5 mM magnesium acetate, 0.5 mM EGTA, 1 mM dithiothreitol, 3.3–20 μM [γ-^{32}P]ATP (4×10^5 cpm) or 3.3–20 μM [γ-^{32}P]GTP (7×10^5 cpm), 2.8 μg of the enzyme, and 800 μg of casein (Hammerstein quality). MLC-kinase and protein kinase C were treated with trypsin, as described by Tanaka *et al.* (20) and Inoue *et al.* (21), respectively. Assays were performed for 1, 3, and 5 min at 30° and in all cases demonstrated linear incorporation of γ-^{32}P-phosphate into substrate over the 5-min assay. The 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 × *g* for 15 min. Activities of adenylate cyclase and guanylate cyclase were determined by the method of Nakazawa *et al.* (22) and Hagiwara *et al.* (23). The standard assay mixture contained 20–200 μM [^{3}H]ATP (2×10^6 cpm) or 20–200 μM [^{3}H]GTP (2×10^6 cpm), 0.5 mM cAMP or cGMP, 15 mM creatine phosphate, 40 μg of creatine kinase, 10 mM $MgCl_2$ or 3 mM $MnCl_2$, 50 mM Tris-HCl buffer (pH 7.7), various concentrations of test drug, and an appropriate amount of the enzyme in a total volume of 0.25 ml. The reaction was started by the addition of the enzyme. Assays were performed for 5, 10, and 15 min at 30° and in all cases demonstrated linear synthesis of cAMP or cGMP over the 15-min assay. The reaction was terminated by boiling for 2 min, following the addition of 40 μl of 1 M HCl. The radioactive cAMP or cGMP produced was isolated by the serial use of a neutral aluminum oxide/AG1-X2 (Bio-Rad Laboratories) column. The assay mixture for adenylate cyclase or guanylate cyclase was adjusted to neutral pH with 1 M Tris solution, and sodium pyrophosphate was added to make a final concentration of 7 mM. Each cyclase mixture was then applied onto a neutral aluminum oxide column (0.5 g, 0.6 × 2.0 cm). When this column was washed with 10 ml of 50 mM Tris-HCl buffer (pH. 7.7), cAMP or cGMP passed through the column while other nucleotides remained absorbed. The washing solutions containing the cycle nucleotides passed directly onto an AG1-X2 column (chloride form 0.7 × 3 cm). The neutral aluminum oxide column was removed, and the resin column was washed with 0.005 M or 0.05 M HCl; cyclic AMP or cyclic GMP was then eluted from this column with 2 ml of 0.05 M or 0.5 M HCl. More than 90% of cAMP or cGMP was always recovered from this column.

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, 0.2–1.0 mM ATP and A-3 or W-7 for myosin (K^+, EDTA)-ATPase; 50 mM Tris-HCl (pH 7.0), 500 mM KCl, 10 mM $CaCl_2$, 0.1 mg/ml of myosin, 0.2–1.0 mM ATP, and A-3 or W-7 for myosin Ca^{2+}-ATPase. Assays were performed for 1, 3, and 5 min at 25° and in all cases demonstrated linear production of inorganic phosphate over the 5-min period. The reaction was started by the addition of ATP and 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 (24).

Results

Naphthalenesulfonamides, such as W-7, which are potent CaM antagonists, inhibit MLC-kinase and cyclic nucleotide phosphodiesterase by inhibiting activation of the enzymes by CaM. When the aminohexyl (C_6) of W-7 was replaced by aminoethyl (C_2), the derivative proved to be a much weaker CaM antagonist (7). As shown in Fig. 1A, A-3 at concentrations up to 100 μM had only a weak effect on Ca^{2+}-CaM-dependent cyclic nucleotide phosphodiesterase activity, whereas W-7 produced nearly 100% inhibition of CaM activation of the enzyme over the same concentration range. Neither A-3 nor W-7 significantly inhibited the basal activity of the enzyme. Conversely, the ability of A-3 to inhibit Ca^{2+}-CaM-dependent MLC-kinase did not correlate with its inhibitory effects on Ca^{2+}-CaM-dependent cyclic nucleotide phosphodiesterase with the same concentration of CaM. Fig. 1B shows that A-3 and

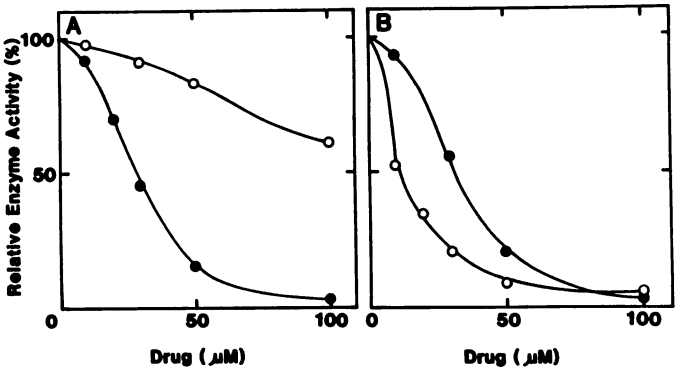

Fig. 1. Effect of W-7 and A-3 on the activities of cyclic nucleotide phosphodiesterase and MLC-kinase activated by CaM. Cyclic nucleotide phosphodiesterase activity (A) and MLC-kinase activity (B) were assayed as described under Materials and Methods, with various concentrations of compounds added as indicated: ●, with W-7, ○ with A-3. The concentration of CaM (0.5 μg/ml) was fixed in both enzyme reactions. MLC-kinase activity was assayed with 30 μM ATP.

W-7 inhibit MLC-kinase in a concentration-dependent manner and that the IC_{50} values of A-3 and W-7 are 13 μM and 34 μM, respectively. Although W-7 inhibited both enzyme activities activated by CaM with a similar concentration dependency, A-3 was much more potent in its ability to inhibit MLC-kinase than cyclic nucleotide phosphodiesterase. These results suggest that A-3 inhibits MLC-kinase through a mechanism differing from that of W-7.

MLC-kinase was reported to be alternatively activated in an irreversible manner by limited proteolysis with trypsin. In this process, the catalytically active fragment produced was entirely independent of Ca^{2+} and CaM. A-3, but not W-7, inhibited both the Ca^{2+}-CaM-dependent and Ca^{2+}-CaM-independent activites of MLC-kinase with similar concentration dependency (Table 1). Kinetic analysis by double-reciprocal plots revealed that the inhibition of MLC-kinase produced by A-3 was competitive with respect to ATP (Fig. 2). Since it has been reported that the CaM antagonists such as trifluoperazine and W-7 bind not only to CaM but also to myosin light chains and that their efficacy to inhibit activation of MLC-kinase depends on the concentration of myosin light chains used (25), we examined the degree of inhibition by A-3 and W-7 at two concentrations of myosin light chain (0.2 and 2 mg/ml). The activity of W-7 to block MLC-kinase activity was changed by changing light chain concentration, but that of A-3 was not (Fig. 3). In light of all these findings, the inhibitory actions of A-3 seem to be the result of direct effects on the active site of the enzyme and not due to effects on the enzyme-activating process and/or protein substrates.

Because A-3 competes with ATP in the MLC-kinase reaction, we investigated the effects of the compound on a wider range of protein kinases and on other ATP- or GTP-utilizing enzymes, specifically, adenylate cyclase and guanylate cyclase from human platelets and myosin ATPase from rabbit skeletal muscle. Tables 1 and 2 summarize the results obtained with A-3 and W-7. A-3 also inhibited cAMP-dependent and cGMP-dependent protein kinases, protein kinase C, casein kinase I, and casein kinase II, competitively with respect to ATP and noncompetitively with respect to protein substrates. We already reported that W-7 inhibits Ca^{2+}-dependent protein phosphorylation by Ca^{2+}-CaM-dependent MLC-kinase and protein kinase C in a competitive fashion with enzyme activators such as CaM and PS (26). To investigate further the mechanism of this inhibition and to compare findings with those of A-3, the effects of W-7 on the activities of Ca^{2+}-CaM-independent MLC-kinase and Ca^{2+}-PS-independent protein kinase C were investigated under conditions of various concentrations of ATP and W-7. As can be seen in Table 1, W-7 inhibited both the protein kinase activities in a competitive fashion with respect to ATP, and K_i values of W-7 for Ca^{2+}-CaM- and Ca^{2+}-PS-

TABLE 1

Effect of A-3 and W-7 on protein kinases

Enzyme	A-3		W-7	
	K_i	Competitive inhibition	K_i	Competitive inhibition
	μM		μM	
MLC-kinase + Ca^{2+}-CaM	7.4	ATP	18	CaM
Trypsin treated + EGTA	7.0	ATP	110	ATP
Protein kinase C + Ca^{2+}-PS	47	ATP	140	PS
Trypsin treated + EGTA	47	ATP	340	ATP
cAMP-dependent protein kinase	4.3	ATP	170	ATP
cGMP-dependent protein kinase	3.8	ATP	130	ATP
Casein kinase I	80	ATP	1200	ATP
Casein kinase II	5.1	ATP	110	ATP

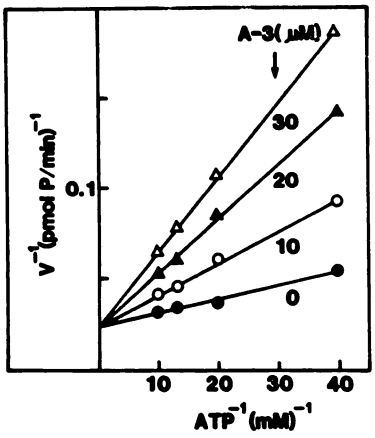

Fig. 2. Inhibition patterns of MLC-kinase by A-3. Reciprocal velocity versus 1/[ATP] at 0.5 μg/ml of CaM with varying A-3. All other conditions are as described under Materials and Methods.

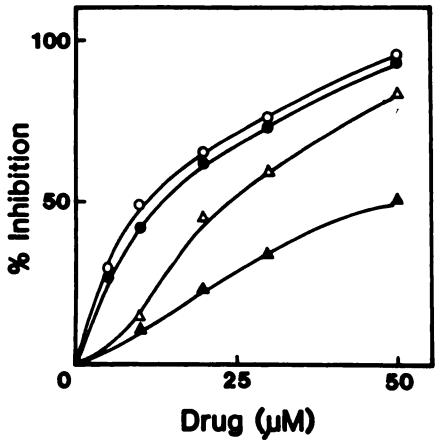

Fig. 3. Effect of myosin light chain on the inhibition of MLC-kinase by A-3 and W-7. The assay of protein kinase activity was performed as described under Materials and Methods with myosin light chain at 0.2 (○, △) or 2 mg/ml (●, ▲), with various concentrations of compounds added as indicated: ○, ●, with A-3; △, ▲, with W-7. The ATP concentration and the CaM concentration were 30 μM and 0.5 μg/ml, respectively.

TABLE 2

Effects of A-3 and W-7 on guanylate cyclase, adenylate cyclase, myosin ATPase and casein kinase II

Enzyme	K_i	
	A-3	W-7
	μM	
Guanylate cyclase	>1000	>1000
Adenylate cyclase	>1000	970
Myosin ATPase		
Ca^{2+}-ATPase	>1000	>1000
K^+, EDTA-ATPase	>1000	>1000
Casein Kinase II		
+ ATP[a]	5.1	110
+ GTP	5.2	65

[a] Casein kinase II + ATP data were taken from Table 1.

independent enzyme activities were 110 μM and 340 μM, much higher than those of both enzymes activated by their co-factors. W-7 acted on the other protein kinases at higher concentration with a mode of action apparently similar to that of A-3. Contrary to the potent abilities of A-3 to inhibit protein kinases, adenylate cyclase, guanylate cyclase activities, and myosin ATPase activities were affected only weakly by A-3 (Table 2). Furthermore, we investigated the ability of A-3 to block casein kinase II activity using GTP as a phosphoryl donor, since casein kinase II can utilize GTP as a phosphoryl donor as well as ATP. The potent ability of A-3 to block this casein kinase II activity is shown in Table 2. Thus, it has been clearly demonstrated that the short chain derivative of naphthalenesulfonamides, A-3, did not work as a simple analogue of ATP and exhibited selective affinity toward the nucleotide-binding sites of protein kinases.

It has been reported that the affinity for CaM and the potency in inhibiting Ca^{2+}-CaM-dependent cyclic nucleotide phosphodiesterase increased with extension of the carbon chain (C_2-C_6) of naphthalenesulfonamides (7). In the present work, we investigated the effect of these naphthalenesulfonamides with various hydrocarbon chain lengths (C_2-C_6) on the catalytically active fragment of MLC-kinase. As shown in Table 3, the K_i values for the enzyme increased with extension of the carbon chain of the derivatives. The K_i values of the compounds of the series for cAMP-dependent protein kinase, cGMP-dependent protein kinase, and the catalytic fragment of protein kinase C also increased with the length of the carbon chain.

Discussion

Protein phosphorylation is an established major, general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli (2–4). The cyclic nucleotides, Ca^{2+}-CaM and diacylglycerol are universal regulators, neither tissue nor species specific, and have an effect on a large number of cellular functions (2, 27). Biological functions of the cyclic nucleotides, Ca^{2+}-CaM and diacylglycerol, seem to be manifest through protein phosphorylations by cyclic nucleotide-dependent protein kinases, Ca^{2+}-CaM-dependent protein kinases, and protein kinase C, respectively.

TABLE 3

Effect on protein kinase of naphthalenesulfonamides with alkyl chain of various lengths

Compound	K_i			
	cAMP-dependent protein kinase	cGMP-dependent protein kinase	Protein kinase C (trypsin treatment)	MLC-kinase (trypsin treatment)
		μM		
N-(6-Aminoethyl)-5-chloro-1-naphthalenesulfonamide	4.3	3.8	47	7.0
N-(6-Aminopropyl)-5-chloro-1-naphthalenesulfonamide	23	15	92	18
N-(6-Aminobutyl)-5-chloro-1-naphthalenesulfonamide	24	44	120	25
N-(6-Aminopentyl)-5-chloro-1-naphthalenesulfonamide	110	55	230	60
N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide	170	130	340	110

We found in our present work that naphthalenesulfonamides inhibit MLC-kinase, not by inhibition of CaM activation of the enzyme but, rather, by competitive inhibition with respect to ATP. This is particularly true of the short alkyl chain derivatives. The relationships of the length of alkyl chain of naphthalenesulfonamides to their direct effects on protein kinases and on Ca^{2+}-CaM were compared. Although the potency in inhibiting CaM functions increased, the direct effects on MLC-kinase decreased with extension of the carbon chain. It has been reported that the affinity of naphthalenesulfonamides for Ca^{2+}-CaM correlated well with their hydrophobicity and their potency in inhibiting Ca^{2+}-CaM-dependent cyclic nucleoteide phosphodiesterase (7). The correlation between their hydrophobicity and affinity for the Ca^{2+}-CaM complex was observed using derivatives with various lengths of alkyl chain, since the hydrophobicity of naphthalenesulfonamides increased with extension of the carbon chain length (7). This evidence suggests that the affinity of naphthalenesulfonamides for MLC-kinase cannot be explained by hydrophobic interactions, and the mechanism of interaction between naphthalenesulfonamides and MLC-kinase may differ from the interaction between the derivatives and CaM.

Many pharmacological agents interact with activators of protein kinases, e.g., cyclic nucleotides, phospholipid, or CaM (7, 28, 29). However, there are few ideal inhibitors of protein kinases which have primary effects on the active sites of the enzyme. Among various inhibitors of protein phosphorylations, the diuretic, amiloride, was found to have primary effects on the protein kinases. This drug was first reported to inhibit cAMP-dependent protein kinase competitively with respect to ATP (30). Amiloride also inhibited other protein kinases, i.e., Ca^{2+}-CaM-dependent glycogen synthase, glycogen synthase kinase-5, casein kinase I, and protein kinase C, by competing at the ATP-binding site (31, 32). We found that naphthalenesulfonamide derivatives also inhibited not only MLC-kinase but also various protein kinases, examined competitively with respect to ATP. These findings can be explained on the basis of similarities of the ATP binding sites of protein kinases or those in close proximity. In addition to this pharmacological evidence, the similarities of amino acid sequences surrounding the ATP-binding region of several protein kinases, i.e., cAMP-dependent protein kinase and oncogenic tyrosine kinase have been reported by Kamps *et al.* (33).

Several protein kinases have been purified in a number of laboratories and characterized with respect to kinetic, physical, and immunological properties. At present, however, little evidence is available concerning the geometry of the active sites of protein kinases or their adjacent sites. Hoppe *et al.* (34) and Baydoun *et al.* (35, 36) have determined the binding constants for a series of nucleotide analogues, in an effort to map the ATP-binding sites of some protein kinases. Their data may help to clarify the general features of how a substrate such as ATP binds and is recognized by its enzymic site. In the present work, we found definite similarities of the interrelation between substitutents of naphthalenesulfonamides and their inhibitory effects on cAMP-dependent protein kinase and cGMP-dependent protein kinase, and the catalytic fragments of protein kinase C and MLC-kinase. The K_i values increased with extension of the carbon chain length of naphthalenesulfonamides. We have

also noted that the K_i values for cyclic nucleotide-dependent protein kinases of isoquinolinesulfonamides with various alkyl chains increased with extension of the hydrocarbon chain of the derivatives (C_2-C_6) (37).

Finally, we analyzed the inhibition of MLC-kinase by naphthalenesulfonamide derivatives of varying alkyl chain lengths. On the basis of differential inhibitions of CaM-activated MLC-kinase and cyclic nucleotide phosphodiesterase activities, it is inferred that the longer chain length derivatives act as inhibitors of CaM function, whereas the shorter chain derivatives bind competitively with ATP at the ATP-binding site of MLC-kinase. Supporting evidence for this hypothesis comes from the effects of the derivatives on other protein kinase activities. This study also indicates that the shorter chain length derivatives are not selective for inhibition of MLC-kinase. However, the present studies suggest not only the notion that CaM antagonists such as naphthalenesulfonamides have many effects which are unrelated to CaM, but also the possibility that naphthalenesulfonamides or certain derivatives may serve as useful tools with which to study directly protein kinases. Clearly, more specific inhibitors for a certain protein kinase need to be developed before *in vivo* studies can be performed.

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