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Hydrophobic Interaction of the Ca^{2+}-Calmodulin Complex with Calmodulin Antagonists

Naphthalenesulfonamide Derivatives

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SUMMARY

Calmodulin antagonists such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), which bind to calmodulin (CaM) in the presence of Ca^{2+} and selectively inhibit CaM-induced enzyme activation, contain a hydrophobic moiety. In this study, the naphthalenesulfonamide derivatives that lacked the chlorine molecule were less hydrophobic than those with chlorine. The chlorine-deficient derivatives also were less able to suppress the fluorescence of the hydrophobic probe (2-*p*-toluidinylnaphthalene-6-sulfonate) in the presence of the Ca^{2+}-CaM complex. The affinity of naphthalenesulfonamides for Ca^{2+}-CaM correlated well with their hydrophobicity and their potency in inhibiting Ca^{2+}-CaM-dependent enzymes such as Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. The correlation between their hydrophobicity and affinity for the Ca^{2+}-CaM complex also was observed when derivatives with various lengths of alkyl chain were used and when bromine, fluorine, or cyanogen was substituted for chlorine. Our observations suggest that these CaM antagonists may bind to the Ca^{2+}-CaM complex through a hydrophobic interaction.

INTRODUCTION

A considerable amount of data suggests that CaM[1] may be an intracellular Ca^{2+}-receptive protein in various cells (1, 2). Evidence for this idea is based on the following experimental evidence. (a) Calcium binds to purified CaM with a high affinity and induces conformational changes. (b) CaM plus calcium activates a number of important enzymes. (c) The Ca^{2+}-CaM complex prevents the *in vitro* assembly of microtubular protein. Despite this biochemical evidence that CaM is an intracellular Ca^{2+}-receptor protein, the complete physiological function of CaM remains obscure. Pharmacological studies using CaM antagonists such as phenothiazines and naphthalenesulfonamides suggested that this cytosolic binding protein may play an important role in platelet function (3), cell proliferation (4), vascular contraction (5), and neutrophil degranulation (6).

It has been reported that phenothiazines and naphthalenesulfonamides bind to CaM in a calcium-dependent manner (5, 7). Recently it was proposed that binding of Ca^{2+} to CaM results in exposure of a hydrophobic domain and this domain may serve as the interface for the Ca^{2+}-dependent binding of CaM to enzymes or to CaM antagonists (8, 9).

We report here studies with three series of naphthalenesulfonamide derivatives which attempt to examine systematically the relationships between the hydrophobicity of each molecule and its affinity for CaM.

EXPERIMENTAL PROCEDURES

Materials. Bovine brain CaM was purified and checked for homogeneity, as previously reported (5, 10). CaM-deficient Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was purified from bovine heart to homogeneity, as outlined by LaPorte *et al.* (11). All derivatives of W-7 and [^{3}H]W-7 were synthesized by the method of Hidaka *et al.* (12). TNS, potassium salt, was purchased from Aldrich Chemical Company (Milwaukee, Wisc.). All other chemicals were of reagent grade or better.

Assay procedure. Cyclic nucleotide phosphodiesterase was measured as reported previously (13). CaM was assayed for its ability to activate a fixed amount of CaM-deficient phosphodiesterase under standard conditions (5). One unit of CaM was defined as the amount activating 50% of the maximal phosphodiesterase activity attainable under standard experimental conditions, and was equivalent to 10 ng of CaM (5). The equilibrium

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[1] The abbreviations used are: CaM, calmodulin; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide.

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binding procedure followed that described by Hummel and Dreyer (14). The displacement of [^{3}H]W-7 from purified CaM in the presence of calcium ion by various concentrations of the compounds under study was measured as previously reported (5). The buffer contained 0.5 μM [^{3}H]W-7, 20 mM Tris-HCl (pH 8.0), 20 mM imidazole, 3 mM magnesium acetate, and 0.1 mM $CaCl_2$ at 25°. Octanol-aqueous buffer partition coefficients were determined experimentally by the method of Leo *et al.* (15). The aqueous phase was 66 mM phosphate buffer (pH 8.0) saturated with *n*-octanol. The organic phase was buffer-saturated, distilled *n*-octanol. Initial substance concentrations in the aqueous phase were between 50 and 300 μM. The volume of each phase was chosen so as to give good sensitivity for UV-spectroscopic determination of the drug concentrations. Whenever possible, experiments were carried out at pH 8.0, as this was the pH of drug-binding and enzymatic assays. Log *P* at pH 8.0 was calculated using the equation described by Yeh and Higuchi (16)

$$p' = P\frac{1}{1 + 10^{(pK_a\text{-}pH)}}$$

where p' is the apparent partition coefficient at the particular pH and P is the intrinsic partition coefficient of the undissociated free base. The pK_a value of each compound was determined by spectrophotometric methods (17).

All fluorescence measurements were made with an Aminco-Bowman spectrofluorometer with the thermostat at 25° ± 1°. Total dilution never exceeded 5%, and relative fluorescence values were uniformly corrected for dilution. Fluorometric titration of CaM with the naphthalenesulfonamide derivatives was performed by preparing samples containing 40 nmoles of CaM and adding the indicated amounts of the naphthalenesulfonamides in 2 ml of 15 μM TNS, 50 mM Tris-HCl (pH 7.6), and 0.1 mM $CaCl_2$. Fluorescence intensity was then determined with excitation at 365 nm, and emission intensity was monitored at 420 nm.

RESULTS

Effect of Ca^{2+} on W-7 binding to CaM. We have demonstrated that [^{3}H]W-7 binds to purified CaM in the presence of 0.1 mM $CaCl_2$ but not in the absence of the calcium ion (1 mM EGTA) (5). Moreover, TNS fluorescence in the presence of CaM was increased when more than 1 μM Ca^{2+} was present and was maximal with 10 μM Ca^{2+} (9). This effect of Ca^{2+} concentration on the interaction between W-7 and CaM in purified bovine brain was studied using a Ca^{2+}-EGTA buffer system (18). W-7 binding to CaM significantly increased with 1 μM Ca^{2+} and was nearly maximal with 10 μM Ca^{2+} (Fig. 1). The concentrations of Ca^{2+} required to bind W-7 to CaM and to activate Ca^{2+}-dependent cyclic nucleotide phosphodiesterase in the presence of purified CaM were the same.

Dechlorination of naphthalenesulfonamide and affinity for CaM. Four pairs of chlorinated and dechlorinated naphthalenesulfonamide derivatives were synthesized by the method of Hidaka *et al.* (12). These pairs were investigated for their capacity to bind to CaM and to inhibit Ca^{2+}-CaM dependent cyclic nucleotide phosphodiesterase activation, as shown in Table 1. We recently have identified the calcium-dependent binding of W-7 to purified CaM and the selective inhibition of Ca^{2+}-CaM-dependent enzymes by this compound (19–21). The capacity of chlorinated and dechlorinated naphthalenesulfonamide derivatives to decrease specific binding of [^{3}H]W-7 to CaM is shown in Table 1. From the concentration of the unlabeled naphthalenesulfonamide derivatives required to decrease the binding of 0.5 μM [^{3}H]W-7 by one-half, one can evaluate the binding affinity of each of those compounds for purified CaM. Chlorine-deficient analogues were from 5–7 times less potent. Table 1 also summarizes the binding affinity of chlorinated and dechlorinated naphthalenesulfonamides for CaM and the potency of these compounds with regard to Ca^{2+}-CaM-induced activation of the phosphodiesterase. Inhibition of the phosphodiesterase by the chlorinated naphthalenesulfonamides was 4- to 18-fold more potent than inhibition by the dechlorinated derivatives. The chlorinated compounds also bound more effectively to purified CaM, suggesting that the actions of these naphthalenesulfonamides as CaM antagonists depended on chlorination of the naphthalene ring.

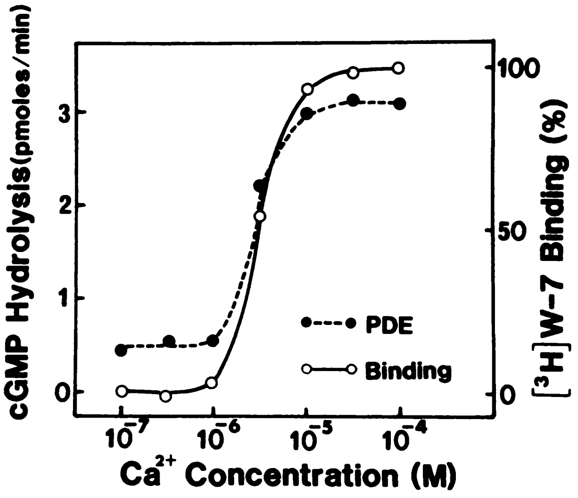


FIG. 1. *[^{3}H]W-7 binding to CaM as function of Ca^{2+} concentration*

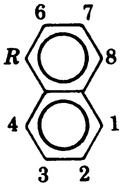
[^{3}H]W-7 binding to CaM and phosphodiesterase (*PDE*) activity with 200 ng of CaM were measured as described under Experimental Procedures. The Ca^{2+}-EGTA buffer system was performed as reported by Bartfai (18).

In addition, the effects of W-7 and W-5 (chlorine-deficient analogue) on the fluorescence of TNS in the presence of CaM and calcium were investigated to determine the affinity of each compound for hydrophobic interaction with CaM (8, 9). As shown in Fig. 2, W-7 was more potent than W-5 in suppressing the fluorescence of the TNS-Ca^{2+}-CaM complex. Similar results were obtained with other pairs of chlorinated and dechlorinated naphthalenesulfonamides (data not shown). The IC_{50} values of W-7 and W-5 were 34 μM and 78 μM, respectively (Fig. 2). The reason for the difference between the IC_{50} value of W-5 fluorescence inhibition and that obtained for direct competition with [^{3}H]W-7 is not clear at present. However, these results can be attributed to different affinities for hydrophobic regions of CaM, as W-5 inter-

TABLE 1

Relationship between affinity for calmodulin and hydrophobicity of naphthalenesulfonamides

Displacement of [^{3}H]W-7 from calmodulin by the compounds under study, phosphodiesterase (PDE) activity, and octanol-aqueous buffer partition coefficients for all of the agents tested were measured as described under Experimental Procedures. The binding of the labeled W-7 was measured in the presence of several concentrations of the drugs under study, and the concentration of unlabeled drug which displaced 50% of the labeled compound (IC_{50}) is given for each drug. The addition of 20 units (200 ng) of calmodulin produced about an 8-fold increase in phosphodiesterase activity. Phosphodiesterase activity was measured in the presence and absence of 200 ng of calmodulin and various concentrations of the compounds under study, using 0.4 µM cyclic GMP as substrate. The IC_{50} value is the concentration of drug required to produce 50% inhibition of the activation of phosphodiesterase. Each value is the mean of three experiments.

	Displacement of [^{3}H]W-7 from calmodulin (IC_{50})		Inhibition of PDE activation by calmodulin (IC_{50})		Octanol-aqueous buffer partition coefficient (log P)	
	$R = Cl$	$R = H$	$R = Cl$	$R = H$	$R = Cl$	$R = H$
	µM		µM			
1-$SO_2NH(CH_2)_5NH_2$	68	440	32	570	0.89	0.11
1-$SO_2NH(CH_2)_6NH_2$	31	210	26	240	1.46	0.13
1-$SO_2NH(CH_2)_8NH_2$	3.6		18		2.57	
1-$SO_2NH(CH_2)_{10}NH_2$	2.3		3.2		2.64	
2-$SO_2NH(CH_2)_4NH_2$	55	280	68	260	3.25	2.35
2-$SO_2NH(CH_2)_6NH_2$	16	180	14	130	4.55	3.00

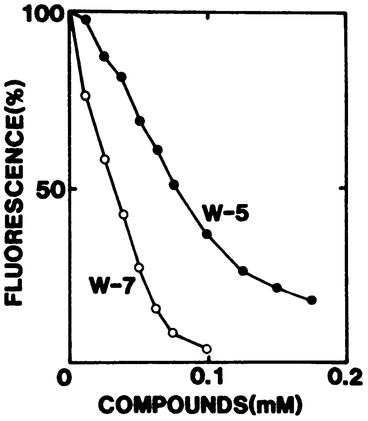


FIG. 2. *Effect of W-7 and W-5 on TNS fluorescence with the Ca^{2+}-CaM complex*

Fluorescence measurements were made as described under Experimental Procedures.

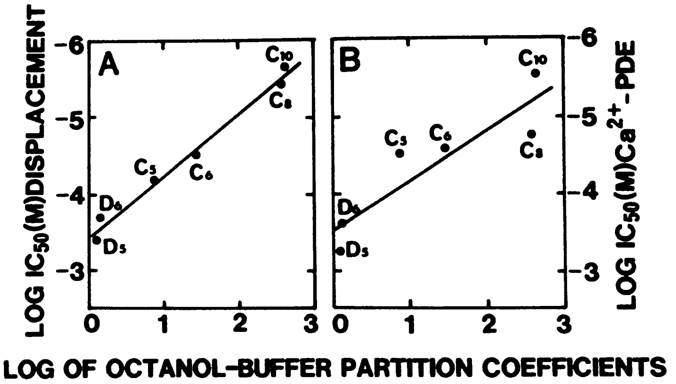


FIG. 3. *Correlation between inhibition of [^{3}H]W-7 binding to CaM and Ca^{2+}-CaM-dependent cyclic nucleotide phosphodiesterase by 1-naphthalenesulfonamides and the octanol-aqueous buffer partition coefficients for these compounds*

Ordinate: the logarithm of the IC_{50} values for inhibition of [^{3}H]W-7 binding to CaM (A) or Ca^{2+}-dependent phosphodiesterase (*PDE*) (B). Abscissa: the logarithm of the partition coefficients for these 1-naphthalenesulfonamides. The line of best fit was determined by regression analysis after logarithmic transformation. Chlorinated (*C*) and dechlorinated (*D*) naphthalenesulfonamides with various lengths of hydrocarbon chain are indicated by the number of carbon atoms.

acted more weakly than W-7 with the Ca^{2+}-CaM complex and inhibited to a lesser extent the activation of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (Table 1).

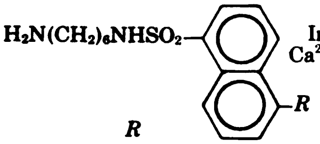
Octanol-water partition coefficients of CaM antagonists; naphthalenesulfonamides. To elucidate mechanisms of potentiation by the chlorination of naphthalenesulfonamide in the antagonistic action, the octanol-aqueous buffer partition coefficients of various naphthalenesulfonamides were determined. As shown in Table 1, the octanol-aqueous partition coefficient values of chlorine-deficient derivatives were always lower than those of chlorinated naphthalenesulfonamides. Moreover, the coefficient values of these agents correlated well with the IC_{50} values obtained for displacement of [^{3}H]W-7 from CaM and for inhibition of CaM-induced enzyme activation (Table 1). This correlation is shown in Fig. 3, where the log IC_{50} values for 1-naphthalenesulfonamide derivatives are plotted against the log of P, the partition coefficients. The correlation coefficients are $r = 0.99$ ($p < 0.01$; Fig. 3A) and $r = 0.90$ ($p < 0.02$; Fig. 3B), respectively, suggesting that the affinity of these compounds for the Ca^{2+}-CaM complex is closely related to the hydrophobicity. Although not statistically significant, a similar correlation between the two was observed in both 1-and 2-naphthalenesulfonamides (data not shown).

The degree to which these naphthalenesulfonamides inhibited the activation of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was directly related to their ability to bind to CaM, which demonstrates a highly significant positive correlation ($r = 0.94$, $p < 0.01$) between these two parameters.

TABLE 2

Relationship between hydrophobic fragment constant and affinity for calmodulin of naphthalenesulfonamides

Phosphodiesterase (PDE) activity was measured as described in Table 1. Fragment constants were taken from Leo *et al.* (23).

	Inhibition of Ca^{2+}-PDE (IC_{50})	Fragment constant ($f\phi$)
	μM	
Cyanogen	130	−0.34
Fluorine	50	0.37
Chlorine	31	0.94
Bromine	25	1.09

Hydrocarbon chain length of naphthalenesulfonamides. We have reported that the capacity of these naphthalenesulfonamide derivatives to prevent [^{3}H]W-7 from specific binding to CaM increased when the hydrocarbon chain was extended (C_5–C_{10}). In addition, the IC_{50} values related to the inhibition of the CaM-induced activation of Ca^{2+}-dependent phosphodiesterase decreased when the chain was lengthened (22). To assess which physical properties of the molecules are responsible for the affinity to CaM and the potency in phosphodiesterase inhibition, we determined the octanol-aqueous buffer partition coefficient of each analogue of naphthalenesulfonamide (Table 1). The degree to which these analogues inhibited the activation of phosphodiesterase and the [^{3}H]W-7 binding to purified CaM was related to their hydrophobicity, determined as the octanol-aqueous buffer partition coefficient of each analogue.

Hydrophobic fragmental constant and affinity for CaM. If these naphthalenesulfonamides do have a hydrophobic interaction with the Ca^{2+}-CaM complex, the change in hydrophobicity of these compounds should correlate well with the change in their affinity for CaM. Thus, cyanogen-, fluorine-, and bromine-substituted naphthalenesulfonamide derivatives were examined for their affinity for CaM, since their hydrophobicity has been well established (23). The affinity of these derivatives for CaM was estimated by their inhibition of the CaM-induced activation of phosphodiesterase, because their potency in this inhibition was demonstrated to correlate well with their ability to bind to CaM. As shown in Table 2, the compound with bromine was the most potent CaM antagonist, derivatives with chlorine or fluorine were intermediate in potency and that with cyanogen was the least potent. The IC_{50} values for these agents were correlated inversely with the fragment constant of hydrophobicity reported by Leo *et al.* (23). These data are consistent with hydrophobic interaction between the Ca^{2+}-CaM complex and these derivatives.

DISCUSSION

The results presented in this paper clearly show that change in the affinity of antagonists for CaM correlates well with change in the hydrophobicity, determined by using three series of naphthalenesulfonamide derivatives: (a) analogues with various lengths of alkyl chain; (b) chlorinated and dechlorinated naphthalenesulfonamides; and (c) derivatives substituted with cyanogen, fluorine, or bromine for chlorine. All of these modifications of compounds produced significant changes not only in the hydrophobicity of each molecule but also in the affinity of each for CaM. The potency of each compound as a CaM antagonist depended on the hydrophobicity, measured experimentally and expected theoretically (23).

Ca^{2+}-CaM-dependent enzymes such as Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (24, 25), myosin light-chain kinase (9), and the erythrocyte Ca^{2+}-ATPase (26) can be activated by the addition of acidic phospholipids or of fatty acids. These phenomena suggested to us that Ca^{2+}-CaM interacts hydrophobically with Ca^{2+}-CaM-dependent enzymes.

With the use of several hydrophobic fluorescent probes, it was found that when Ca^{2+} binds to the high-affinity sites of CaM it induces a conformational change which exposes hydrophobic groups; CaM-dependent enzymes or CaM antagonists such as trifluoperazine and naphthalenesulfonamides may bind to this hydrophobic domain (8, 9, 27).

There is an apparent correlation between the two properties of antipsychotic agents of inhibiting CaM-induced activation of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase and stabilizing membranes (28) and their hydrophobicity determined as the log of P, the partition coefficient (29). Moreover, we reported a correlation between the affinity of local anesthetics for CaM and their octanol-water partition coefficients (30). However, as the correlation was assessed by using a variety of structurally unrelated neuroleptic agents (28, 29) and local anesthetics (30), it remained obscure which structural elements of the molecules are responsible for their affinity for CaM and their hydrophobicity. In our study, we demonstrated clearly that three structural elements of naphthalenesulfonamide derivatives are responsible for their affinity for CaM and their hydrophobicity. Among the 1-naphthalenesulfonamide derivatives we studied, we found a definite correlation between their hydrophobicity and affinity for CaM. However, among the 1- and 2-naphthalenesulfonamide derivatives, the significance of the correlation between the two decreased. This suggests that other factors governing drug binding also may be present.

In summary, the data presented in this study indicate that the CaM antagonists, naphthalenesulfonamide derivatives, interact with the hydrophobic domain of CaM exposed by Ca^{2+}-binding, and that this may play an important role in CaM-enzyme interaction (8, 9). It seems likely that the major forces contributing to the binding of these agents to CaM are hydrophobic interactions, although polar or electrostatic interaction may make some contribution. Systematically synthesized naphthalenesulfonamides make feasible a quantitative characterization of the hydrophobic domain of CaM; this domain possibly may be responsible for the function of CaM.

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