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CGS 9343B, a Novel, Potent, and Selective Inhibitor of Calmodulin Activity

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SUMMARY

1,3-Dihydro-1-[1-[(4-methyl-4H,6H-pyrrolo[1,2-*a*][4,1]-benzoxazepin-4-yl)methyl]-4-piperidinyl]-2H-benzimidazol-2-one (1:1) maleate was synthesized in six steps from methyl anthranilate and designated CGS 9343B. CGS 9343B inhibited calmodulin-stimulated cAMP phosphodiesterase activity with an IC_{50} value of 3.3 μM. CGS 9343B was 3.8 times more potent than trifluoperazine (IC_{50} = 12.7 μM) as an inhibitor of calmodulin activity. CGS 9343B did not inhibit protein kinase C activity at concentrations up to 100 μM, whereas trifluoperazine inhibited protein kinase C activity with an IC_{50} value of 43.9 μM. CGS 9343B weakly displaced [^{3}H]spiperone from postsynaptic dopamine receptors with an IC_{50} value of 4.8 μM while the value for trifluoperazine, a potent antipsychotic agent, was 0.018 μM. It is concluded that CGS 9343B is a novel, potent, and selective inhibitor of calmodulin activity. Unlike trifluoperazine, CGS 9343B does not inhibit protein kinase C activity and does not possess potential antidopaminergic activity.

Calcium has long been known to be important for maintaining or controlling diverse cellular functions and is considered to be a second messenger mediating the action of external stimuli (1). However, the intracellular mechanism of action of calcium has only recently become understood. One pathway of calcium action is through the class of calcium-binding proteins which can directly activate intracellular enzymes. Calmodulin is the most abundant and versatile Ca^{2+}-binding protein which is capable of activating a variety of enzymes *in vitro* when complexed with Ca^{2+} (2, 3). The Ca^{2+}/calmodulin complex can activate certain protein kinases resulting in the subsequent phosphorylation and modification of enzyme activity (4, 5). An alternative pathway for the intracellular action of Ca^{2+} is through the binding and activation of protein kinase C which is also dependent on phospholipids for activity (6). Since calmodulin and protein kinase C have an affinity for calcium in the range of cytoplasmic free calcium (10^{-7}–10^{-5} M) and can affect protein phosphorylation systems in many cell types, these pathways are considered to be the known mediators of intracellular calcium action.

The development of specific inhibitors of either calmodulin or protein kinase C activity is needed to elucidate the physiological role of each pathway in response to different external stimuli. Inhibitors of calmodulin have been known to exist since the observation by Levin and Weiss (7) that many neuroleptic drugs bind to calmodulin and inhibit its activity. Although the interaction of neuroleptic agents such as trifluoperazine with calmodulin is unrelated to the clinical efficacy of these agents (8), this observation has provided investigators with a probe for calmodulin function in isolated cell systems (9–11). However, the interpretation of results with trifluoperazine in isolated cell systems became complicated when Schatzman *et al.* (12) reported that trifluoperazine as well as other neuroleptic agents are inhibitors of protein kinase C activity. The use of trifluoperazine as a probe for calmodulin function was also complicated by the well established activity of this agent as a potent dopamine receptor antagonist.

This report describes a novel inhibitor of calmodulin activity (CGS 9343B) which was approximately 4 times more potent than trifluoperazine and did not inhibit the activity of protein kinase C at concentrations up to 100 μM. Additionally, CGS 9343B showed only a weak interaction with dopamine receptors and was 270-fold less potent than trifluoperazine. CGS 9343B is a more selective probe for calmodulin function than other inhibitors reported to date.

Materials and Methods

Bovine heart and brain tissues were obtained from local slaughterhouses. Mice, Mbf:(SW) (18–20-g males), were obtained from Marland Farms (Hewitt, NJ). [^{3}H]cAMP (30 Ci/mmol), [^{3}H]spiperone (20–40 Ci/mmol), and [^{32}P]ATP (10–40 Ci/mmol) were purchased from NEN, Dupont. Trifluoperazine was a gift of Smith, Kline and French Laboratories (Philadelphia, PA).

Synthesis of CGS 9343B. 1,3-Dihydro-1-[1-[(4-methyl-4H,6H-pyrrolo[1,2-*a*][4,1]-benzoxazepin-4-yl)methyl]-4-piperidinyl]-2H-ben-

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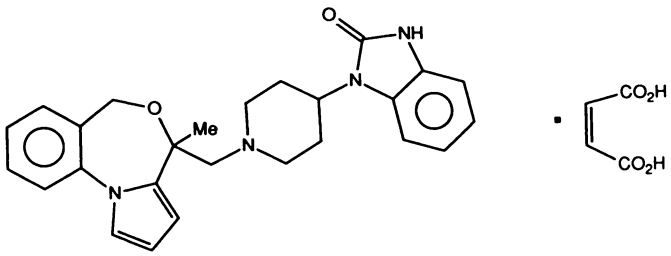
ABBREVIATIONS: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetate.

zimidazol-2-one (1:1) maleate, designated as CGS 9343B, was prepared in six steps from methyl anthranilate by the following reactions. Methyl anthranilate was condensed with 2,5-dimethoxytetrahydrofuran in glacial acetic acid at reflux temperature to yield methyl 2-(1H-pyrrol-1-yl)benzoate which was then reduced with lithium aluminum hydride in ether to yield 2-(1H-pyrrol-1-yl)benzenemethanol. Condensation of this alcohol with ethyl pyruvate in acetic acid followed by hydrolysis of the ester with sodium hydroxide yielded 4-methyl-4H,6H-pyrrolo[1,2-*a*][4,1]benzoxazepine-4-carboxylic acid. Condensation of this carboxylic acid with 4-(2-keto-1-benzimidazolinyl)piperidine using carbonylidiimidazole produced 1,3-dihydro-1-[1-[(4-methyl-4H,6H,-pyrrolo[1,2-*a*][4,1]benzoxazepin-4-yl)carbonyl]-4-piperidinyl]-2H-benzimidazol-2-one. This amide was reduced in tetrahydrofuran with lithium aluminum hydride to yield 1,3-dihydro-1-[1-[(4-methyl-4H,6H-pyrrolo[1,2-*a*][4,1]benzoxazepin-4-yl)-methyl]-4-piperidinyl]-2H-benzimidazol-2-one which was then converted to a 1:1 maleate salt (CGS 9343B). All compounds at each stage of synthesis gave satisfactory microanalysis and were characterized by infrared, NMR, and/or mass spectroscopy indicating a final purity greater than 99.5%.

Assay of calmodulin-stimulated cAMP phosphodiesterase activity. Calmodulin activity was measured by the ability of this protein to stimulate the activity of a partially purified preparation of cAMP phosphodiesterase. Calmodulin-dependent cAMP phosphodiesterase was partially purified from bovine heart by the method of Ho *et al.* (13) Calmodulin was purified from bovine brain by the procedure of Dedman *et al.* (14). Calmodulin and phosphodiesterase activities were measured as previously described (8). All reactions were carried out for 10 min at 30° in a total volume of 0.4 ml at pH 8.0 containing 5 mM $MgCl_2$, 40 mM Tris, 0.1 mM EGTA, 4.0 mM 2-mercaptoethanol, 0.01 mM cAMP including 200,000 cpm [^{3}H]cAMP, 10.2 ng of calmodulin-dependent phosphodiesterase, and inhibitors at the indicated concentrations. Tubes receiving calmodulin (100 ng/ml) also contained 0.3 mM $CaCl_2$. Reactions were terminated by the addition of 1.0 ml of methanol. Adenosine was separated from unhydrolyzed cAMP on a 1.0-ml column of Bio-Rad resin, AG 1-X2. All samples were counted by liquid scintillation and corrected for per cent recovery of adenosine which ranged from 85 to 95%. Background values for tubes containing no enzyme ranged from 1.0 to 1.5% of total counts added.



CGS 9343 B

Fig. 1. The structure of CGS 9343B.

Assay of protein kinase C activity. Protein kinase C was purified from the cytosolic fraction of mouse brain using a two-step protocol involving DE52 and phenyl Sepharose chromatography, essentially as described by Kikkawa *et al.* (15). After elution from the phenyl Sepharose column, the enzyme was adjusted to 0.01% Triton X-100, frozen in liquid nitrogen, and stored at −70°. Approximately 0.1 μg of chilled enzyme was assayed in a total volume of 250 μl containing cofactors, activators, and substrate at the following final concentrations: histone (lysine rich), 100 μg/ml; phosphatidylserine, 10 μg/ml; diolein (15% 1,2-isomer), 1 μg/ml; and ^{32}P-labeled ATP, 5 μM. Endogenous calcium levels were 20 μM as measured by atomic absorption spectroscopy. Tubes were incubated at 30° for 10 min and then returned to 4° and adjusted to 5 mM EDTA. Forty μl of the assay mixture were spotted onto phosphocellulose paper, washed extensively with water, and counted by liquid scintillation.

Assay of specific binding of [^{3}H]spiperone to dopamine D-2 receptors. Membrane suspensions prepared from calf caudate nucleus were incubated with [^{3}H]spiperone by a modification of the procedure described by Creese *et al.* (16). Caudate nuclei were dissected from freshly obtained calf brains and stored at −70°. For each binding

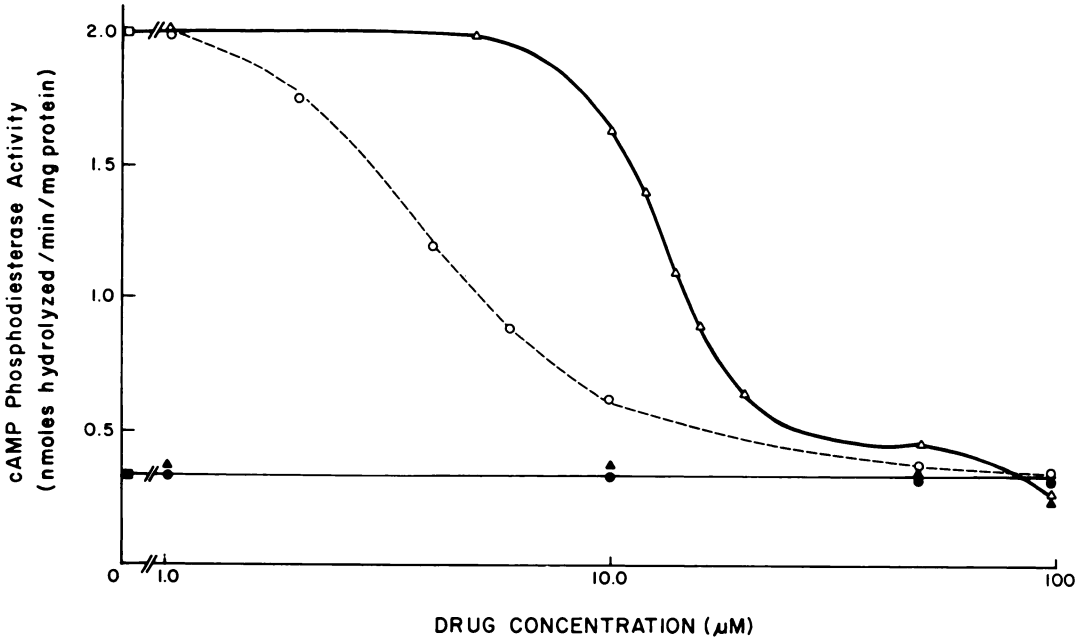


Fig. 2. The inhibition of calmodulin-stimulated cAMP phosphodiesterase activity by CGS 9343B and trifluoperazine. cAMP phosphodiesterase activity was measured as described in Materials and Methods. Drugs were dissolved in distilled water and diluted into the assay mixture at the indicated concentration. □, calmodulin-stimulated cAMP phosphodiesterase activity in the absence of drug; ○, calmodulin-stimulated cAMP phosphodiesterase activity in the presence of CGS 9343B; △, calmodulin-stimulated cAMP phosphodiesterase activity in the presence of trifluoperazine; ■, basal cAMP phosphodiesterase activity in the absence of drug; ●, basal cAMP phosphodiesterase activity in the presence of CGS 9343B; ▲, basal cAMP phosphodiesterase activity in the presence of trifluoperazine.

TABLE 1

Summary of the *in vitro* pharmacological profile of CGS 9343B and trifluoperazine

The IC_{50} values for CGS 9343B and trifluoperazine in the indicated assays are given with the standard errors. The number of times that the experiment was repeated is given in parentheses.

Assay	IC_{50}	
	CGS 9343B	Trifluoperazine
	μM	
Inhibition of calmodulin-stimulated PDE activity	3.3 ± 0.35 (3)	12.7 ± 0.89 (3)
Inhibition of protein kinase C activity	inactive ~100 μM (7)	43.9 ± 7.8 (7)
Inhibition of [^{3}H]spiperone binding	4.8 ± 1.2 (4)	0.018 ± 0.006 (4)

experiment, tissue was homogenized with a Brinkmann Polytron (setting 6, 20 sec) in 50 volumes (w/v) of ice-cold 50 mM Tris-HCl buffer (pH 7.7) containing 10 mM dithiothreitol. The homogenate was centrifuged at 48,000 × *g* for 10 min. The above homogenization and centrifugation steps were repeated once more with the resulting pellet. The final pellet was suspended in 100 volumes (based on original tissue weights) of ice-cold 50 mM Tris-HCl buffer (pH 7.6 at 25°) containing 0.01 mM pargyline, 0.1% ascorbic acid, 120 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM MgCl, and 10 mM dithiothreitol. The suspension was incubated at 37° for 5 min and returned to ice. Aliquots of the final tissue suspension were added to triplicate tubes containing [^{3}H] spiperone (0.2 nM final concentration) plus test compound in 0.1% ascorbic acid. Nonspecific binding was determined in the presence of 1.0 μM (+)-butaclamol. Tubes were incubated at 37° for 10 min and the suspensions were immediately filtered under vacuum through Whatman GF/B glass fiber filters. The filters were washed with 10 ml of cold 50 mM Tris-HCl buffer (pH 7.7 at 25°) and counted by liquid scintillation. IC_{50} values (concentration of compound required to displace 50% of specific binding) were determined by log-logit analysis of specific binding data.

Results

Inhibition of calmodulin-stimulated cAMP phosphodiesterase activity by CGS 9343B and trifluoperazine. cAMP phosphodiesterase activity was assayed at a basal specific activity of 0.3 nmol/min/mg of protein. In the presence of Ca^{2+} and calmodulin, the enzyme was stimulated 6.7-fold to 2.0 nmol/min/mg of protein. CGS 9343B (Fig. 1) inhibited calmodulin-stimulated cAMP phosphodiestase activity in a dose-dependent manner (Fig. 2) with an IC_{50} value of 3.3 μM (Table 1). Basal cAMP phosphodiesterase activity was unaffected at concentrations up to 100 μM. CGS 9343B was not tested at higher concentrations due to limitations of solubility. Trifluoperazine had an IC_{50} value of 12.7 μM for the inhibition of calmodulin-stimulated cAMP phosphodiesterase activity (Table 1). Unlike CGS 9343B, trifluoperazine inhibited basal cAMP phosphodiesterase activity by 35% at 100 μM (Fig. 2). This result suggested that, at higher concentrations, trifluoperazine was also a phophodiesterase inhibitor. The IC_{50} of 12.7 μM for trifluoperazine compared closely to the value of 10 μM originally reported by Levin and Weiss (7).

Effects of CGS 9343B and trifluoperazine on protein kinase C activity. Protein kinase C activity was assayed under conditions that were linear with respect to time and protein concentrations (Fig. 3). These conditions of linearity were used in the subsequent inhibitor studies and demonstrate that the activity of the enzyme was not limited by any of its cofactors, activators, or substrate. Protein kinase C had a specific activity of 500 pmol of P incorporated/min/μg of protein in the subsequent inhibitor studies. CGS 9343B did not inhibit protein kinase C activity at concentrations up to 100 μM (Fig. 4). Trifluoperazine inhibited protein kinase C activity in a dose-dependent manner (Fig. 4) with an IC_{50} value of 43.9 μM (Table 1) which compared closely to the reported IC_{50} value of 45 μM by Schatzman *et al.* (12).

Effect of CGS 9343B and trifluoperazine on spiperone binding to dopamine receptors. [^{3}H]Spiperone bound to postsynaptic dopamine receptors with high affinity (Fig. 5). Specific binding, defined by displacement with butaclamol, represented 79% of total bound radioactivity. Scatchard analysis of these binding data (Fig. 5, *inset*) revealed a dissociation constant (K_d) of 0.16 nM and a maximal number of binding sites (B_{max}) of 23 pmol/g of tissue (17). Trifluoperazine, a potent D_2 receptor antagonist, showed a dose-dependent displacement of specific binding (Fig. 6) with an IC_{50} value of 18 nM (Table 1). In this same assay, CGS 9343B had an IC_{50} value of 4800 nM which is 270 times weaker than trifluoperazine.

Discussion

CGS 9343B is a novel and potent inhibitor of calmodulin activity that does not inhibit protein kinase C activity at concentrations up to 100 μM, and only weakly interacts with the dopamine (D_2) receptor. Other structurally diverse inhibitors of calmodulin have been described but have not been pharmacologically characterized to the same extent as CGS 9343B or do not selectively inhibit calmodulin activity. W-7, a naphthalenesulfonamide inhibitor of calmodulin, also inhibits protein kinase C activity with a similar potency (18). Several other inhibitors of calmodulin have been described such as cetiedil (19), ophiobolin A (20), and calmidazolium (21), but their activities as protein kinase C inhibitors or dopamine receptor antagonists have not been established. Although these structurally diverse agents all inhibit calmodulin activity with IC_{50} values in the 5–20 μM range, a common structure-activity relationship is not evident. Prozialeck and Weiss (22) have suggested that a hydrophobic ring structure separated by two to four carbon atoms from a charged nitrogen is a common feature of phenothiazine calmodulin inhibitors. In this respect, CGS 9343B also has a hydrophobic domain consisting of an *N*-phenyl pyrrole separated from a basic nitrogen by at least two carbon atoms. Additionally, the 4-substituent on the piperidine in CGS 9343B is identical to that found in pimozide, one of the most potent neuroleptic inhibitors of calmodulin (8). Additionally, several peptide inhibitors of calmodulin have been described such as β-endorphin (23) and melittin (24). Although these peptides are potent calmodulin inhibitors, it is not clear whether they bind to the same site on calmodulin as the low molecular weight nonpeptide inhibitors. However, it is known that phenothiazines inhibit the binding of these peptides to calmodulin, suggesting that some common inhibitory features do exist. The drug-binding site in calmodulin appears to reside in the long central helix IV region which connects the Ca^{2+} binding domains 2 and 3 (25). Newton *et al.* (26) have covalently attached a phenothiazine to the Ca^{2+}/calmodulin complex and the primary site of attachment appears to be Lys 75 in the helix IV region (25). This region is also important for the interaction of calmodulin with target enzymes. Walsh and Stevens (27) have shown that carboxymethylation of methio-

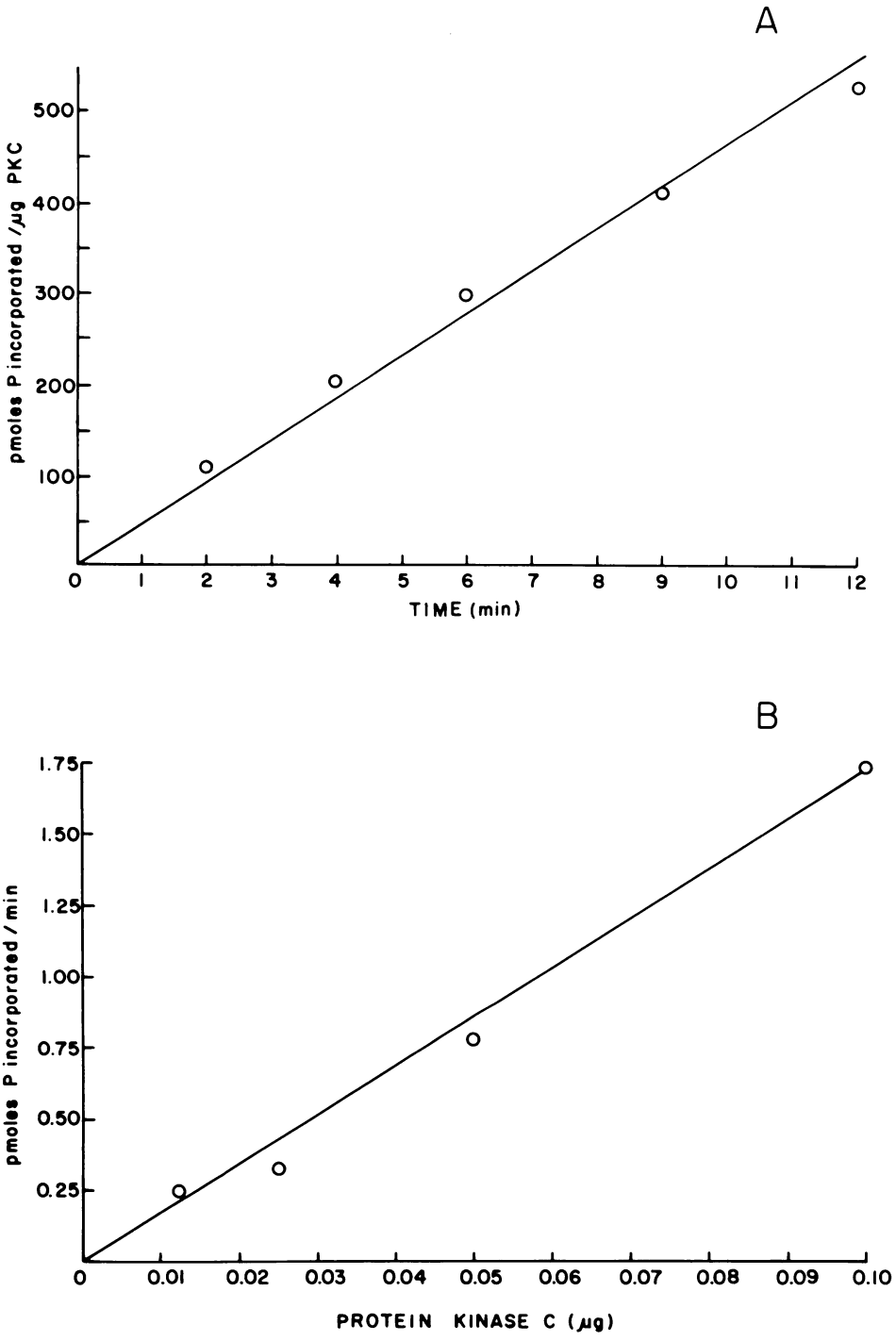


Fig. 3. Protein kinase C activity as a function of time, and protein concentration. Protein kinase C was purified as described in Materials and Methods and assayed using 0.1 μg of enzyme for the indicated times (A). Protein kinase C was assayed for 3 min using the indicated amount of enzyme (B).

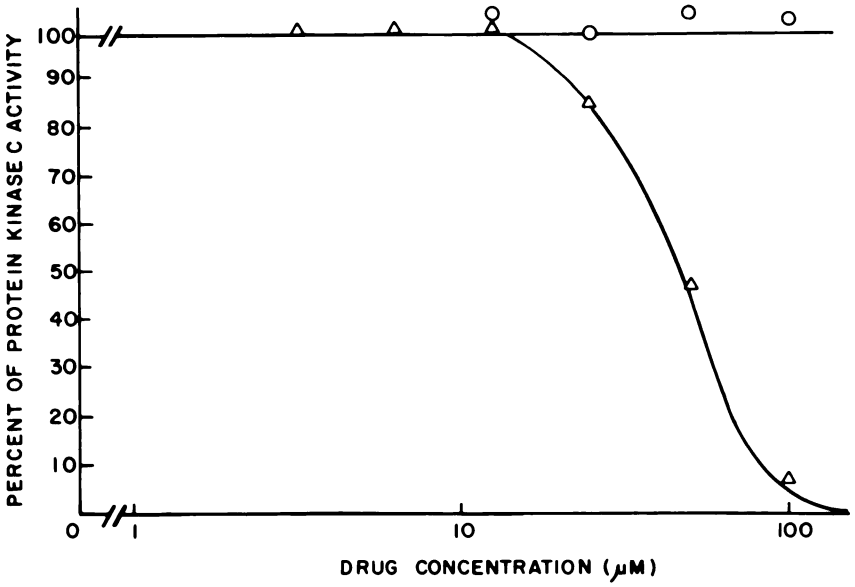


Fig. 4. The effect of CGS 9343B and trifluoperazine on protein kinase C activity. Protein kinase C was assayed under conditions that were linear with respect to time and enzyme concentration as described in the legend to Fig. 3. Drugs were dissolved in distilled water and diluted into the assay at the indicated concentration. ○, CGS 9343B; △, trifluoperazine.

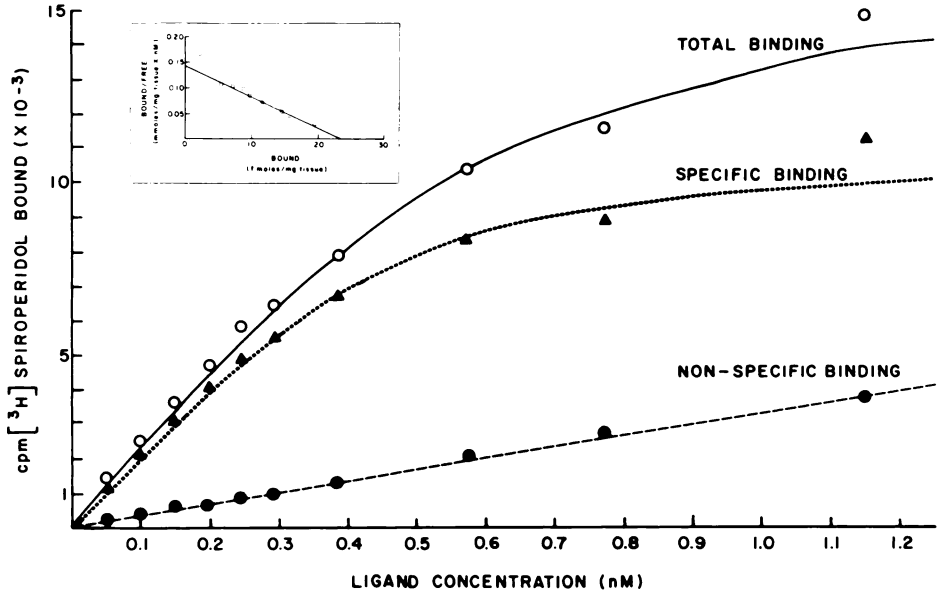


Fig. 5. Saturation isotherm for [^{3}H]spiroperidol binding to calf caudate membranes. Nonspecific binding was determined in the presence of 1 μM (+)-butaclamol. *Inset:* Scatchard plot of the above binding data calculated as described in Materials and Methods.

nine residues 71, 72, and 76 prevents the activation of cAMP phosphodiesterase, and these residues are contained within the helix IV region which serves as the backbone of calmodulin.

The specificity of CGS 9343B as a calmodulin inhibitor with no protein kinase C-inhibitory activity makes it a useful probe for calmodulin function in isolated cells. Calmodulin inhibitors such as trifluoperazine and W-7 have been used extensively to block the Ca^{2+}/calmodulin pathway in cells that have been treated with various external stimuli. However, the results are ambiguous since these inhibitors can also block the Ca^{2+}/protein kinase C pathway (9–11). Specific pharmacological probes such as CGS 9343B will complement the genetic approaches to elucidating the intracellular function of calmodulin. Recently, a paramecium mutant has been described which lacks a Ca^{2+}-dependent K^+ current (28). This current could be restored by the injection of wild-type calmodulin, suggesting that calmodulin is required for the regulation of this Ca^{2+}-dependent K^+ current. This observation could be confirmed by pharmacological means if CGS 9343B inhibited the Ca^{2+}-dependent K^+ current in the wild-type paramecium. Further genetic analysis of calmodulin function has been attempted by site-directed mutagenesis of cloned and expressed calmodulin genes. Putkey *et al.* (29) have produced a mutant calmodulin with 16 amino acid substitutions that will fully activate cAMP phophodiesterase and myosin light chain kinase but will only half-maximally activate calcineurin and calmodulin-dependent multiprotein kinase. These results suggest that the interaction of calmodulin with various target proteins may involve different functional domains. The use of genetically altered calmodulins with potent and specific inhibitors such as CGS 9343B may also elucidate whether there are different inhibitor-binding domains within calmodulin.

The lack of dopamine antagonism found with CGS 9343B may provide an opportunity to assess the clinical efficacy of this compound as an antisecretory agent. The dopamine antagonists trifluoperazine and chlorpromazine have been shown to be efficacious in reversing the severe secretory diarrhea in cholera patients (30, 31), but their neuroleptic properties pre-

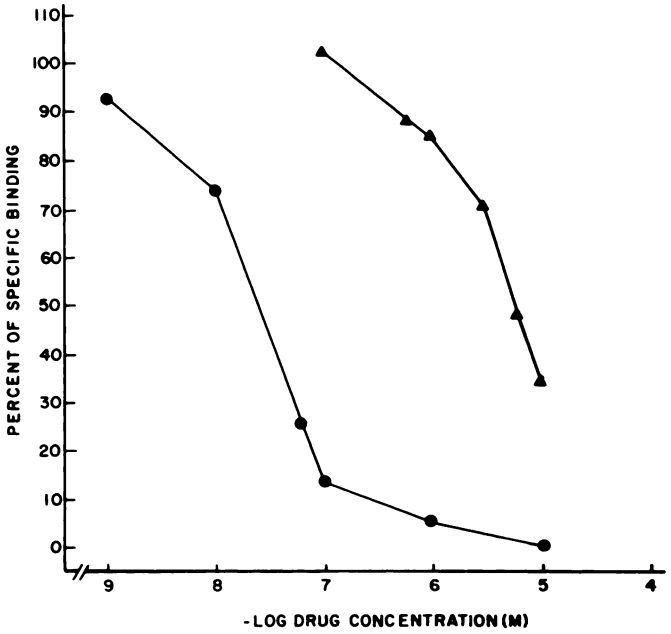


Fig. 6. The inhibition of specific [^{3}H]spiroperidol binding to calf caudate membrane by CGS 9343B and trifluoperazine. The binding of [^{3}H] spiroperidol was performed as described in the legend to Fig. 5. Each point represents the mean of three separate experiments. ▲, CGS 9343B; ●, trifluoperazine.

clude their general use. Several lines of evidence suggest that the efficacy of trifluoperazine is due to its activity as a calmodulin inhibitor. Calmodulin is highly concentrated in intestinal microvilli (32) and has been shown to mediate the Ca^{2+}-dependent inhibition of NaCl-coupled transport in purified intestinal brush border membrane vesicles (33). This coupled electroneutral transport is a fundamental mechanism for fluid absorption in the intestine (34). Since trifluoperazine can block this Ca^{2+}/calmodulin inhibition of NaCl transport (33), this may explain its efficacy in the treatment of secretory diarrhea. CGS 9343B is an acceptable calmodulin inhibitor whose therapeutic potential should be investigated.

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