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DIHYDROPYRIDINE RECEPTORS: POSSIBLE ALLOSTERIC
REGULATION BY TREMORGENIC (U) TEXAS UNIV HEALTH
SCIENCE CENTER AT SAN ANTONIO DEPT OF PHARM

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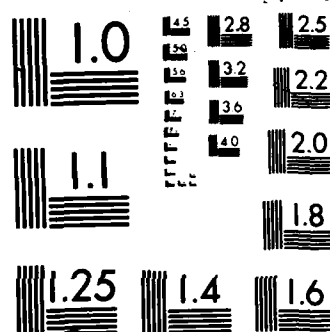
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**CHEMICAL
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**DIHYDROPYRIDINE RECEPTORS:
POSSIBLE ALLOSTERIC REGULATION
BY TREMORGENIC TOXINS**

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November 1986

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Dihydropyridine (DHP) receptors appear to be coupled to voltage-sensitive calcium channels that mediate Ca^{++} flux in neural tissue. A number of toxins known to interact with these channels induce tremors and seizures and modulate the ability of DHP compounds to alter the gating properties of Ca^{++} channels. It is therefore likely that tremorgenic myco-toxins, an economically important group of fungal toxins, may modulate Ca^{++} channels either directly or by their ability to act at DHP receptors. Tremorgenic doses of aflatrem, cyclopiazonic acid, and verruculogen increase the number and decrease the affinity of DHP receptors in rat cortex. These results suggest that the Ca^{++} channel and its associated receptors are important targets for several classes of fungal toxins,					
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PREFACE

The work described in this report was authorized under Project No. 1L162706A553C, WA04, Reconnaissance, Detection and Identification, Advanced CB Detection/Reconnaissance Systems. This work was started in February 1985 and completed in September 1985. The experimental data are contained in laboratory notebooks 85-0121 and 85-0146.

In the conducting the work described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Revision of the Guide for Laboratory Animals Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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This report has been cleared for release to the public.

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DIHYDROPYRIDINE RECEPTORS: POSSIBLE ALLOSTERIC REGULATION BY TREMORGENIC TOXINS

1. INTRODUCTION

Calcium ion fluxes through voltage-sensitive channels appear to be the coupling factor between excitation and contraction in smooth and cardiac muscle, and excitation and secretion in nerve terminals. These conclusions are based, in part, on observations that cardiac contractions and nervous conduction cease in a Ca^{++} free medium.¹ Sites which bind ^3H -nitrendipine appear to recognize antagonists pharmacologically relevant to the dihydropyridine (DHP) Ca^{++} channel.² The highest density of nitrendipine sites is in the brain,³ and these sites are especially concentrated in regions rich in synaptic connections,⁴ suggesting a role in the modulation of neurotransmission. Although Ca^{++} channels in muscle and nervous tissue may differ pharmacologically in their degree of coupling to DHP receptors and sensitivity to antagonists of the organic Ca^{++} channel;⁵ nevertheless, the binding to DHP receptor has utility for the identification of compounds with potential for either direct activity in the Ca^{++} channel or allosteric regulation of channel conformation and function, and subsequent incapacitating and lethal properties.

A number of toxins are known to interact with Ca^{++} channels.⁶ For example, the steroidal alkaloids batrachotoxin and veratridine appear to modulate the ability of DHP compounds to alter the gating properties of Ca^{++} channels, either by direct action at the DHP binding site or at an allosteric site that regulates DHP binding to the receptor.⁷

Recently, economically and, possibly, militarily important mycotoxins from the fungal genera Penicillium, Aspergillus, and Claviceps have been found to induce tremors and seizures in humans and cattle.^{8,9} These tremorgens belong to several chemically distinct groups, including the fumitremorgens, paspalitrems and the tetramic acids. The best known compounds within these groups are verruculogen (P. verruculosum), aflatrem (A. flavus), and cyclopiazonic acid (P. cyclopium). Though chemically distinct, all three share the ability to induce tremors and seizures that are similar in some respects to those induced by the naturally occurring marine toxin and Ca^{++} channel agonist maitotoxin,¹⁰ which has been shown to stimulate Ca^{++} flux in a manner that may be blocked by DHP antagonists.

Therefore, it is likely that tremorgenic mycotoxins modulate Ca^{++} channels either directly or by their effects at DHP receptors, and that DHP receptor binding would be sensitive to compounds with tremorgenic properties despite chemical dissimilarities. We tested this hypothesis by assessing the effects of representative toxins from three classes of tremorgens, given either in vivo or in vitro, on ^3H -nitrendipine binding to rat cortical synaptic membranes.

2. MATERIALS AND METHODS

2.1 Subjects.

Male albino (Fischer 344; N = 30) rats weighing 200-250 gm were individually housed in hanging wire cages and allowed ad libitum access to laboratory chow (Purina Rat Chow) and tap water. They were maintained under conditions of controlled temperature (24 ± 2 °C) and humidity (30-70 percent) on a 12-hr light/dark schedule and allowed to acclimate to their surroundings for 1 week prior to the experiments.

2.2 Materials.

Toxins were prepared by fermentation from Aspergillus flavus cultures and, following purification, were determined to be greater than 98 percent pure based on their UV extinction coefficients. Nifedipine was purchased from Sigma Chemical Co., and ^3H -nitrendipine was purchased from New England Nuclear Inc.

2.3 Dosing Regimen.

The rats designated for in vivo exposure to toxin were randomly divided into one control and three treatment groups. The three treatment groups received either a mildly tremorgenic dose of verruculogen, aflatrem, or cyclopiazonic acid (1 mg/kg, ip), and were paired with control rats receiving an equal volume of DMSO. On the days of the experiments, paired rats from the control and treatment groups were injected as described and decapitated 1 hr after injection. Rats designated for the in vitro exposure to toxin were decapitated on the days of the experiments and used as their own controls.

2.4 Receptor Binding Assay for the In Vivo Exposure.

Rats were treated and decapitated as described, and the cortex (COR) rapidly dissected on ice. The tissue was weighed and homogenized on ice in 10 ml of 50 mM of Tris-HCl buffer (pH 7.7) using a smooth glass homogenizer with a matched teflon pestle (Wheaton, setting 3, 10 strokes). The homogenate was centrifuged ($1000 \times g$, 10 min, 6 °C), the pellet discarded, and the supernatant recentrifuged ($20,000 \times g$, 10 min, 6 °C). The supernatant (S_2) was discarded and the pellet (P_2) was resuspended by hand homogenization in 3 ml of Tris-HCL (pH 7.7). Concentration of protein was determined by the method of Bradford.¹¹ The receptor-binding assay was carried out by combining in test tubes: 1600 μl of Tris-HCl, 100 μl of CaCl_2 (1 mM final concn), 100 μl of either tris-HCL or nifedipine (New England Nuclear, Inc., 77.4 Ci/mM, 75 pM 1 nM final concn), and 100 μl of tissue suspension. The contents were mixed by vortex and incubated in the dark for 90 min at 25 °C, then aspirated onto GF/B filters using a Brandel Harvester. The filters were washed three times with 5 ml of cold Tris-HCl and the disks removed to Hang-in vials (United Technologies Packard) to which 5 ml of Formula 947 (New England Nuclear Inc.) were added. Counting was performed in a Packard 300-C scintillation spectrometer (62 percent efficiency).

2.5 Receptor Binding Assay for the In Vitro Exposure.

Untreated rats were decapitated and the P₂ tissue fraction prepared as previously described. The P₂ fraction was suspended in 3 ml of Tris-EGTA (50 mM Tris, 10 mM EGTA, pH 7.7), incubated on ice for 30 min, and centrifuged (20,000 x g, 20 min, 6 °C). The pellet was resuspended in Tris-EDTA (50 mM Tris, 10 mM EDTA, pH 7.7), incubated on ice for 30 min, and centrifuged as before. The pellet was then resuspended in Tris-HCl and incubated and centrifuged as before. This final, washed pellet was resuspended in Tris-HCl, and the binding assays were performed by combining in test tubes: 1480 µl of Tris-HCl, 100 µl of CaCl₂ (1 mM final concn), 100 µl of MgCl₂ (1 mM final concn), 20 µl of DMSO or toxin (aflatrem, cyclopiazonic acid, or verruculogen; 1 or 10 µM final concn in DMSO), 100 µl of ³H-nitrendipine (New England Nuclear Inc., 77.4 Ci/mM, 75 pM-1 nM final concn), and 100 µl of tissue suspension. The contents were mixed by vortex, and incubation and counting were performed as previously described.

2.6 Data Analysis.

Specific binding was determined by subtracting non-nifedipine displaceable ³H-nitrendipine binding from total binding. The control data from the *in vivo* experiments were combined, and all data were expressed as moles of specific ³H-nitrendipine binding per milligram of protein. Scatchard¹² analysis was performed for visual representation of the binding data, but kinetic constants K_d and B_{max} were determined mathematically by computer.

3. RESULTS

3.1 In Vivo Exposure to Toxin.

Cyclopiazonic acid induced a 40 percent increase in the number of ³H-nitrendipine binding sites (B_{max} = 70 fMol/mg protein) relative to controls (B_{max} = 50 fMol/mg protein), and a 21 percent decrease in the affinity (K_d = 1.08 nM) compared to controls (K_d = 0.80 nM) (Figure 1). Aflatrem induced a large, 100 percent increase in number (B_{max} = 100 fMol/mg protein) and a 70 percent decrease in affinity (K_d = 1.52 nM) relative to these same controls (Figure 2), while verruculogen induced only a small 20 percent increase in number (B_{max} = 60 fMol/mg protein) and a large 88 percent decrease in affinity (K_d = 1.68 nM) (Figure 3).

3.2 In Vitro Exposure to Toxin.

The table shows the kinetic constants for nifedipine-displaceable ³H-nitrendipine binding obtained in the presence of cyclopiazonic acid, aflatrem, or verruculogen. Verruculogen (10⁻⁶M) induces an increase in the number of binding sites (B_{max} = 239 fMol/mg protein) relative to control (B_{max} = 156 fMol/mg protein) and an apparent decrease in affinity at 10⁻⁶M (K_d = 6.45 nM) and 10⁻⁵M (K_d = 6.28 nM) relative to controls (K_d = 3.66 nM). No consistent effects of cyclopiazonic acid or aflatrem were observed.

Table. Kinetic Constants for Nifedipine-Displaceable ^3H -Nitrendipine Binding in the Cortical Tissue of Rats Treated with Cyclopiazonic Acid (CPA), Verruculogen (VER) or Aflatrem (AFL)

		Control	10 ⁻⁶	10 ⁻⁵
CPA	Kd	5.82	5.75	5.17
	Bmax	252	258	237
Ver	Kd	3.66	6.45	6.28
	Bmax	156	239	199
AFL	Kd	6.35	5.22	4.80
	Bmax	300	355	247
Kd=nM				

Note: Each value represents the mean of three tests, each run in duplicate. Tests of each toxin were run separately with their own controls.

CYCLOPIAZONIC ACID
(in vivo 1mg/kg i.p.)

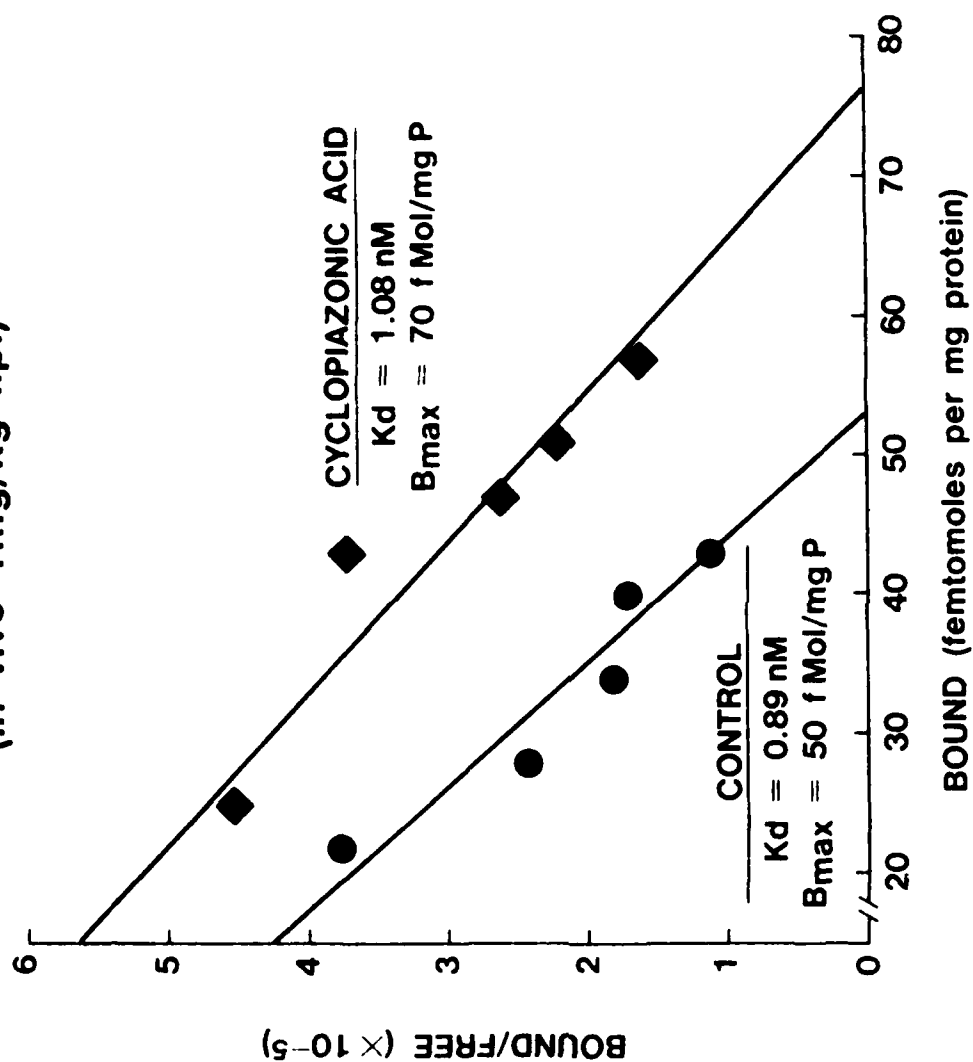


Figure 1. Scatchard Plot of Nifedipine-Displaceable ^3H -Nitrendipine Binding in Cortical Tissue From Rats Treated With Cyclopiazonic Acid or DMSO Vehicle

Each data point represents the mean of at least three separate experiments run in duplicate.

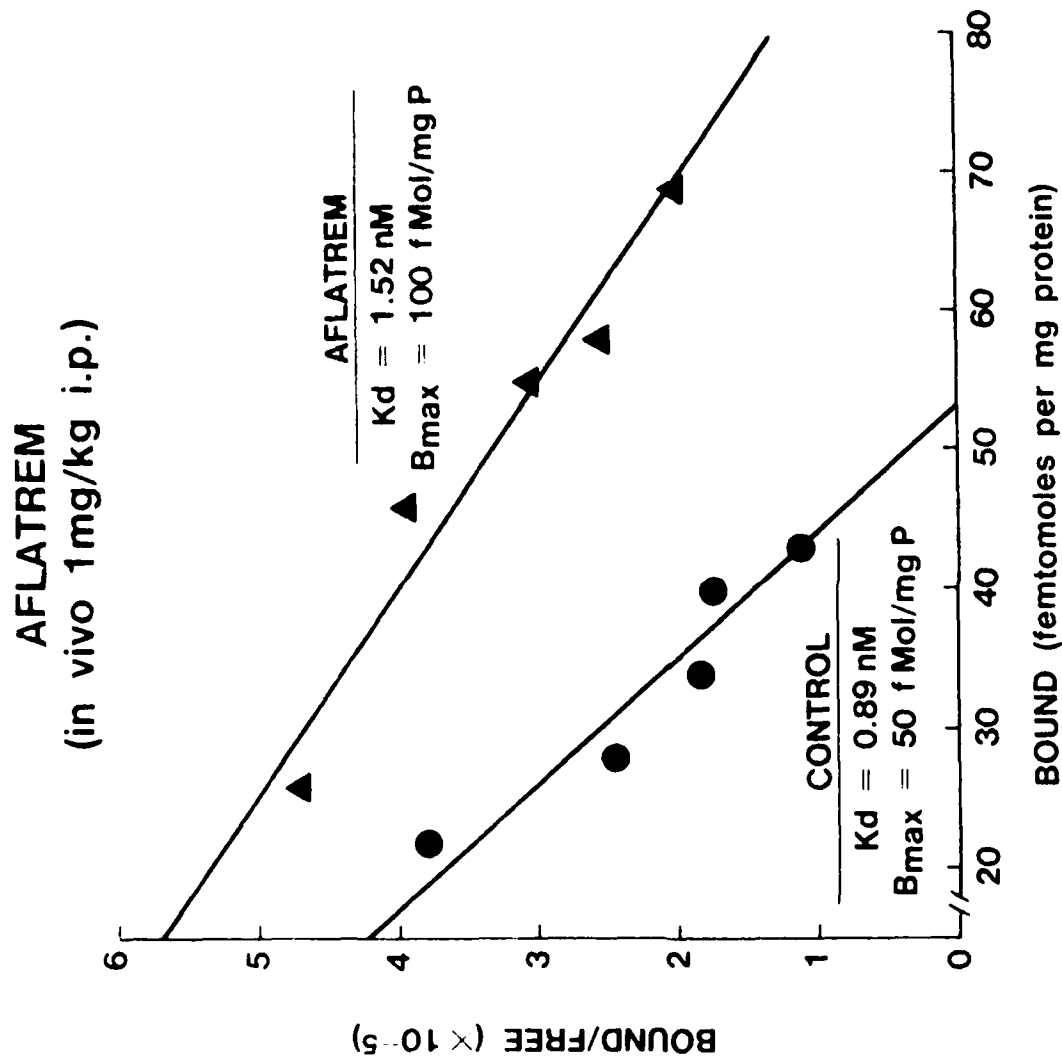


Figure 2. Scatchard Plot of Nifedipine-Displaceable ³H-Nitrendipine Binding in Cortical Tissue From Rats Treated With Aflatrem or DMSO Vehicle

Each data point represents the mean of at least three separate experiments run in duplicate.

VERRUCULOGEN (in vivo 1mg/kg i.p.)

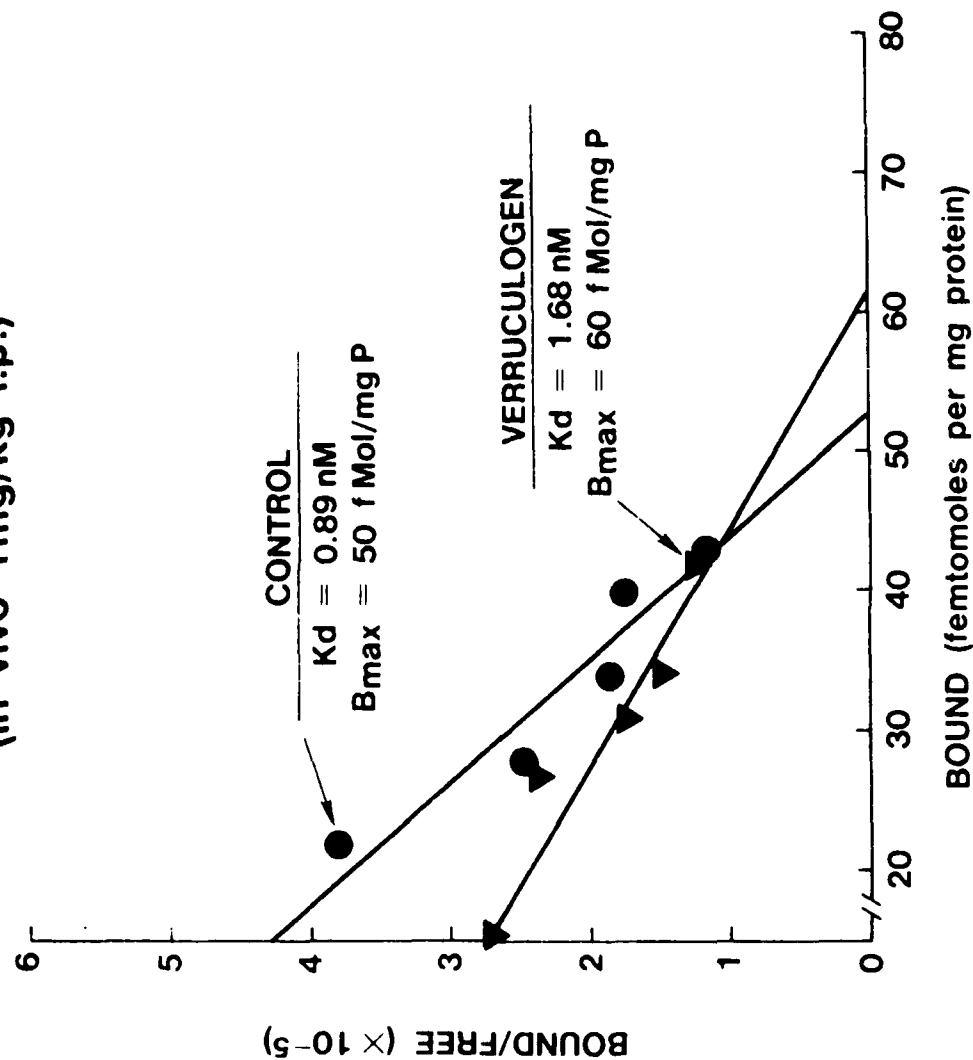


Figure 3. Scatchard Plot of Nifedipine-Displaceable ^3H -Nitrendipine Binding in Cortical Tissue From Rats Treated With Verruculogen or DMSO Vehicle

Each data point represents the mean of at least three separate experiments run in duplicate.

4. DISCUSSION

Current through voltage-sensitive Ca^{++} channels (VSCC) provides Ca^{++} to intracellular stores, a major purpose of which is the coupling of neuronal excitation to neurotransmitter secretion.¹ Receptors coupled to the VSCC are differentially sensitive to agonists and antagonists as a function of channel conformation, or mode. DHP compounds have the ability to modify the mode of the channel and, conversely, drugs that interact with the channel may have facilitatory or inhibitory properties depending on the conformation of the channel.

DHP antagonists appear to stabilize the channel in a closed configuration and agonists in an open one by acting as allosteric modulators of the channel. Thus, drugs that act as agonists tend to increase Ca^{++} flux while antagonists inhibit flux. For example, maitotoxin (MTX) stimulates $^{45}\text{Ca}^{++}$ influx into cultured NG108-15 neuroblastoma X glioma cells¹⁰ and rat pheochromocytoma cells,¹³ an action blocked by organic Ca^{++} channel blockers such as nitrendipine. This suggests a direct activation of the VSCC by MTX, and the observation that MTX fails to inhibit nitrendipine binding¹⁰ further supports the interpretation that it does not directly interact with DHP receptors.

Tremorgenic mycotoxins administered in vivo increase the number, i.e., B_{max} , and decrease the apparent affinity, i.e., K_d , of DHP antagonist receptors labeled by ^3H -nitrendipine. The reciprocal alterations in number and affinity may reflect a compensatory response to the actions of the tremorgens, though it is unclear which is the primary action of intoxication and which the reaction to it. In any event, the data are subject to two mutually exclusive interpretations: Tremorgens may stabilize the channel in the open configuration, exposing a greater number of DHP receptors, or they may fix the channel in the closed state, a mode more sensitive to channel blockers such as nitrendipine. The effects of batrachotoxin (BTX) on the voltage-sensitive Na^+ channel are instructive since this channel shares many similarities to the VSCC. Besides altering the voltage dependency of the Na^+ channel, BTX inhibits channel inactivation and alters ion permeability,¹⁴ indicative of a change in channel conformation. By analogy, the ability of tremorgens to alter the interaction of nitrendipine with the DHP receptor may, as in the case of BTX and the Na^+ channel, indicate altered conformation of the VSCC, in this instance, to the closed configuration.

The relative inability of tremorgenic mycotoxins to modulate nitrendipine binding when given in vitro supports the position that they do not directly interact with the DHP receptor but achieve their effects allosterically. The reconstituted system used in the in vitro studies may preclude the necessary coupling of the DHP receptor to the allosteric site.

5. CONCLUSIONS

Tremorgenic mycotoxins appear to allosterically regulate the binding of DHP antagonists to their receptors. One viable interpretation is that these toxins stabilize the VSCC in its closed configuration, but a definitive explanation of their mechanism awaits the study of ion transport to assess their effects on voltage-dependent Ca^{++} uptake into synaptosomes. These results suggest that VSCC and its associated receptors are important targets for several classes of economically and militarily significant fungal toxins.

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