Tremorgenic Indole Alkaloids Potently Inhibit Smooth Muscle High-Conductance Calcium-Activated Potassium Channels

Hans-Günther Knaus,[‡,§] Owen B. McManus,[‡] Seok H. Lee,[∥] William A. Schmalhofer,[‡] Margarita Garcia-Calvo,[‡,⊥] Lisa M. H. Helms,[‡] Manuel Sanchez,[‡,#] Kathleen Giangiacomo,[‡] John P. Reuben,[‡] Amos B. Smith, III,[○] Gregory J. Kaczorowski,[‡] and Maria L. Garcia*[,‡]

Department of Membrane Biochemistry and Biophysics and Department of Natural Products Chemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, and Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323

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ABSTRACT: Tremorgenic indole alkaloids produce neurological disorders (e.g., staggers syndromes) in ruminants. The mode of action of these fungal mycotoxins is not understood but may be related to their known effects on neurotransmitter release. To determine whether these effects could be due to inhibition of K^+ channels, the interaction of various indole diterpenes with high-conductance Ca^{2+}-activated K^+ (maxi-K) channels was examined. Paspalitrem A, paspalitrem C, aflatrem, penitrem A, and paspalinine inhibit binding of [^{125}I]charybdotoxin (ChTX) to maxi-K channels in bovine aortic smooth muscle sarcolemmal membranes. In contrast, three structurally related compounds, paxilline, verruculogen, and paspalicine, enhanced toxin binding. As predicted from the binding studies, covalent incorporation of [^{125}I]ChTX into the 31-kDa subunit of the maxi-K channel was blocked by compounds that inhibit [^{125}I]ChTX binding and enhanced by compounds that stimulate [^{125}I]ChTX binding. Modulation of [^{125}I]ChTX binding was due to allosteric mechanisms. Despite their different effects on binding of [^{125}I]ChTX to maxi-K channels, all compounds potently inhibited maxi-K channels in electrophysiological experiments. Other types of voltage-dependent or Ca^{2+}-activated K^+ channels examined were not affected. Chemical modifications of paxilline indicate a defined structure–activity relationship for channel inhibition. Paspalicine, a deshydroxy analog of paspalinine lacking tremorgenic activity, also potently blocked maxi-K channels. Taken together, these data suggest that indole diterpenes are the most potent nonpeptidyl inhibitors of maxi-K channels identified to date. Some of their pharmacological properties could be explained by inhibition of maxi-K channels, although tremorgenicity may be unrelated to channel block.

Tremorgenic mycotoxins are metabolites produced by fungi belonging to the genera *Penicillium*, *Aspergillus*, and *Claviceps* which elicit intermittent or sustained tremors in vertebrate animals (Cole & Cox, 1981). These agents can be divided into four groups based on chemical similarity: paspalitrem, penitrem, fumitremorgin-verruculogen, and tryptoquivaline structural classes (Selala et al., 1989). All tremorgens in the above classes have in common an indole moiety derived from tryptophan. Also included in these groups are several metabolites that are chemically related but not tremorgenic, such as paspaline, paspalicine, and cyclopiazonic acid. The clinical symptoms typically observed during mycotoxin-induced intoxication include diminished activity and immobility, followed by hyperexcitability, convulsions, muscle tremor, ataxia, and tetanic seizures (Cole, 1980). However, death seldom occurs, and these symptoms are reversible if the affected animal is removed from the toxic-feed source. Tremorgenic mycotoxins are lipophilic molecules that may cross the blood–brain barrier and gain access to the central nervous system.

The mechanism by which the fungal tremorgens act is not well-defined, but biochemical and clinical studies indicate that these compounds affect neurotransmitter release. Using synaptosomal preparations derived from sheep and rat, it was shown that penitrem A increases the spontaneous release of glutamate, γ-aminobutyric acid (GABA),[1] and aspartate from cerebrocortical synaptosomes but not from spinal cord or medullary synaptosomes (Norris et al., 1980). Verruculogen also increases spontaneous glutamate and aspartate release. Similar effects have been noted *in vivo* with verruculogen (Petersen et al., 1982). In guinea pig ileum preparations, paxilline, penitrem B, and verruculogen cause an increase in contractile responses due to electrical field stimulation, and this effect was attributed to enhanced release of acetylcholine from presynaptic nerve terminals (Selala et al., 1991). Verruculogen was shown to increase presynaptic neurotransmitter release at the locust neuromuscular junction (Norris et al., 1980), while penitrem A produces similar effects in rat neuromuscular junction preparations (Wilson et al., 1972). It has been postulated that because a rigid GABA-like conformation exists within the structure of the fungal tremorgens, these agents could act as surrogates of GABA (Selala et al., 1989). In support of this proposal, aflatrem potentiates GABA-induced chloride currents through $GABA_A$ receptor

* Author to whom correspondence should be addressed.
‡ Department of Membrane Biochemistry and Biophysics.
§ Recipient of Erwin Schrödinger Fellowships P-0574-MED and P-0795-MED of the Austrian Research Foundation. Permanent address: Institute for Biochemical Pharmacology, University of Innsbruck, Innsbruck, Austria.
∥ Department of Natural Products Chemistry.
⊥ Recipient of a Fullbright Fellowship.
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[1] Abbreviations: maxi-K channel, high-conductance Ca^{2+}-activated K^+ channel; ChTX, charybdotoxin; [^{125}I]ChTX, monoiodotyrosine charybdotoxin; GABA, γ-aminobutyric acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TAPS, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid.

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chloride channels heterologously expressed in *Xenopus* oocytes (Yao et al., 1989). Thus, it appears that the indole alkaloid tremorgens have multiple effects on receptors and on neurotransmitter release mechanisms at both central and peripheral levels.

We considered whether the effects of these tremorgenic mycotoxins on neurotransmitter release might be due, in part, to effects on potassium channels. High-conductance Ca^{2+}-activated K^+ (maxi-K) channels have been shown in some neuronal preparations to regulate neurotransmitter release by controlling the duration of action potentials (Robitaille & Charlton, 1992; Robitaille et al., 1993). Discovery of peptide toxins, such as charybdotoxin, iberiotoxin, and limbatustoxin (Miller et al., 1985; Garcia et al., 1991), has provided tools to examine the biochemical properties of these channels and to screen for other compounds that bind to these channels. [^{125}I]ChTX binds to a single class of sites in bovine aortic and tracheal smooth muscles that is associated with maxi-K channels (Slaughter et al., 1989a; Vazquez et al., 1989; Garcia-Calvo et al., 1991). Using this binding assay as a monitor of receptor activity, the maxi-K channel has been purified to homogeneity from tracheal smooth muscle (Garcia-Calvo et al., 1994), and the subunit composition of this channel was elucidated (Knaus et al., 1994). The [^{125}I]ChTX binding assay has also previously been used to identify novel modulators of maxi-K channels derived from natural product sources (McManus et al., 1993). We have now used this binding assay and electrophysiological experiments to determine whether tremorgenic mycotoxins interact with maxi-K channels.

In the present study, we report that tremorgenic mycotoxins interact in a defined fashion with maxi-K channels from smooth muscle. These mycotoxins modulate binding of [^{125}I]ChTX to maxi-K channels by either positive or negative allosteric mechanisms. Despite differences in the allosteric interactions with the ChTX binding site, these agents block maxi-K channels with high potency. Tremorgenic mycotoxins are the most potent nonpeptidyl inhibitors of maxi-K channels identified to date, and they appear to define a new site of drug interaction on the maxi-K channel. It is possible that some of the pharmacological properties observed with these compounds could be due to their ability to inhibit maxi-K channels.

MATERIALS AND METHODS

Materials. ChTX was obtained from Peninsula Laboratories and [^{125}I]ChTX (2200 Ci/mmol) from New England Nuclear Corp. Paxilline, penitrem A, verruculogen, and cyclopiazonic acid were purchased from Sigma Chemical Co. Paspalinine and paspalicine were obtained through total synthesis as previously outlined (Smith et al., 1992). GF/C glass fiber filters were bought from Whatman. Precast 12% acrylamide gels were from Novex. All other reagents were obtained from commercial sources and were of the highest purity commercially available.

Isolation of Paspalitrems A and C and Aflatrem. Paspalitrems A and C were isolated from a *Phomopsis* species of enophytic fungi as previously described (Bills et al., 1992). Briefly, extraction of solids from 30 flasks of millet fermentation three times with methyl ethyl ketone yielded 4.4 g of brown oily residue after solvent removal. A portion of CH_2Cl_2 was added to the residue, and then the mixture was triturated, sonicated, and filtered through sintered glass, and the filter cake was washed twice with additional solvent. Filtrates were pooled, and the solvent was removed *in vacuo* to give 3.01 g of residue. CH_2Cl_2-soluble material was then subjected to silica flash column chromatography (silica gel 60, particle size 0.040–0.063 mm, 230–400 mesh ASTM; EM Science) using a glass column (37 mm × 450 mm). Compounds were eluted stepwise with CH_2Cl_2–CH_3OH, the effluent was monitored at 240 nm, and 20-mL fractions were collected. Fractions were analyzed by reversed-phase HPLC on Partisil 10 ODS-3 (Whatman; 4.6 mm × 25 cm; 80% aqueous CH_3OH, isocratic; flow rate, 1 mL/min; ambient temperature) to monitor the presence of paspalitrem C and paspalitrem A. The retention times for these two compounds were 20.1 and 21.8 min, respectively. Fractions rich in the major components were recovered (1.2 g) and subsequently subjected to preparative reversed-phase HPLC (Partisil 10 ODS-3, 9.4 mm × 50 cm; Whatman) using 70% aqueous CH_3OH (isocratic; flow rate, 10 mL/min; ambient temperature) to provide the major component, paspalitrem C (72 mg), and a minor component, paspalitrem A (5 mg). Paspalitrem C: $C_{32}H_{39}O_4N$; MW 501.2879 (calcd), 501.2894 (found); UV (CH_3OH) λ_{max} (nm) 236 and 282, λ_{min} (nm) 218 and 267. Paspalitrem A: $C_{32}H_{39}O_4N$; MW 501.2879 (calcd), 501.2891 (found); UV (CH_3OH) λ_{max} (nm) 238, λ_{min} (nm) 216.

Aflatrem was isolated in the following fashion. A fermentation broth (*Aspergillus flavus*; ATCC 16875; 4 L) was extracted with the same volume of methyl ethyl ketone, and then the solvent was removed to give 120 g of tarlike crude solid. Subsequent treatment with CH_2Cl_2 provided 61 g of reddish brown oil after removal of the solvent, which contained a large quantity of polyunsaturated fatlike material that solidified upon refrigeration. Successive flash column chromatographies on SiO_2 and Bakerbond C_{18} were carried out. Final purification was accomplished by reversed-phase HPLC on Partisil 10 ODS-3 (22 mm × 50 cm; Whatman) using 60% CH_3CN–H_2O (flow rate 10 mL/min). Aflatrem (96 mg) was eluted at 105 min. Aflatrem: $C_{32}H_{39}O_4N$; MW 501.2879 (calcd), 501.2861 (found); UV (CH_3CN) λ_{max} (nm) 236 and 285, λ_{min} (nm) 216 and 268.

Synthesis of Paxilline Analogs. (1) Paxillinols. To a solution of paxilline (20 mg) in ethanol was added $NaBH_4$ in ethanol in slight excess. The reaction was allowed to proceed for 1 h, and then chips of ice were added to quench the reaction. The mixture was transferred to a separatory funnel, crystalline NaCl was added to saturate the aqueous layer, and the products were extracted three times with fresh portions of diethyl ether. The ether layer was washed with saturated aqueous NaCl and dried over anhydrous $MgSO_4$. Removal of ether gave paxillinols in quantitative yield [paxillin-16β-ol (15.8 mg) and paxillin-16α-ol (5.3 mg) in a ratio of 3:1]. R_f values were 0.54 for paxillinols and 0.62 for paxilline on silica gel 60 F-254 when developed with 10% CH_3OH–CH_2Cl_2. The final purification of paxillinols was accomplished by reversed-phase HPLC on Partisil 10 ODS-3 (9.4 mm × 50 cm; Whatman) using 60% CH_3OH–H_2O (flow rate 5 mL/min), for which the retention times of the β and α isomers were 48 and 87 min, respectively. Paxillin-16β-ol: UV (CH_3OH) λ_{max} (nm) 233 and 286, λ_{min} (nm) 213 and 257. Paxillin-16α-ol: UV (CH_3OH) λ_{max} (nm) 233 and 286, λ_{min} (nm) 213 and 257.

Paxillinamine in which the carbonyl oxygen was replaced by an amino group was prepared in two steps: First, the paxilline ketoxime [$C_{27}H_{34}O_4N_2$, MW 450.2518 (calcd), 450.2517 (found)] was prepared by treating paxilline with NH_2OH·HCl in C_2H_5OH. This was then purified by preparative TLC on silica gel 60 F-254 (20 cm × 20 cm, 2-mm thickness) using 5% CH_3OH–CH_2Cl_2 to give 18.6 mg (60% yield). The paxilline ketoxime was reduced by $NaBH_4$ with $TiCl_4$ in 1,2-dimethoxyethane to give paxillinamine

[$C_{27}H_{36}N_2O_3$; MW 436.2726 (calcd), 436.2707 (found); m/z 436 ($M^{\bullet+}$), 421 (M – CH_3), 182].

(2) 1,3-Dihydroxy-2-methylpaxilline. Treatment of paxilline using trichloramine [prepared from $Ca(OCl)_2$, NH_4Cl, and HCl] and $AlCl_3$ in CH_2Cl_2 resulted in the *N*-hydroxypaxilline derivative in 39% yield after purification [$C_{28}H_{37}NO_6$; MW 483.2621 (calcd), 483.2609 (found)].

(3) Paxizoline. Paxilline ketoxime was treated with diphenyl disulfide and tributylphosphine in dry tetrahydrofuran at room temperature overnight under nitrogen, and then the solvent was removed. The crude product was purified by HPLC on Partisil 10 ODS-3 using 70% CH_3OH–H_2O to give paxizoline [$C_{27}H_{32}N_2O_3$; MW 432.2413 (calcd), 432.2403 (found); MS (m/z) 432 (M^+), 417 (M – CH_3, base peak), 399 (M – CH_3 – H_2O), 182, 130; UV (CH_3OH) λ_{max} (nm) 234 and 263, λ_{min} 252] in 75% yield.

Spectroscopic Measurements. All NMR spectra were recorded on a Varian XL-300 NMR spectrometer at ambient temperature with samples in either CD_3OD or CD_2Cl_2. The solvent peaks δ 3.30 and 5.32 were used as internal references for CD_3OD and CD_2Cl_2, respectively, for ^1H NMR; chemical shifts are given in parts per million (ppm). ^{13}C NMR spectra were recorded using the solvent peaks δ 49.0 and 53.8 for CD_3OD and CD_2Cl_2, respectively. Mass spectra were obtained on a Finnigan-MAT 212 mass spectrometer at 90 eV or a Finnigan-MAT TSQ 70 at 70 eV. Fast atom bombardment (FAB) was run on a VG 20–253 or a Finnigan-MAT 90 spectrometer using dithiothreitol–dithioerythritol (MB) or MB containing cesium iodide. The compounds were derivatized using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine (1:1) at 50 °C for 30 min. UV spectra were obtained on a Beckman DU-70 spectrophotometer.

Preparation of Bovine Aortic Smooth Muscle Sarcolemmal Membrane Vesicles. Highly purified sarcolemmal membrane vesicles were prepared from bovine aortic smooth muscle as previously described (Slaughter et al., 1989b). The membrane fraction at the 8–30% interface of the final sucrose density gradient was resuspended in 160 mM NaCl, 20 mM Tris-HCl, pH 7.4, rapidly frozen in liquid N_2, and stored at –70 °C. Binding activities were stable for at least 1 year.

Binding Assays. The interaction of [^{125}I]ChTX with either membrane vesicles or solubilized material was monitored as previously outlined (Garcia-Calvo et al., 1991; Vazquez et al., 1989). Briefly, ChTX receptor preparations were incubated with [^{125}I]ChTX in a medium consisting of 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% digitonin, and 0.1% bovine serum albumin, in the absence or presence of other added agents, at room temperature until equilibrium was achieved. Separation of free from bound ligand was accomplished by filtration through GF/C glass fiber filters that had been presoaked in 0.5% poly(ethylenimine). Nonspecific binding was determined in the presence of 10 nM ChTX. Triplicate assays were routinely performed under each experimental condition, and the data were averaged. The standard error of the mean of these results was typically less than 3%. Stock solutions of the drugs were prepared in dimethyl sulfoxide. The concentration of this agent was never allowed to exceed 0.25%, a concentration that by itself had no effect on [^{125}I]ChTX binding. The binding of [^{125}I]ChTX to voltage-dependent K^+ channels in brain was determined as before (Vazquez et al., 1990).

Cross-Linking Experiments. Covalent incorporation of [^{125}I]ChTX into aortic sarcolemmal membrane vesicles was carried out as previously described (Garcia-Calvo et al., 1991). Briefly, membranes were incubated with 110 pM [^{125}I]ChTX in a medium consisting of 20 mM NaCl, 20 mM Hepes–NaOH, pH 7.4, 0.1% digitonin, and 0.04% bovine serum albumin, in the absence or presence of other agents, for 1 h at room temperature. Membrane vesicles were collected by centrifugation, pellets were resuspended in 200 mM NaCl and 10 mM TAPS–NaOH, pH 9.0, and disuccinimidyl suberate was added at a final concentration of 0.18 mM. Samples were incubated at room temperature for 1 min, and the reaction was stopped by addition of Tris-HCl, pH 7.4, at a final concentration of 500 mM. Membranes were collected by centrifugation, washed, and resuspended in SDS–PAGE sample buffer containing 100 mM β-mercaptoethanol. Samples were subjected to SDS–PAGE using 12% acrylamide gels. Gels were dried and exposed to Kodak XAR-5 film for 2 days at –70 °C.

Analysis of Data. Data from saturation experiments were subjected to a Scatchard analysis, and linear regression was performed to determine the equilibrium dissociation constant (K_d) and maximum receptor concentration (B_{max}). Correlation coefficients for these determinations were typically greater than 0.95. The dissociation rate constant for ChTX (k_{-1}) was determined directly from a first-order plot of ligand dissociation vs time.

Electrophysiological Recordings. Patch clamp recordings of maxi-K channels in membrane patches excised from cultured bovine aortic smooth muscle cells (Williams et al., 1988) were made using conventional techniques (Hamill et al., 1981). Pipets were typically filled with solutions containing 150 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, and 0.01 mM $CaCl_2$ and adjusted to pH 7.20 with 3.7 mM KOH. The inside face of the membrane patch was bathed in a solution similar to the pipet solution except where differences in calcium, magnesium, and EGTA are noted. Maxi-K channels were identified by their large single-channel conductance (~250–300 pS) and sensitivity of channel open probability to membrane potential and intracellular Ca^{2+} concentration. Experiments were done at room temperature (23–25 °C).

Planar lipid bilayers were formed from a solution of 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) in a 7/3 molar ratio dissolved in decane (50 mg/mL). This lipid solution was painted across a small hole (250 μm) separating two aqueous compartments, and bilayers with capacitances of 200–250 pF readily formed. Currents flowing across the bilayer were measured with standard voltage clamp techniques. Aortic sarcolemmal membranes were added to the cis side of the bilayer under a concentration gradient of 150 mM KCl cis and 15–25 mM KCl trans until channel incorporation occurred. The orientation of maxi-K channels after insertion into the bilayer was determined from the Ca^{2+} and voltage sensitivity of the channel. After channel incorporation, the solutions on both sides of the bilayer were equalized to contain 150 mM KCl, 10 mM Hepes, 3.7 mM KOH, pH 7.20, and 1–100 μM Ca^{2+}.

Data were stored either on a Racal store 4DS FM tape recorder (Racal Recorders, Vienna, VA) or on digital video tape using a video cassette recorder after the signal was digitized with a VR-10 PCM digital encoder (Instrutech Corp., Belmont, NY). For quantitative analysis, the data were played into a DEC 11–73 (Digital Equipment Corp., Maynard, MA) after digitization with a DT2782-8D1A analog to digital converter (Data Translation Inc., Marlboro, MA) or played into a Mac IIx or Quadra 700 computer (Apple Computers) after digitization with an ITC-16 interface (Instrutech).

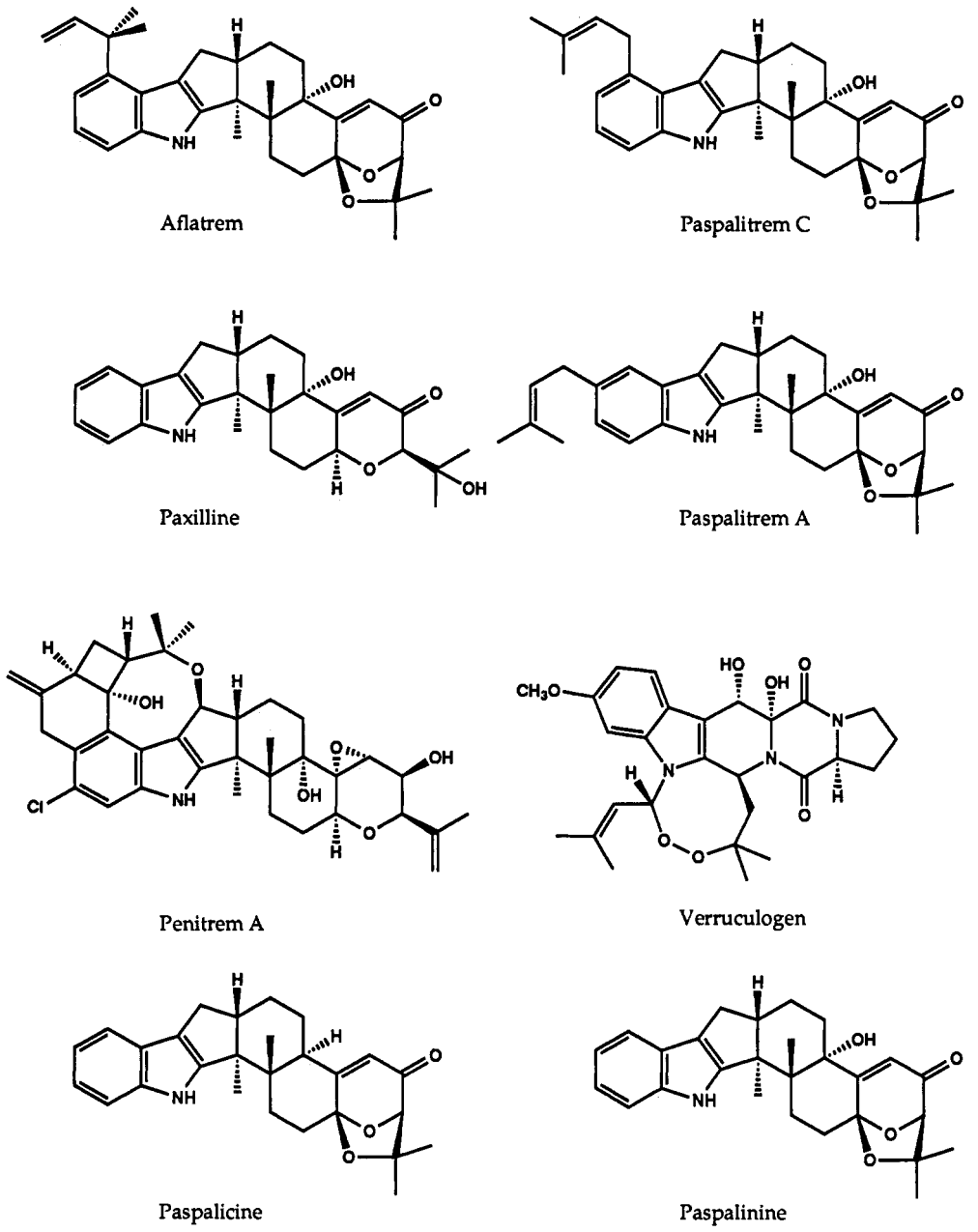


FIGURE 1: Structures of tremorgenic mycotoxins.

Protein Determination. Protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as standard.

RESULTS

Indole Diterpene Alkaloids Modulate [^{125}I]ChTX Binding to Maxi-K Channels. The chemical structures of the naturally occurring indole diterpene alkaloids investigated in the present study are illustrated in Figure 1. The effects of these tremorgenic mycotoxins on maxi-K channels were first evaluated by studying their interaction with the ChTX receptor in bovine aortic smooth muscle sarcolemmal membrane vesicles. It has previously been demonstrated that [^{125}I]ChTX binds to a single class of receptor sites in smooth muscle sarcolemma that are part of the maxi-K channel complex (Slaughter et al., 1989a; Vazquez et al., 1989; Garcia-Calvo et al., 1991, 1994; Knaus et al., 1994). When sarcolemmal membranes were incubated with 30 pM [^{125}I]ChTX, a concentration of toxin that gives 25% occupancy of receptor sites, in the presence of increasing concentrations of different tremorgenic compounds, a complex pattern of interactions was observed. Agents such as paspalitrems A and C, penitrem A, aflatrem, and paspalinine caused concentration-dependent inhibition of [^{125}I]ChTX binding (Figure 2). The potencies with which the various compounds inhibited toxin binding were different, as were the degrees of inhibition produced (Table 1). The most potent of these compounds is aflatrem, which displayed a $K_{1/2}$ of ca. 170 nM and which inhibited the binding reaction almost completely. Paspalitrem C and penitrem A were weaker inhibitors of [^{125}I]ChTX binding as judged by the maximal level of inhibition observed and by the concentrations required to cause half-maximal inhibition. In contrast to the inhibitory effects of these mycotoxins, paxilline and verruculogen markedly enhanced [^{125}I]ChTX binding in a concentration-dependent fashion. The $K_{1/2}$ values (170 nM) for stimulation of [^{125}I]ChTX binding by paxilline and verruculogen were similar to the $K_{1/2}$ value observed for inhibition of [^{125}I]ChTX binding by aflatrem. Paspalicine caused a smaller increase in [^{125}I]ChTX binding than did paxilline or verruculogen, although this effect occurred at

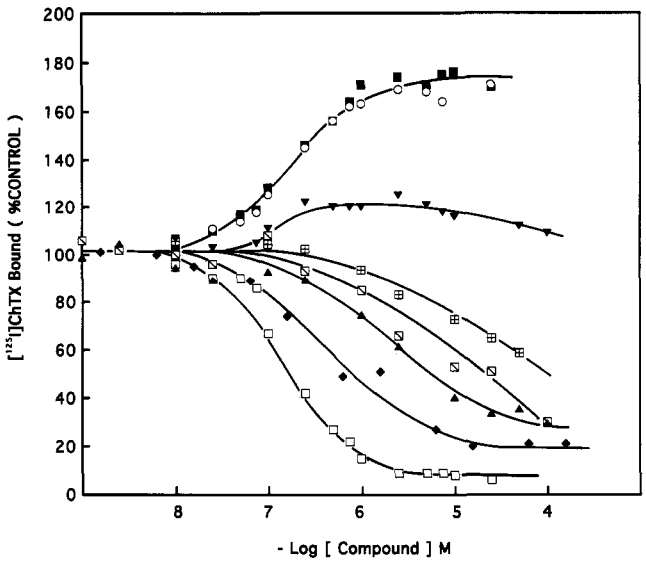


FIGURE 2: Interaction of indole diterpene alkaloids with the ChTX receptor in bovine aortic smooth muscle sarcolemmal membranes. Membrane vesicles were incubated with 30 pM [^{125}I]ChTX in a medium consisting of 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, and 0.1% digitonin, in the absence or presence of increasing concentrations of paxilline (■), verruculogen (○), paspalitrem C (◆), paspalitrem A (⧅), aflatrem (□), penitrem A (▲), paspalinine (⊞), or paspalicine (▼), until equilibrium was achieved. Nonspecific binding was determined in the presence of 10 nM ChTX. Specific binding data are presented relative to an untreated control. Under control conditions, about 25% of the receptor sites are occupied by [^{125}I]ChTX.

Table 1: Tremorgenic Mycotoxins Block Maxi-K Channels and Modulate ChTX Binding to Maxi-K Channels[a]

	fraction current blocked ($x \pm$ SE)	max effect on ChTX binding (%)	$K_{1/2}$ ChTX binding (nM)
paxilline	70 ± 15	+170	170
verruculogen	95 ± 5	+170	170
paspalicine	83 ± 4	+120	100
paspalinine	100		
paspalitrem A	98 ± 2		
penitrem A	100	−66	1700
paspalitrem C	99.9 ± 0.1	−78	410
aflatrem	100	−91	170

[a] Fraction current blocked measures the fractional block of maxi-K channels in electrophysiological experiments by 10 nM of the indicated compound. Maxi-K channel gating was measured in inside-out membranes excised from bovine aortic smooth muscle with each patch containing one to five maxi-K channels. Experimental conditions are as described in Figures 6 and 7. Channel open probability was measured during a 2–3-min interval starting 3 min after exposure to the compound and compared to channel open probability measured during control. Values are mean ± standard error of mean for 3–11 experiments for each value with 33 total experiments. The maximum effect on ChTX binding was determined from the data in Figure 2, and the $K_{1/2}$ was determined from interpolation of the data in Figure 2.

slightly lower concentrations. Paspalinine and paspalitrem A weakly inhibited [^{125}I]ChTX binding. This observed profile is an indication that modulation of ChTX binding most likely occurs through an allosteric mechanism in which both positive and negative interactions are possible. A structurally related compound, cyclopiazonic acid, had no observed effect on toxin binding (data not shown).

The pattern of effects on [^{125}I]ChTX binding produced by the tremorgenic mycotoxins has also been observed with solubilized and partially purified ChTX receptor preparations, which suggests that this phenomenon is not due to physical perturbation of the membrane environment in which the receptor is located. As a measure of the specificity of this

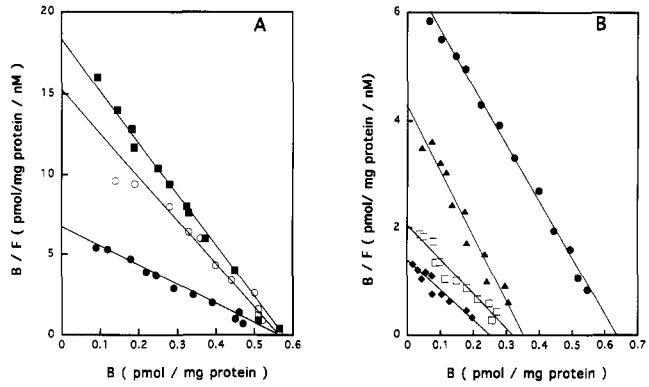


FIGURE 3: Scatchard analyses of the effects of indole diterpene alkaloids on [^{125}I]ChTX binding to bovine aortic sarcolemmal membranes. Membrane vesicles were incubated with increasing concentrations of [^{125}I]ChTX in the absence (●) or presence of (A) 3 μM verruculogen (○) or 2.5 μM paxilline (■) or (B) 2.5 μM paspalitrem C (◆), 300 nM aflatrem (□), or 5 μM penitrem A (▲). The incubation medium consisted of 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, and 0.1% digitonin, and incubations were carried out at room temperature until equilibrium was achieved. Nonspecific binding was determined in the presence of 10 nM ChTX. Specific binding data in each case are presented in the form of a Scatchard representation.

interaction, the action of these agents on [^{125}I]ChTX binding to rat brain synaptic plasma membranes was also investigated. Under these experimental conditions, ChTX binding to rat brain membranes reflects the interaction of toxin with voltage-dependent potassium channels that are distinct from the maxi-K channel (Vazquez et al., 1990; Giangiacomo et al., 1993). None of the compounds described in the present investigation had any observed effect on [^{125}I]ChTX binding in brain when tested at concentrations up to 100 μM, suggesting specificity in the interaction of these agents with different types of K^+ channels.

Indole Diterpene Alkaloids Allosterically Modulate [^{125}I]-ChTX Binding. To investigate the mechanisms by which tremorgenic mycotoxins modulate binding of [^{125}I]ChTX to smooth muscle maxi-K channels, saturation binding experiments were carried out with increasing concentrations of [^{125}I]-ChTX in the absence or presence of fixed amounts of selected agents. Under control conditions, [^{125}I]ChTX bound to a single class of sites that displayed a K_d of 86 pM and a B_{max} of 0.56 pmol/mg of protein. In the presence of either 2.5 μM paxilline or 3 μM verruculogen, there was an apparent increase in toxin affinity (K_d values of 31 and 37 pM, respectively) with no significant change in the maximum density of receptor sites present (Figure 3A). Therefore, those compounds that enhance [^{125}I]ChTX binding appear to act allosterically to increase toxin affinity. In contrast, inhibitors of the [^{125}I]-ChTX binding reaction, such as paspalitrem C, aflatrem, and penitrem A, caused a marked reduction in the maximum density of receptor sites, with very little change in toxin affinity (Figure 3B). It is unlikely that the reduction of receptor site density reflects an irreversible action of these compounds. For example, the inhibition caused by aflatrem can be reversed either by washing the membranes or by adding increasing concentrations of paxilline. These data suggest that the indole diterpene alkaloids are likely to bind to a unique site(s) on the maxi-K channel from where they can affect binding of ChTX to its receptor near the external mouth of the channel through either positive or negative heterotropic interactions. From these data, it is impossible to determine if all of the mycotoxins share a common binding site on the maxi-K channel or whether several binding sites are overlapping. However, it is important

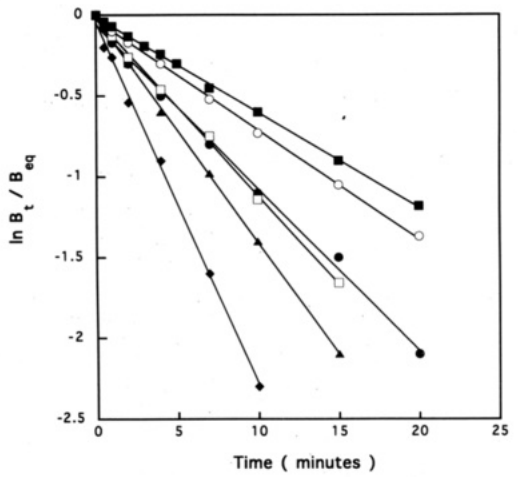


FIGURE 4: Effect of indole diterpene alkaloids on the kinetics of dissociation of [^{125}I]ChTX from bovine aortic sarcolemmal membranes. Membrane vesicles were incubated with 30 pM [^{125}I]ChTX at room temperature until equilibrium was achieved. Dissociation rates were initiated by addition of 10 nM ChTX alone (●) or by repeating the experiment in the presence of 10 μM paspalitrem C (◆), 10 μM penitrem A (▲), 10 μM aflatrem (□), 10 μM paxilline (■), or 3 μM verruculogen (○) and incubating the membranes at room temperature for the indicated time periods.

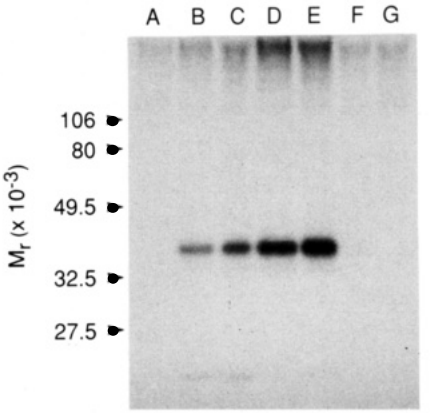


FIGURE 5: Effect of paxilline and aflatrem on covalent incorporation of [^{125}I]ChTX into the β subunit of the maxi-K channel. Sarcolemmal membrane vesicles were incubated with 110 pM [^{125}I]ChTX in a medium consisting of 20 mM NaCl, 20 mM Hepes–NaOH, pH 7.4, 0.04% bovine serum albumin, and 0.1% digitonin, in the absence (lane B) or presence of either 10 nM ChTX (lane A), 100 nM, 1.0 μM, or 10 μM paxilline (lanes C–E), or 1.0 or 5.0 μM aflatrem (lanes F and G) at room temperature until equilibrium was achieved. Samples were subjected to a cross-linking reaction in the presence of disuccinimidyl suberate and to SDS–PAGE as described under Materials and Methods. The migrations of molecular weight standards are shown.

to note that stimulation of [^{125}I]ChTX binding caused by either paxilline or verruculogen was prevented by aflatrem and that inhibition of binding by aflatrem is reversed by paxilline, suggesting that these agents might compete for the same receptor site (data not shown).

As a further test of the hypothesis that the tremorgenic mycotoxins are allosteric modulators of [^{125}I]ChTX binding, the rates of [^{125}I]ChTX dissociation from its receptor were determined in the absence or presence of selected compounds. Under control conditions, toxin dissociation occurred as a monoexponential function with a $t_{1/2}$ of ca. 6.5 min (Figure 4). In the presence of either 10 μM paxilline or 3 μM verruculogen, the rates of toxin dissociation were decreased ($t_{1/2}$ of 11.5 and 10 min, respectively; Figure 4). This pattern is consistent with the finding that these particular compounds increase the affinity of the ChTX receptor for [^{125}I]ChTX, and it suggests that such action occurs mainly through an effect on toxin off-rates. Paspalitrem C, an inhibitor of the [^{125}I]ChTX binding reaction, caused a marked enhancement of toxin dissociation kinetics, whereas two other inhibitors of binding, penitrem A and aflatrem, produced either small or no significant effects on the rate of toxin dissociation, respectively (Figure 4).

An alternative means of investigating the interaction of the indole diterpene alkaloids with maxi-K channels is to monitor the pattern of covalent incorporation of [^{125}I]ChTX into the 31-kDa β subunit of these channels in the presence of the bifunctional cross-linking reagent disuccinimidyl suberate. It has previously been shown that toxin is specifically incorporated into this glycoprotein and that incorporation of radioactivity is prevented by other agents which inhibit the [^{125}I]ChTX binding reaction (Garcia-Calvo et al., 1991, 1994). When the [^{125}I]ChTX cross-linking reaction was performed with bovine aortic sarcolemmal membranes under control conditions, followed by SDS–PAGE analysis, the 31-kDa subunit of the channel was labeled and this process was prevented by 10 nM ChTX (Figure 5). Consistent with the hypothesis that tremorgenic mycotoxins interact in a specific fashion with the smooth muscle ChTX receptor, paxilline caused a concentration-dependent increase in the level of [^{125}I]-ChTX incorporated into this protein, whereas aflatrem blocked the cross-linking reaction (Figure 5). Thus, the pharmacological effects of the tremorgenic mycotoxins on the [^{125}I]-ChTX binding reaction are mirrored in the pattern of labeling of the β subunit of the maxi-K channel.

Positive and Negative Allosteric Modulators of ChTX Binding Block Maxi-K Channels. The tremorgenic indole alkaloids shown in Figure 1 had complex effects on binding of [^{125}I]ChTX to aortic membranes in that some compounds stimulated [^{125}I]ChTX binding, while others inhibited binding of toxin to the channel. We examined the effects of these compounds on maxi-K channels from bovine aortic smooth muscle in electrophysiological experiments. Figure 6 compares the effects of an inhibitor of [^{125}I]ChTX binding, paspalitrem C, with a stimulator of [^{125}I]ChTX binding, paxilline. Application of 10 nM paspalitrem C to the inside face of an excised membrane patch caused nearly complete block of the maxi-K channels in that patch within 2 min (Figure 6A). Channel block was not reversed after a 20-min wash with drug-free solution. Figure 6B shows a parallel experiment in which application of 10 nM paxilline caused a rapid block of maxi-K channels in the patch by 44% that was mostly reversed after 10 min of washout. Although these compounds have opposite effects on [^{125}I]ChTX binding, both are potent inhibitors of maxi-K channels in electrophysiological experiments.

The potencies of the indole diterpenes shown in Figure 1 for blocking maxi-K channels are compared in Table 1. Paxilline was the weakest blocker under these conditions, with 10 nM blocking maxi-K channels, on average, 70% of the time. The compounds that enhanced [^{125}I]ChTX binding (paxilline, verruculogen, and paspalicine) were less potent at blocking maxi-K channels in electrophysiological experiments than were the compounds that inhibited [^{125}I]ChTX binding. The $K_{1/2}$ value for the effect of each indole diterpene on [^{125}I]-ChTX binding was not predictive of the potency of that compound in electrophysiological experiments. For instance, paxilline and verruculogen had nearly identical effects on [^{125}I]-ChTX binding, while verruculogen was a more potent channel blocker. Also, penitrem A was comparatively weak in binding experiments but was potent in blocking maxi-K channels in electrophysiological experiments.

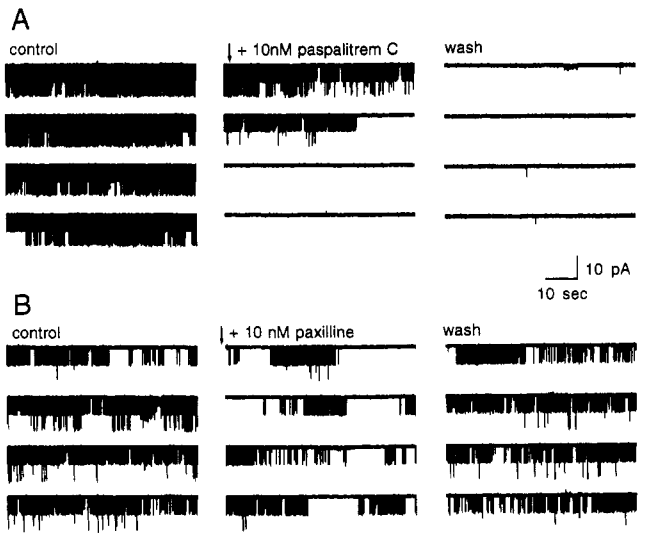


FIGURE 6: Positive and negative allosteric modulators of ChTX binding block maxi-K channels. Effects of paspalitrem C (A) and paxilline (B) on maxi-K channels in excised inside-out membrane patches from bovine aortic smooth muscle are shown for two experiments done under identical conditions. Channel opening causes downward deflections in the current trace. The arrows at the top of the middle panels indicate when perfusion with paspalitrem C (A) or paxilline (B) started. The wash shown in (A) was recorded immediately after beginning perfusion with control solution, and the wash shown in (B) was recorded after 10 min of perfusion with control solution. The membrane potential was held at −20 mV, and the bath solution contained 1 mM Mg^{2+} and 10 μM Ca^{2+}.

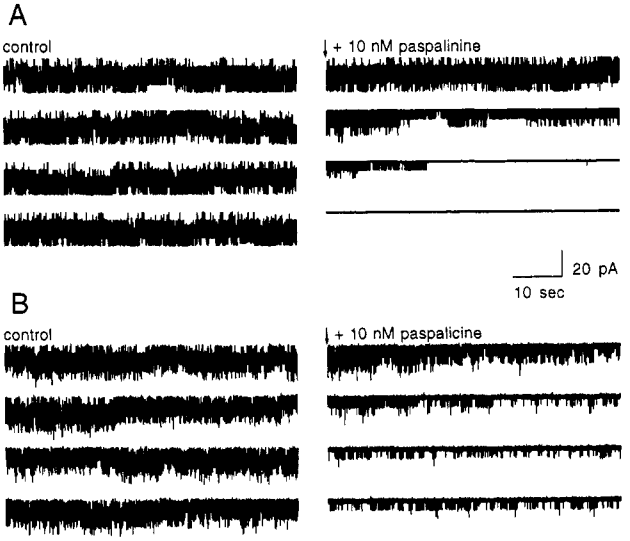


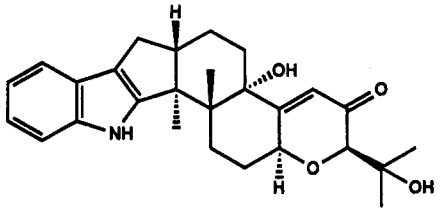
FIGURE 7: Both tremorgenic and nontremorgenic indole alkaloids block maxi-K channels. Effects of paspalinine (A) and paspalicine (B) on maxi-K channels in excised inside-out membrane patches from bovine aortic smooth muscle are shown for two experiments done under identical conditions. Channel opening causes downward deflections in the current trace. The membrane potential was held at −20 mV, and the bath solution contained 1 mM Mg^{2+} and 10 μM Ca^{2+}.

Most of the indole alkaloids listed in Table 1 caused nearly complete block of maxi-K channels at 10 nM, and this block was not significantly reversed after a brief (10–15 min) washout. Significant recovery of channel open probability to within half of the control value after a 10–15-min washout was only observed with paxilline (Figure 6) and paspalicine. The faster rates of washout of channel block by paxilline and paspalicine compared to those of the other indole alkaloids shown in Figure 1 might be expected on the basis of the smaller fractions of current blocked by paxilline and paspalicine.

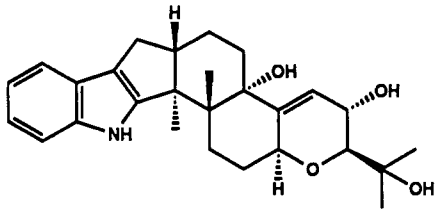
The ability of the indole alkaloids to affect maxi-K channels was also monitored after single channels from bovine aortic sarcolemmal membranes were reconstituted into planar lipid bilayers. These compounds blocked channel activity when applied from either side of the bilayer, although application at the internal channel surface usually produced more potent channel block than did external application. In addition, the activities of the different agents appear distinct. Exposure of a single maxi-K channel to paxilline caused appearance of long silent periods devoid of channel activity, which is reminiscent of the pattern of channel block caused by ChTX. On the other hand, paspalitrem C exhibited a more complex pattern of channel block. Low concentrations (3–10 nM) of this compound applied to the inside face of the channel caused the appearance of numerous brief (<100 mS) interruptions in channel current. The ability of these indole alkaloids to block maxi-K channels also appears related to the state of the channel. When internal Ca^{2+} was elevated and channel open probability was high, the indole diterpenes were weaker channel blockers.

Both Tremorgenic and Nontremorgenic Indole Alkaloids Block Maxi-K Channels. Most of the indole alkaloids described in this paper are tremorgenic. The ability of these molecules to induce tremors is critically dependent on a hydroxyl group at position 19 of the diterpene nucleus (Cole, 1980; Selala et al., 1989). This distinction is illustrated by comparing paspalicine and paspalinine (Figure 1). The only structural difference between these two compounds is the presence of the C-19 hydroxyl group, which causes paspalinine to be tremorgenic, while its deshydroxy analog, paspalicine, is nontremorgenic. Application of either paspalinine or paspalicine at 10 nM caused clear reductions in maxi-K channel activity (Figure 7), demonstrating that both compounds effectively block maxi-K channels. Examination of Figure 7, where paspalicine blocked maxi-K channel activity 95% and paspalinine blocked 100%, and Table 1 reveals that paspalinine is a more potent blocker of maxi-K channels in these experiments. Could this difference in channel blocking potency serve as an explanation for the lack of tremorgenicity of paspalicine? This seems unlikely because paxilline is tremorgenic and is less potent than paspalicine as a maxi-K channel blocker (Table 1). Thus, block of maxi-K channels may not be related to the tremorgenic effects of these compounds. However, we cannot rule out the possibility that block of maxi-K channels in neural tissue, which could display a pharmacological profile different from that observed with the smooth muscle channel, may contribute to the tremorgenic effects of these indole alkaloids.

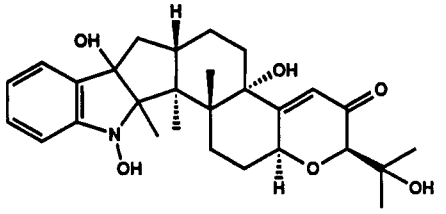
Specificity of Indole Alkaloid Blockers for Maxi-K Channels. We examined the ability of the indole alkaloids shown in Figure 1 to block other ion channels. Human T-lymphocytes contain small-conductance calcium-activated potassium channels (Leonard et al., 1992; Grissmer et al., 1993). These channels resemble maxi-K channels in that both are activated by calcium and blocked by charybdotoxin. They differ in that the lymphocyte channel has a smaller conductance than the maxi-K channel and because gating of the lymphocyte channel is independent of membrane potential, while maxi-K channel gating depends on membrane potential. Paspalitrem C (100 nM applied to the internal side) did not inhibit the lymphocyte small-conductance calcium-activated potassium channel. Human T-lymphocytes also have a ChTX-sensitive, voltage-dependent potassium channel that is not activated by calcium (Lewis & Cahalan, 1988). Extracellular application of up to 1 μM of either paxilline or paspalitrem C had no



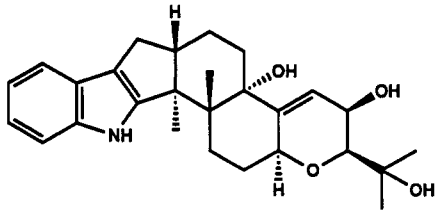
Paxilline



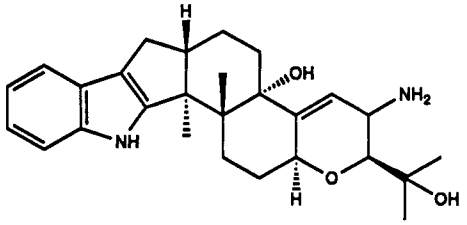
Paxillin-16α-ol



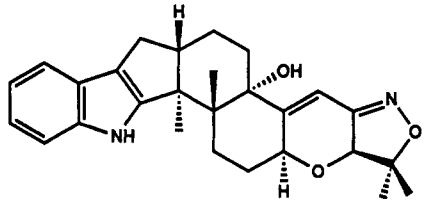
1,3-Dihydroxy-2-methyl paxilline



Paxillin-16β-ol



Paxillinamine



Paxizoline

FIGURE 8: Structures of paxilline analogs.

effect on the whole-cell current due to this channel. We also assayed the effects of these indole alkaloids on ChTX-sensitive, voltage-dependent potassium channels in rat brain. In binding experiments, these compounds had no effect on the binding of [^{125}I]ChTX to voltage-dependent potassium channels in brain synaptic plasma membrane vesicles (see above). The delayed rectifier potassium channel in mouse pancreatic β cells was weakly blocked by paxilline, with half of the current inhibited at 2 μM (C. Cullinan and M. Leibowitz, personal communication). Paxilline (10 μM) also had no effect on calcium flux through L-type calcium channels (Felix et al., 1992) in GH_3 cells (J. Felix and R. Slaughter, personal communication) or on voltage-dependent Na^+ channel activity in GH_3 cells.

Structure–Activity Relationship Studies with Paxilline Derivatives. To explore the structural determinants that govern the interaction of indole diterpene alkaloids with the maxi-K channel, chemical modifications of paxilline were performed (Figure 8). Reduction of the carbonyl group at C-16 yields two stereoisomers, paxillin-16β-ol and paxillin-16α-ol. Neither of these compounds had any effect on [^{125}I]-ChTX binding to maxi-K channels in bovine aortic smooth muscle membranes up to concentrations of 100 μM, and their activity as inhibitors of maxi-K channels in electrophysiological experiments was reduced when compared with that of paxilline (half-block of maxi-K channels in excised inside-out patches occurred at 0.1–1 μM). Substitution at the double bond of the indole B ring to yield the *N*-hydroxypaxilline derivative, 1,3-dihydroxy-2-methylpaxilline, produces a compound that was also devoid of activity (100 μM) in binding experiments. In attempts to introduce an amino group at position 16, the intermediate paxilline ketoxime was synthesized. This compound, as well as paxillinamine, was inactive in binding experiments. Cyclization of paxilline ketoxime yields paxizoline, which modulated toxin binding in a biphasic fashion. At low concentrations, paxizoline caused a small, but reproducible, stimulation of [^{125}I]ChTX binding, while partial inhibition of toxin binding was observed at higher concentrations (data not shown). Paxizoline was also an effective inhibitor of maxi-K channels, and its potency was similar to that of paxilline itself. These data suggest that the interaction of paxilline with maxi-K channels occurs through a defined structure–activity relationship and that most of the paxilline analogs prepared in this study display a significant loss of activity when compared to the parent compound.

DISCUSSION

The results presented in this paper demonstrate that a series of indole diterpene alkaloids previously characterized as tremorgenic mycotoxins are potent inhibitors of maxi-K channels. These compounds modulated binding of [^{125}I]ChTX to receptor sites associated with maxi-K channels in bovine aortic smooth muscle sarcolemmal membranes through allosteric mechanisms manifesting both positive and negative interactions. Covalent incorporation of [^{125}I]ChTX into the 31-kDa β subunit of the maxi-K channel in the presence of a bifunctional cross-linking reagent was affected in the same fashion by the alkaloids as was toxin binding, further supporting the notion that the interaction of these agents with the maxi-K channel is a specific process. These compounds inhibited maxi-K channel activity either in excised patches from cultured bovine aortic smooth muscle cells or after reconstitution of the aortic smooth muscle channel into planar lipid bilayers. Effects on some other types of ion channels were not observed at the low concentrations used to block maxi-K channels. Despite the structural diversity of the alkaloids found to block channel activity in this study, the interaction of these molecules with the maxi-K channel appears

specific since several simple derivatives of paxilline were inactive as channel inhibitors.

The binding site for the indole diterpene alkaloids on the maxi-K channel is likely to differ from the binding site for ChTX. ChTX blocks maxi-K channels only when applied to the external side of the channel and the peptide binds near the entrance to the pore and thereby blocks the ion conduction pathway (Anderson et al., 1988; MacKinnon & Miller, 1988). In contrast, the indole alkaloids block maxi-K channels in electrophysiological experiments when applied to either the inner or outer surface of the channel, although the compounds appear more potent when added at the cytoplasmic face. This difference suggests that the binding site(s) for the indole diterpene alkaloids may be located in a position different from the receptor for ChTX.

Results of binding experiments performed with [^{125}I]ChTX further support the idea of distinct locations for receptor sites for the indole diterpene alkaloids and ChTX. An interesting property of these alkaloids is their ability to affect the binding of [^{125}I]ChTX to maxi-K channels through either positive or negative allosterism. While most of these compounds inhibited [^{125}I]ChTX binding with a defined rank order of potency, three of them, paxilline, verruculogen, and paspalicine, stimulated toxin binding in a concentration-dependent fashion. Stimulation was due to an increase in [^{125}I]ChTX affinity, and this effect appears to result, at least in part, from a decrease in the rate of toxin dissociation. These data imply that distinct allosterically linked binding sites exist on the channel for ChTX and for the indole diterpene alkaloids. This hypothesis is further supported by the pattern of inhibition of [^{125}I]ChTX binding that was observed. Most indole diterpenes only partially inhibited [^{125}I]ChTX binding, and in saturation experiments, they displayed noncompetitive behavior. Whether all of these alkaloids share a common or overlapping site on the maxi-K channel cannot be determined with certainty from the present studies. However, stimulation of [^{125}I]ChTX binding by paxilline or verruculogen was antagonized by aflatrem, whereas paxilline reversed the inhibition caused by aflatrem, suggesting that the various molecules may share the same receptor.

Recently, another site of interaction was described on the smooth muscle maxi-K channel that is also allosterically coupled to the ChTX receptor (McManus et al., 1993). At this site, which is located on the intracellular face of the channel, a series of potent glycotriterpene maxi-K channel agonists bind and destabilize ChTX bound to a different site on the external side of the channel. These agents, like the indole diterpene alkaloids, alter the kinetics of ChTX dissociation from its receptor. When radiolabeled derivatives of the glycotriterpene analogs become available, it will be interesting to determine whether the sites for these agonists are allosterically coupled to the receptors for the indole diterpenes. If so, the maxi-K channel will display the characteristics of a multidrug receptor, which is typical of voltage-gated Na^+ and Ca^{2+} channels but which has not yet been well documented for K^+ channels.

Despite the differences manifested in the binding of [^{125}I]-ChTX, all of the indole diterpene alkaloids shown in Figure 1 are potent inhibitors of the maxi-K channel in electrophysiological experiments. They blocked channel activity in excised membrane patches, or when applied from either side of the membrane in lipid bilayer experiments. However, the abilities of the various compounds to modulate [^{125}I]ChTX binding and inhibit channel activity do not correlate well (cf. Table 1). There are several likely explanations to account for this discrepancy. First, the binding and electrophysiological determinations are done under different experimental conditions. In addition, unlike their effects on channel activity, the effects of the alkaloids on [^{125}I]ChTX binding are not direct but are allosteric in nature. Furthermore, the hydrophobic properties of these molecules create a problem when we try to define their apparent affinity constants. They bind to most surfaces and may concentrate within the membrane, making it difficult to determine absolute concentrations at their binding sites. The extent to which this occurs may also be different under different experimental protocols. Nevertheless, it is not surprising that such a discrepancy exists since similar differences have been noted in the binding and functional characteristics of other ion channel modulators (e.g., the interaction of dihydropyridines with L-type Ca^{2+} channels).

Blocking of maxi-K channels by these indole diterpene alkaloids appears to be a specific and often reversible phenomenon. At concentrations at which complete inhibition of maxi-K channel activity was observed, these agents had little effect on other types of ion channels, including small-conductance Ca^{2+}-activated K^+ channels, voltage-dependent potassium channels found in human T-lymphocytes, and delayed rectifier K^+ channels from pancreatic β-cells. In addition, these compounds did not affect either Ca^{2+} or Na^+ channels in GH_3 cells. These data suggest that this group of tremorgenic mycotoxins are the most potent and selective nonpeptidyl inhibitors of the maxi-K channel discovered to date. They define a new high-affinity receptor site on the maxi-K channel.

Some of the pharmacological effects ascribed to the indole diterpenes may result from block of maxi-K channels. Inhibition of maxi-K channels could cause broadening of action potentials at nerve terminals by preventing repolarization, thereby leading to an increase in release of neurotransmitters. This mechanism could explain the observed increases in neurotransmitter release from neuromuscular preparations caused by some of the indole alkaloids characterized in the present study. Depolarization resulting from block of maxi-K channels by indole alkaloids might also contribute to the observed increases in neurotransmitter release from cerebrocortical synaptosomes caused by these compounds. However, the tremorgenic properties of this structural class do not appear to be due to inhibition of maxi-K channels since paspalicine, which is not tremorgenic, also blocks maxi-K channels. Paspalicine lacks the essential hydroxyl group at C-19 that is a common component of the indole diterpene alkaloids that produce tremors. Therefore, the *in vivo* pharmacological profile associated with the indole diterpene alkaloids cannot strictly be related to inhibition of maxi-K channel activity. However, some of the *in vitro* effects of these compounds in promoting neurotransmitter release from synaptosomes or from nerve terminals at the neuromuscular junction may be due to blocking of maxi-K channels.

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