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IN VITRO EFFECTS OF TREMORGENIC MYCOTOXINS

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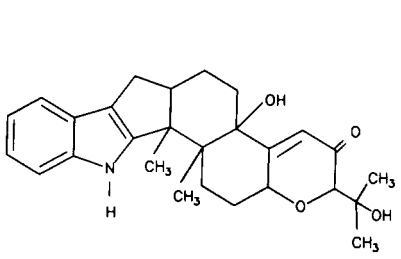
ABSTRACT.—Paxilline [**1**] was isolated from *Penicillium paxilli* (NRRL 6110). It was studied together with penitrem B [**2**] and verruculogen [**3**] in the electrically stimulated guinea pig ileum. All three mycotoxins enhanced the electrically induced twitch contractions, without influencing the contractions provoked by exogenous acetylcholine. The effect of the mycotoxins could be greatly diminished by hyoscine. The possible mechanism of action of these substances in this in vitro model is discussed. The electrically stimulated guinea pig ileum could be useful in the detection and estimation of the biological activity of tremorgenic mycotoxins.

"Mycotoxins" is a convenient generic term applied to a large versatile group of substances that are formed during growth of fungi by consecutive series of enzyme-catalyzed reactions from a few biochemically simple intermediates of primary metabolism, e.g., acetate, mevalonate, malonate, and certain amino acids (1,2). These secondary metabolites are capable of eliciting toxic reactions (mycotoxicoses) in humans, animals, plants, and other cellular living forms including cultured cells either through ingestion or direct contact. Fungi are ubiquitous in nature, being capable of growing on almost any plant product. As such, these metabolites can associate intimately with every food or animal feed regardless of climatic or geographic region. They can be produced in the field, at every stage of harvesting, processing, storage, or transportation of a given commodity.

Fungal tremorgens or tremorgenic mycotoxins (3–5) are a unique class of intracellular indolic metabolites with significant central nervous system activity. They are produced by a variety of fungi including the ergot species *Claviceps paspali* as well as certain strains of the saprophytic genera *Aspergillus* and *Penicillium*. Fungi that belong to the latter two genera are organisms of contamination, being often involved in the spoilage of foodstuffs, even in home refrigerators. Tremorgenic strains of these genera have been isolated from a wide variety of commodities, including cereal products such as oats, barley, wheat, millet, corn, rice, and pasture forage.

Tremorgenic mycotoxins act on vertebrate nervous systems, inducing sustained tremors, hyperexcitability, convulsions, and ataxia. They have become generally associated with a variety of mycotoxicoses, commonly referred to as staggers syndromes (ryegrass, marsh, and paspalum staggers, migram disease, and Bermuda grass tremors) in ruminants. These diseases are known in many parts of the world for inflicting huge economic losses in agriculture. The nature of the tremor and accompanying symptoms produced by these toxins in laboratory animals is clinically indistinguishable from staggers syndromes. When administered orally or parenterally in small doses to test animals, fungal tremorgens characteristically cause sustained body tremors followed by spasticity of the limbs (6–9). Depending on the dose administered, animals may convulse and eventually die. Acute mycotoxicoses in humans caused by these toxins have not so far been observed, but they have been implicated in the etiology of extrinsic allergic alveolitis or wood trimmer's disease (10).

Although research on the fungal tremorgens has progressed rapidly in recent years, their mechanism of action is not known and continues to be a subject of research in many laboratories. We have investigated the effects of paxilline [**1**], penitrem B [**2**], and verruculogen [**3**], all members of the fungal tremorgens, on the isolated ileum of the guinea pig and report on the possible mode of action of these metabolites in this tissue.



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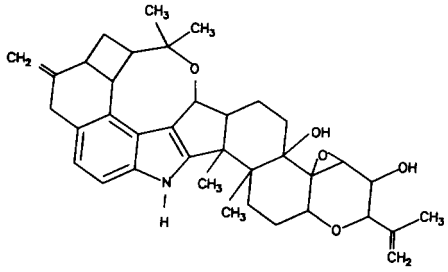

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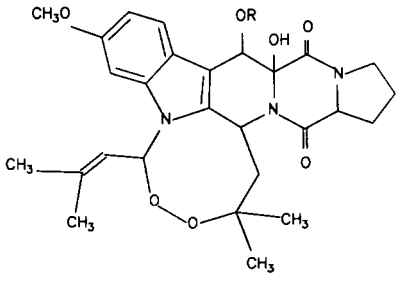

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RESULTS

All tested mycotoxins gave a concentration-dependent transient contraction of the guinea pig ileum. This contraction was followed by a destabilization of the baseline and an enhancement of the electrical twitch contractions (Figure 1).

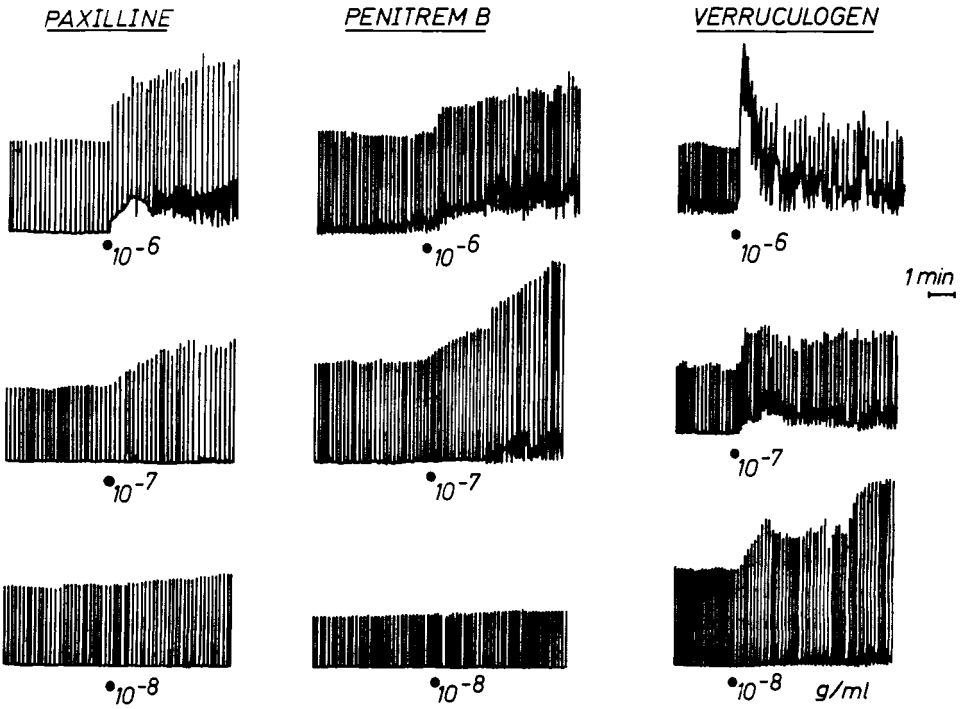

FIGURE 1. Original tracings of electrically stimulated guinea pig ileum behavior after adding verruculogen [3], penitrem B [2], or paxilline [1]. Stimulation characteristics: 40 mA, 1 msec, 0.1 Hz. Final bath concentrations are expressed as g/ml.

Paxilline and verruculogen exhibited dose-dependent contractions. Weaker contractions were seen for penitrem B (Figure 2).

All three mycotoxins significantly enhanced the electrically induced twitch contractions. Only verruculogen gave a significant enhancement in the lowest concentration applied (10^{-8} g/ml) (Figure 3).

The mycotoxins did not influence the contractions elicited by exogenous acetylcholine significantly (Table 1).

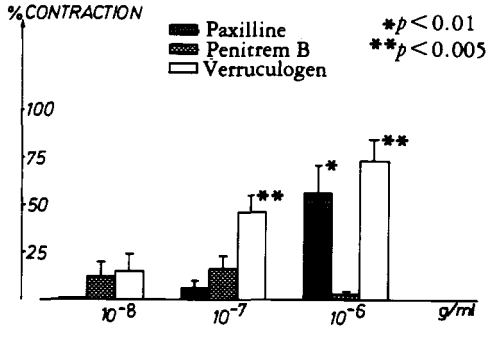

FIGURE 2. Contractile effect of mycotoxins on non-stimulated guinea pig ileum strips. Each column represents the mean ± SEM expressed as a percentage of the acetylcholine control contraction. $N = 7$ for each column. Significance calculated as compared to DMSO controls.

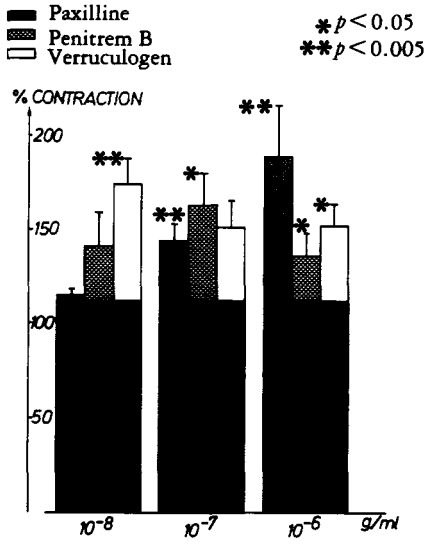

FIGURE 3. Mycotoxin enhancement of electrically induced twitch contractions. Each column represents the mean ± SEM expressed as % of the column. Black part of each column = twitch contraction after DMSO. Significance calculated as compared to DMSO controls.

TABLE 1. Influence of Mycotoxins on Contractions Elicited by Exogenous Acetylcholine (3.10^{-8} g/ml).[a]

Concentration of mycotoxin (g/ml)	Compound			
	3	2	1	DMSO
10^{-8}	127 ± 26	111 ± 7	121 ± 10	120 ± 13
10^{-7}	123 ± 23	96 ± 17	118 ± 7	
10^{-6}	109 ± 9	117 ± 8	126 ± 23	

[a]N = 7. Results are expressed in % ± SEM of control acetylcholine contractions.

After two washings, the isolated organs were still under the influence of the mycotoxins, as could be seen from the irregular baseline and enhanced twitch contractions. Hyoscine (10^{-8} g/ml) inhibited by about 90% the electrically induced twitch contractions (Table 2).

TABLE 2. Influence of Hyoscine (10^{-8} g/ml) on Post-Mycotoxin Electrically Provoked Twitch Contractions.[a]

Concentration of mycotoxin (g/ml)	Compound			
	3	2	1	DMSO
10^{-8}	7 ± 5	12 ± 6	9 ± 5	16 ± 6
10^{-7}	11 ± 9	10 ± 5	11 ± 3	
10^{-6}	14 ± 7	10 ± 4	2 ± 2	

[a]N = 7. Results are expressed in % ± SEM of control twitch contractions. Stimulation characteristics: 40 mA; 0.1 Hz; 1 msec.

DISCUSSION

Tremorgenic mycotoxins reportedly interfere with the excitatory amino acid neurotransmission in the central nervous system (6,11,12). There also exists evidence for a partial inhibition of γ-aminobutyric acid (GABA) neurotransmission by these toxins in CNS systems (11, 13–15).

Paxilline [**1**], penitrem B [**2**], and verruculogen [**3**] significantly enhanced the twitch contractions of the electrically stimulated guinea pig ileum used here as an in vitro model.

The twitch contractions seen after every pulse are due to acetylcholine released in the synaptic cleft (16). Because hyoscine inhibited by more than 90% the mycotoxin-enhanced twitch contractions, the results point mainly to a cholinergic effect.

The enhanced contractions could be due to either a postsynaptic sensitization of the muscarinic receptors or to availability of higher amounts of acetylcholine in the synaptic cleft.

The mycotoxins studied did not potentiate exogenous acetylcholine contractions. By consequence, a postsynaptic sensitizing effect is thus less probable.

Higher amounts of acetylcholine can be made available in the synaptic cleft by inhibition of the cholinesterase or enhancement of transmitter release. Sobotka *et al.* (17) could not find experimental evidence for a cholinesterase inhibition. Furthermore, the continuous baseline enhancement characteristic for cholinesterase inhibitors was not observed in our experiments, so the increased amounts of transmitter could be due to an enhanced release mechanism. Efforts to quantify the amounts of acetylcholine released by coaxial stimulation are currently being investigated (18).

Our results point to the electrical stimulation of the guinea pig ileum as a useful peripheral in vitro preparation to test physiological effects of fungal tremorgens or similar metabolites. Verruculogen [3] was the most potent compound in our test system. This correlates well with in vivo results, the tremorgenic ED_{50} of this toxin (0.39 mg/kg, ip, mice) being lower than that of penitrem B [2] (5 mg/kg, ip, mice) and paxilline [1] (25 mg/kg, ip, mice) (17,19,20). Mycotoxins play an important role in veterinary and food science, and the electrically stimulated guinea pig ileum could partially replace the more expensive in vivo models often used in investigating these compounds. Furthermore, the electrically stimulated guinea pig ileum is currently being used as bioassay to guide extractions and to examine structure-activity relationships within this group of mycotoxins (21).

EXPERIMENTAL

MYCOTOXINS.—Penitrem B was obtained from the Division of Food Science and Technology (CSIR), Pretoria, South Africa. Verruculogen was a gift from Dr. R.J. Cole, National Peanut Research Laboratory, Department of Agriculture, 600 Forrester Drive, Dawson, Georgia 31742. Paxilline was isolated according to the protocol described below.

Fungal cultivation and extraction of paxilline.—Paxilline was isolated from *P. paxilli* (NRRL 6110). This strain was obtained from the U.S. Department of Agriculture, Peoria, Illinois. For inoculum, the fungus was maintained on modified agar slants consisting of glucose (1 g), KH_2PO_4 (1 g), and $MgSO_4$ (20 g/liter of deionized H_2O). Autoclaved shredded wheat (500 g) in five 2-liter conical flasks was inoculated and incubated in the dark at 25° for 27 days. The cultures were harvested by centrifugation and lyophilized. The combined dried mass was briefly extracted in a Waring blender with Me_2CO and filtered under reduced pressure. The residual homogenate was further extracted in a Soxhlet for 6 h using Me_2CO. The extracts were combined, brought to dryness, and redissolved in Et_2O. This was washed with H_2O, dried over Na_2SO_4, and then allowed to evaporate slowly in the dark. The toxin (101 mg) was obtained as yellow crystals.

Physical and chemical analysis.—An MeOH solution of these crystals was spotted on a tlc plate (Si gel F_{254}, 0.25 mm thickness, E. Merck, Darmstadt) and developed in toluene-EtOAc-HCO_2H (5:4:1). Three spots were observed under uv light. The major component, paxilline [1] (R_f 0.75) gave a green color after spraying with van Urk's reagent (1% 4-dimethylaminobenzaldehyde in ethanolic H_2SO_4) followed by oven-heating at 120° for 1–2 min. The product was further purified by preparative tlc (Si gel F_{254}, 1 mm plates) using $CHCl_3$-MeOH (95:5) as developing solvent. Paxilline [1] was obtained as light yellowish needles, mp 247.3–249.5° (dec), after recrystallization with Et_2O. The mp (uncorrected) was obtained using open tubes on a Büchi SMP. Uv spectra of the toxin in MeOH were recorded on a Perkin-Elmer Lambda 5 UV spectrophotometer. Ir spectra were recorded on a Beckman Acculab 4. The natural abundance, proton-decoupled ^{13}C (50.1 MHz) spectrum was recorded on a JEOL FX 200 spectrometer with the sample dissolved in $CDCl_3$. The ^1H- (199.5 MHz) nmr spectrum with TMS as internal standard was obtained from the same instrument. Mass spectral data were obtained by the direct inlet probe (DIP) method with a Hewlett-Packard 5988A gc-ms system with the ion source temperature kept at 250°. Ionization was effected by electron impact at 70 eV. All spectra were matched with published data (5).

BIOASSAY STUDIES.—Male and female guinea pigs (400–600 g) were killed by exsanguination after a blow on the head. The terminal ileum was excised, and a ±10-cm proximal section (closest to the ileocecal junction) was discarded. The organ was cleared of adjacent tissue and the contents gently flushed out. Strips of 6–7 cm were mounted on a coaxial electrode and transferred to an organ bath (37°) containing a modified Krebs solution of the following composition in g/liter (mM): $CaCl_2 \cdot H_2O$ 0.37 (2.5), KCl 0.35 (4.7), KH_2PO_4 0.16 (1.2), $MgSO_4 \cdot 7H_2O$ 0.29 (1.17), NaCl 6.9 (118), $NaHCO_3$ 2.1 (25.0), glucose 1.82 (10.10). All salts were of "pro analysi" quality (E. Merck, Darmstadt); glucose was of "reinst" quality (Merck).

The bathing fluid was continuously gassed with carbogen (95% O_2 and 5% CO_2). Contractions were auxotonically measured with adapted Harvard Smooth Muscle transducers and recorded on a Watanabe 6-channel recorder. Electrical stimulation was performed with a Student Stimulator (Janssen Scientific Instruments). Pulse characters were 40 mA, 1 msec duration, and 0.1 Hz.

Stock solutions (10^{-3} g/ml) of mycotoxins were prepared in DMSO and kept at −28° until use. These solutions were diluted with distilled H_2O. Final concentrations of DMSO in the organ bath did not exceed 0.1%. Final concentrations (g/ml) were: mycotoxins 10^{-8}–10^{-6}, acetylcholine 3×10^{-8}, and hyoscine 10^{-8}. The concentration of acetylcholine used here gave 70% of the maximal contraction (22).

Experimental protocol.—The ileum strips were allowed to stabilize for 15–20 min (1 g tension) before acetylcholine injection. Injections of acetylcholine were repeated until stable contractions were seen. Electrical stimulation was then started and continued until a steady state of the evoked heights of contraction was obtained (usually 15 min). A time schedule is presented in Table 3.

TABLE 3. Protocol for Coaxial Stimulation

Time (min)	Event
−15–−20	stabilization of the ileum
0	exogenous acetylcholine + washing
5	exogenous acetylcholine + washing
10	electrical stimulation
25	mycotoxin or DMSO
40	stop electrical stimulation
45	exogenous acetylcholine + washing
50	electrical stimulation
65	hyoscine
80	stop electrical stimulation + change ileum strip

LITERATURE CITED

1. J.D. Bu'Lock, in: "The Biosynthesis of Mycotoxins." Ed. by P.S. Steyn, Academic Press, New York, 1971, Chapter 1, pp. 1–16.
2. P.S. Steyn, in: "Mycotoxins in Human and Animal Health." Ed. by J.V. Rodricks, C.W. Hesseltine, and M.A. Mehlman, Pathotox Publishers, Park Forest South, Illinois, 1977, pp. 422–432.
3. A. Ciegler, R.F. Vesonder, and R.J. Cole, *Adv. Chem. Ser.*, **49**, 163 (1976).
4. R.T. Gallagher, R.G. Keogh, G.C.M. Latch, and C.S.W. Reid, *N.Z. J. Agric. Res.*, **20**, 431 (1977).
5. R.J. Cole and R.H. Cox, in: "Handbook of Toxic Fungal Metabolites." Academic Press, New York, 1981, pp. 355–509.
6. D.W. Peterson, H.F. Bradford, and P.G. Mantle, *Biochem. Pharmacol.*, **31**, 2807 (1982).
7. B.J. Wilson and C.H. Wilson, *Science*, **144**, 177 (1964).
8. M. Yamazaki, S. Suzuki, and K. Miyaki, *Chem. Pharm. Bull. (Tokyo)*, **19**, 1739 (1971).
9. S.J. Cysewski, *Am. J. Vet. Res.*, **36**, 159 (1975).
10. J.C. Land, K. Hult, R. Fuchs, S. Hagelberg, and H. Lundstrom, *Appl. Environ. Microbiol.*, **53**, 787 (1987).
11. P.J. Norris, C.C.T. Smith, J. De Belleroche, H.F. Bradford, P.G. Mantle, A.J. Thomas, and R.H.C. Penny, *J. Neurochem.*, **34**, 33 (1980).
12. P.G. Mantle, *Res. Vet. Sci.*, **34**, 373 (1983).
13. L.J. Hotujac and P. Stern, *Acta Med. Iugosl.*, **28**, 223 (1974).
14. L.J. Hotujac, R.H. Muftic, and N. Filipovic, *Pharmacology*, **14**, 297 (1976).
15. D.B. Gant, R.J. Cole, J.J. Valdes, M.E. Eldefrawi, and A.T. Eldefrawi, *Life Sci.*, **41**, 2207 (1987).
16. W.D.M. Paton, *J. Physiol. (London)*, **127**, 40 (1955).
17. T.J. Sobotka, R.E. Brodie, and S.L. Paid, *Pharmacology*, **16**, 287 (1978).
18. I.A. Deckers, M. Rampart, H. Bult, and A.G. Herman, *Eur. J. Pharmacol.*, **167**, 415 (1989).
19. R.J. Cole, J.W. Kirksey and J.M. Wells, *Can. J. Microbiol.*, **20**, 1159 (1974).
20. D.S.P. Patterson, B.A. Roberts, B.J. Shreeve, S.M. MacDonald, and A.W. Hayes, *Appl. Environ. Microbiol.*, **37**, 172 (1979).
21. M.I. Selala, F. Daelemans, and P.J.C. Schepens, *Drug Chem. Toxicol.*, **12**, 237 (1989).
22. G.M. Laekeman and A.G. Herman, *Prostaglandins*, **15**, 829 (1978).

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