Interaction of Purified Ergovaline from Endophyte-Infected Tall Fescue with Synaptosomal ATPase Enzyme System†

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Ergovaline (EV), the primary alkaloid implicated as a causative agent in fescue toxicosis, was extracted from endophyte-infected tall fescue seed (E+), and extraction procedures were developed to purify EV since it is not commercially available. The E+ seed was extracted with 5% lactic acid, initially cleaned up using liquid–solid extraction with Bio-Beads SM-2, and further purified using reversed-phase C-18 HPLC with a gradient of acetonitrile, 2.6 mM ammonium carbonate, and methanol. Incubation of purified ergovaline with rat brain homogenates resulted in a dose-dependent inhibition of Na^+/K^+ ATPase activity, with 50% inhibition occurring at 50 μM concentration of EV. Mg^{2+} activated ATPase was not significantly affected by the same treatment using doses up to 200 μM EV. Kinetic data indicated that EV interacted (uncompetitively) and inhibited synaptosomal Na^+/K^+ ATPase activities. This study demonstrates that EV isolated from E+ fescue seed can alter brain Na^+/K^+ ATPase activity and may be a contributing causative factor in fescue toxicosis.

INTRODUCTION

The relationship of fungal endophyte infestation in tall fescue, *Festuca arundinacae*, to the clinical signs of fescue toxicosis was first suggested by Bacon et al. (1977). Porter et al. (1979) demonstrated laboratory cultures of *Epichloe typhina*, reclassified as *Acremonium coenophialum* by Morgan-Jones and Gams (1982), produced clavine and ergot alkaloids. Using the mass spectrometry/mass spectrometry technique developed by Plattner et al. (1983), Yates et al. (1985) provided proof that ergovaline (EV), an ergot alkaloid, was present in endophyte-infested tall fescue pastures where animal toxicity (fescue foot) was noted. Ergovaline has been shown to be the predominant ergopeptine alkaloid in infested tall fescue (Yates et al., 1985; Lyons et al., 1986). Cornell et al. (1990) reported rations containing infested tall fescue seed as the source of toxins produced fescue toxicosis symptomatology when ergovaline levels of 50 ppb were fed under controlled climatic conditions of 30 °C and 60% relative humidity. Although the symptomatology of animals with fescue toxicosis is consistent with that of ergot alkaloid induced toxicity, definitive proof that ergovaline is the principal causative agent has not been accomplished because purified ergovaline is not available. We previously reported a method for extracting EV from fescue seed using chloroform as an extraction solvent (Moubarak et al., 1989). Due to the hazardous nature of handling and disposing of large quantities of chloroform, this method was not suitable for large-scale extraction. The objectives of this study were to develop a technique, using diluted lactic acid, to purify milligram quantities of pure EV and test its potency on synaptosomal ATPase activity.

EXPERIMENTAL PROCEDURES

Ergovaline Extraction. Five kilograms of ground E+ tall fescue seed was extracted in 25 L of 5% lactic acid (Testereci et al., 1990) overnight on an orbital shaker (90 rpm). The extract was filtered through a 20-L bucket with multiple 0.5-cm holes at the bottom and the filtrate centrifuged at 8000*g* for 20 min. The ergot alkaloids were absorbed on 500 g of SM-2 Bio-Beads (Bio-Rad) while mixing for 2 h at 4 °C. The Bio-Beads were removed from the extract by centrifugation at 8000*g*. The ergot alkaloids were then extracted by incubation of the Bio-Beads with 1000 mL of methanol for 2 h at 4 °C and filtered. The Bio-Beads were washed with deionized water in preparation for reuse. Methanol extracts were concentrated on a rotary evaporator under reduced pressure at 37 °C to 5 mL. The extract was placed on a 2 × 30 cm C_{18} column (20–30 μm, Vydac) and eluted with acetonitrile/2.6 mM ammonium carbonate (1/2) at 2 mL/min flow rate and collected in 5-mL fractions. The ergovaline fractions were identified using analytical HPLC, pooled, extracted with chloroform (1/1), dried on a rotary evaporator, and brought up in 1.0 mL of methanol. Final purification of ergovaline was made on a 0.4 × 25 cm C_{18} HPLC column using acetonitrile/2.6 mM ammonium carbonate (1/2) as a mobile phase. The combined ergovaline fractions were tested for purity using fluorescence, UV detection, and GC for possible contamination with loline alkaloids and freeze-dried into respective aliquots. The purity of EV was estimated to be 95% when the EV peak area was compared with the peak area of other contaminants, including ergovalinine. To prevent isomerization, EV was kept freeze-dried at −4 °C for up to 1 year.

Liquid Chromatography (HPLC). The final fractions were examined for purity by modification of the HPLC method described by Rottinghaus et al. (1991) and Scott and Lawrence (1980). A 20-μL sample loop was fitted to an ISCO 2350 pump and 2360 gradient programmer, and detection was accomplished by a Kratos Spectroflow (Applied Biosystem) 980 programmable fluorescence detector (excitation at 310 nm and 370-nm long pass filter). Separation was conducted on a 3 × 3 CR C_{18} cartridge column using linear gradient elution as follows: acetonitrile (A), 2.6 mM ammonium carbonate (B), and methanol (C).

time, min	flow rate, mL/min	event
0.0	1.0	20% A, 70% B, 10% C
5.0	1.0	50% A, 40% B, 10% C
10.0	1.0	20% A, 55% B, 25% C
16.0	1.0	20% A, 70% B, 10% C
16.1	0.3	end

This gradient program was used to separate and quantitate the ergovaline peaks of each fraction with no carry-over effects from run to run.

Enzyme Assays. Male Sprague-Dawley rats weighing 200–250 g were housed in an animal care facility with alternating 12-h light and dark cycles. Free access was given to laboratory chow and water. Adenosine 5′-triphosphate (ATP) bovine serum

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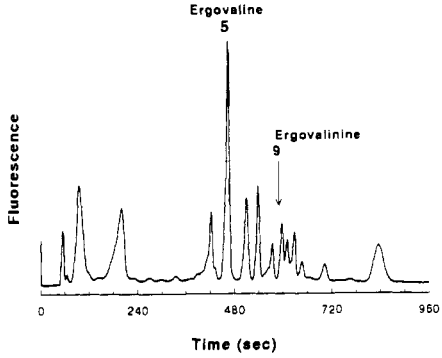

Figure 1. HPLC separation of typical lactic acid extract of endophyte-infected tall fescue seed. Peaks: 5, ergovaline; 9, ergovalinine.

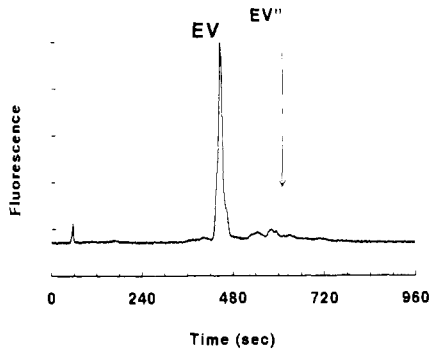

Figure 2. HPLC chromatogram of ergovaline fraction purified from endophyte-infected tall fescue seed. EV, ergovaline; EV", ergovalinine.

albumin (BSA), ergotamine (ET), and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO). The EV standard was a gift from Dr. G. Rottinghaus (University of Missouri). ET was used as an internal standard in the HPLC analysis. Rats were sacrificed with CO_2 anesthesia and decapitated, and the forebrain was isolated from the cerebellum and brain stem. Homogenates were prepared (Jones and Matus, 1974), stored in 10% sucrose at −90 °C, and used within 10–15 days. Protein concentration was determined (Lowry et al., 1951) using BSA as the standard.

Na^+/K^+ ATPase (ATP-phosphohydrolase, EC 3.6.1.3) activity in brain homogenates was determined spectrophotometrically (Forbush, 1983). The reaction medium contained 25 mM KCl, 120 mM NaCl, 4 mM $MgCl_2$, 4 mM ATP, 60 mM Tris buffer (pH 7.4), and 0.025 mg of protein homogenate. Ergovaline fractions were dissolved in methanol and added to the reaction tubes. Methanol was evaporated with a stream of nitrogen at 37 °C, and the synaptosomal homogenate was incubated with EV for 10 min at 37 °C. The reaction was initiated by addition of a mixture of ions and ATP to brain homogenates, with or without ergovaline fractions. After incubating at 37 °C for 10 min, the reaction was stopped with sodium dodecyl sulfate. The inorganic phosphate–molybdate complex was measured spectrophotometrically at 706 nm. Total ATPase activity was measured in the presence of Na^+, K^+ and Mg^{2+} ions, and Mg^{2+} ATPase activity with both the ions and 5 mM ouabain. The Na^+/K^+ ATPase activity was calculated as the difference between the total ATPase and the Mg^{2+} ATPase activity.

RESULTS AND DISCUSSION

A typical HPLC chromatogram of lactic acid extract from E+ fescue seed is shown in Figure 1. The identity of the ergovaline peak has been verified using the same ergovaline standard used by Yates and Powell (1988) and Rottinghaus et al. (1991). A purified fraction of ergovaline is shown in Figure 2. In the enzyme assays, the pure EV fraction used was less than 5% isomerized to ergovalinine. The degree of isomerization of ergovaline to ergovalinine is enhanced by exposure to UV light, moisture, and elevated temperatures. There is no evidence to indicate the presence of ergovalinine in freshly extracted E+ tall fescue seed, and its presence is presumed to be the result of isomerization during the extraction process. No attempt was made to separate or test ergovalinine at this time. Five percent lactic acid removed 65–70% (as compared to chloroform extraction, no data presented) of the ergovaline from the seed after shaking overnight. Aqueous lactic acid is a more environmentally compatible solution and reduces the amount of interfering substances, such as plant pigments, when compared to chloroform extraction. The liquid–solid extraction of the lactic acid extract with Bio-Beads SM-2 is a fast, convenient means of removing most (90–95%, no data presented) of the ergopeptides. The amount of ergovaline produced using this procedure was determined by the initial concentration of EV in the infected seed, which ranged from less than 1.0 to 6.0 ppm. The ergovaline and ergovalinine fraction purified according to the described procedure did not contain any significant contaminants as judged by fluorescence (Figure 2), UV detection, and GC procedures for detection of other alkaloids such as loline alkaloids.

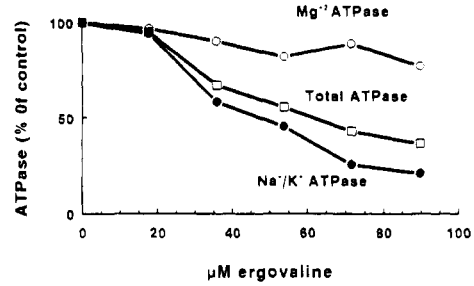

Figure 3. Effect of increasing concentrations of ergovaline on synaptic Mg^{2+} activated, total, and Na^+/K^+ ATPase activity.

The ergovaline fraction elicited a dose-dependent inhibition of brain Na^+/K^+ ATPase in vitro with 50% inhibition occurring at 50 μM concentration. Mg^{2+} activated ATPase was not significantly affected by the same treatment using doses up to 200 μM of EV (Figure 3). A similar inhibition of Na^+/K^+ ATPase using ergotamine and ergonovine (available commercially) has been reported earlier (Moubarak et al., 1990). Ergotamine and ergonovine are ergot alkaloids that are produced by fungi of the genus *Claviceps* and are in part responsible for ergot poisoning in humans and livestock. The similarity of the chemical structures of ergovaline and ergotamine (Figure 4) suggests parallel toxic effects. Inhibition of Na^+/K^+ ATPase activity was further examined by measuring enzyme activity at various concentrations (0.1–1.4 mM) of the enzyme substrate, ATP, in the presence and absence of ergovaline (75 μM). The linearity of the Lineweaver–Burk plot of $1/V$ vs $1/S$ shows that the interaction between the substrate and Na^+/K^+ ATPase in both the presence and absence of ergovaline follows Michaelis–Menten kinetics (Figure 5). Furthermore, the slope of the plots remained constant at increasing concentrations of ergovaline, but the V_{max} decreased, indicating uncompetitive inhibition. Thus, ergovaline interacts with the enzyme–substrate complex given an inactive complex. On the other hand, the kinetic data (Moubarak et al., 1990) from the interaction of ergonovine with Na^+/K^+ ATPase showed that ergonovine can interact with either the free enzyme or the enzyme–substrate complex in a noncompetitive fashion. Such enzyme kinetic data provide valuable information on the mode of action of one toxin as compared to another. The presence of the tricyclic amino acid portion of the molecule in ergovaline and its absence in ergonovine may play a role in the differences in kinetic behavior of the two compounds.

Fescue toxins have been shown to alter brain dopam-

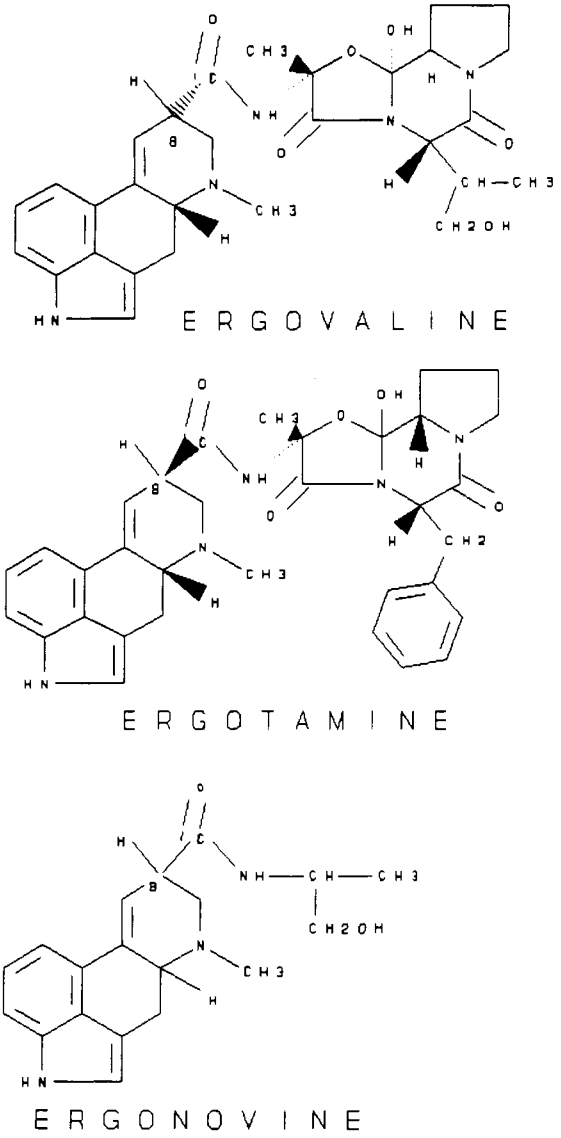

Figure 4. Chemical structures of ergovaline, ergotamine, and ergonovine.

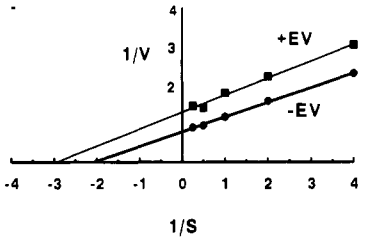

Figure 5. Lineweaver–Burk plots of Na^+/K^+ ATPase activity in the absence of ergovaline (●) and with 75 μM ergovaline (■).

inergic (Schillo et al., 1988) and serotoninergic (Porter et al., 1990) mechanisms. The ATPase enzyme system plays a key role in maintenance of neuronal membrane potential (Pillai and Ross, 1986). Inhibition of Na^+/K^+ ATPase activity by the ergopeptine alkaloids may be directly related to neural changes associated with consumption of endophyte-infected fescue and may be a causative factor in fescue toxicosis.

LITERATURE CITED

Bacon, C. W.; Porter, J. K.; Robbins, J. D.; Luttrell, E. S. *Epichloe typhina* from toxic tall fescue grasses. *Appl. Environ. Microbiol.* **1977**, *34*, 526–581.

Cornell, C. N.; Lueker, J. V.; Garner, G. B.; Ellis, J. L. Establishing Ergovaline Levels For Fescue Toxicosis, With And Without Endoparasites, Under Controlled Climatic Conditions. *Proceedings of the International Symposium on Acremonium/Grass Interactions*, New Orleans, LA; Quisenberry, S. S., Joost, R. E., Eds.; Louisiana Agricultural Experiment Station: Baton Rouge, 1990; pp 75–79.

Forbush, B. Assay of Na^+/K^+-ATPase in Plasma Membrane Preparation; Increasing the Permeability of Membrane Vesicles Using Sodium Dodecyl Sulfate Buffered with Bovine Serum Albumin. *Anal. Biochem.* **1983**, *128*, 159–163.

Jones, D. H.; Matus, A. I. Isolation of Synaptic Plasma Membrane from Brain by Combined Flotation-Sedimentation Density Gradient Centrifugation. *Biochim. Biophys. Acta* **1974**, *356*, 276–287.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein Measurements with Folinphenol Reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Lyons, P. C.; Plattner, R. D.; Bacon, C. W. Occurrence of Peptide and Clavine Ergot Alkaloids in Tall Fescue Grass. *Science* **1986**, *232*, 487–489.

Morgan-Jones, G.; Gams, W. Notes on Hyphomycetes, XLI. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloe typhina* a new taxa in one of two sections of *Acremonium*. *Mycotaxon* **1982**, *15*, 311–318.

Moubarak, A.; Piper, E. L.; West, C. P. Ergopeptine Purification from Endophyte-Infected Tall Fescue Seed. In *Proceedings of Arkansas Fescue Toxicosis Conference*; West, C. P., Ed.; Arkansas Agricultural Experiment Station Special Report 140; Arkansas Agricultural Experiment Station: Fayetteville, 1989; pp 11–16.

Moubarak, A. S.; Piper, E. L.; West, C. P. Interaction of Ergot Alkaloids With the Synaptosomal ATPase System. *Med. Sci. Res.* **1990**, *18*, 709–710.

Pillai, N. P.; Ross, D. H. Effects of Opiates on High-Affinity Ca^{2+}, Mg^{2+}-ATPase in Brain Membrane Subfractions. *J. Neurochem.* **1986**, *47*, 1642–1646.

Plattner, R. D.; Yates, S. G.; Porter, J. K. Quadrupole Mass Spectrometry/Mass Spectrometry of Ergot Cycl—Alkaloids. *J. Agric. Food Chem.* **1983**, *31*, 785–789.

Porter, J. K.; Bacon, C. W.; Robbins, J. D. Ergosine, Ergosinine, and Chanoclavine I from *Epichloe typhina*. *J. Agric. Food Chem.* **1979**, *27*, 595–598.

Porter, J. K.; Stuedemann, J. A.; Thompson, F. N., Jr.; Lipham, L. B. Neuroendocrine Measurements in Steers Grazed on Endophyte-Infected Fescue. *J. Anim. Sci.* **1990**, *68*, 3285–3292.

Rottinghaus, G. E.; Garner, G. B.; Cornell, C. N.; Ellis, J. L. HPLC Method For Quantitating Ergovaline in Endophyte-Infested Tall Fescue: Seasonal Variation of Ergovaline Levels in Stems With Leaf Sheaths, Leaf Blades, and Seed Heads. *J. Agric. Food Chem.* **1991**, *39*, 112–115.

Schillo, K. K.; Leshin, L. S.; Bolling, J. A.; Gay, N. Effects of Endophyte-Infected Fescue on Concentration of Prolactin in Blood Sera and the Anterior Pituitary and Concentrations of Dopamine and Dopamine Metabolites in Brains of Steers. *J. Anim. Sci.* **1988**, *66*, 713–718.

Scott, P. M.; Lawrence, G. A. Analysis of Ergot Alkaloids in Flour. *J. Agric. Food Chem.* **1980**, *28*, 1258–1261.

Testereci, H.; Garner, G. B.; Rottinghaus, G. E.; Cornell, C. N.; Andersen, M. P. A Method for Large Scale Isolation Of Ergovaline From Endophyte Infected Tall Fescue Seed (*Festuca Arundinacea*). *Proceedings of the International Symposium on Acremonium/Grass Interactions*, New Orleans, LA; Quisenberry, S. S., Joost, R. E., Eds.; Louisiana Agricultural Experiment Station: Baton Rouge, 1990; pp 100–103.

Yates, S. G.; Powell, R. G. Analysis of Ergopeptine Alkaloids in Endophyte-Infested Tall Fescue. *J. Agric. Food Chem.* **1988**, *36*, 337–340.

Yates, S. G.; Plattner, R. D.; Garner, G. B. Detection of Ergopeptine Alkaloids in Endophyte Infected, Toxic KY-31 Tall Fescue by Mass Spectrometry/Mass Spectrometry. *J. Agric. Food Chem.* **1985**, *33*, 719–722.

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