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ACTIONS OF A TREMORGENIC MYCOTOXIN ON AMINO ACID TRANSMITTER RELEASE *IN VIVO*

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Abstract—The tremorgenic mycotoxin verruculogen was administered directly into the brain of freely moving rats by the use of cannula systems that superfused either the cortical surface or the lateral ventricular space. The tremor produced by these CNS routes was compared with that produced by i.p. administration of the toxin or the dried mycelium of the fungus that synthesizes the verruculogen. The nature and degree of tremor produced by the central vs peripheral routes suggest that the site of action of verruculogen is not immediately adjacent to the cannula sites in the brain. Measures of the amino acids in the superfusates collected during the verruculogen-induced tremor showed an increase in the excitatory neurotransmitters, glutamate and aspartate in superfusates from the lateral ventricle but not in superfusates from the cortical surface. This differential effect on transmitter release suggests that a subcortical action of verruculogen is responsible for its tremorgenic activity.

Verruculogen is one of a group of fungal secondary metabolites which produces sustained tremor and muscular incoordination in laboratory and farm animals. Research on verruculogen and related mycotoxins has been prompted by the suggestion that they may be the causative agents of certain neurological disorders in farm animals [1]. These tremorgenic mycotoxins do not appear to produce any histological evidence of their toxic action [2, 3], and the neurological signs that are produced are completely reversible [4]. Therefore, an action limited to the biochemical level probably underlies the mechanism for the tremorgenic effects of verruculogen.

No malfunction of cholinergic or catecholaminergic systems in the brains of verruculogen treated animals have been detected [5, 6], though more detailed studies of turnover and release may yet show effects on these neurotransmitters. On the other hand, changes in amino acid transmitters following administration of tremorgenic mycotoxins have been demonstrated in studies employing synaptosomes [6], in tissue studies of amino acid levels [7] and in pharmacological studies on whole animals [8]. These reports suggest involvement of the excitatory amino acids, glutamate and aspartate, and the inhibitory amino acids, glycine and GABA, in the biochemical mechanism causing the tremor induced by these agents.

In the present study, *in vivo* amino acid release was measured directly by superfusion of rat brain cortex or a lateral ventricle after application of verruculogen to these areas. A comparison was made between the tremorgenic effects of toxin administration by these routes with those produced by i.p. injection to assess possible sites of action of verruculogen.

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MATERIALS AND METHODS

Toxin preparations. Penicillium simplicissimum isolated from New Zealand soil [10] was grown in submerged cultures as described previously [11]. The mycelia from these cultures were freeze-dried and pulverized by hand to provide a powdery material. This material was suspended in saline for i.p. injection to rats.

The same freeze-dried mycelium was also used to provide the purified verruculogen. An acetone:chloroform (1:1 v/v) extract of the mycelium was applied to preparative layer plates (silica gel, GF 254, Merck) and developed in chloroform:acetone (93:7 v/v). The band containing verruculogen was eluted with acetone, dried and dissolved in methanol:water (5:1 v/v) for injection into a reverse-phase analytical HPLC column (Ultrasphere ODS, 10 × 250 mm). Four peaks were eluted from this column, the largest corresponding to authentic verruculogen, a second peak corresponding to the less potent tremorgen, fumitremorgen B, and two unidentified peaks.

Animals. Male Sprague-Dawley rats, 350–400 g, were used throughout this study.

Tremorgenic potency. The powdered mycelium was injected i.p. in a saline suspension (50 mg/ml) at doses of 50 and 100 mg/kg. The rats were then placed in individual cages for a 90 min observation period, and the degree of tremor was quantified on a 5 point rating scale (Table 1). The verruculogen was first dissolved in acetone (5–10 μl) and then dispersed in 2% (v/v) Mulgofen EL 620 (G.A.F. Ltd., Manchester, U.K.) saline.

This was injected at a dose of 0.67 mg/kg and the tremorgenic effects assessed as for the mycelium. Mulgofen is a mixture of polyoxyethylated vegetable oils which emulsifies a wide range of hydrophobic substances.

Superfusion of the lateral ventricle. Concentric

Table 1. Tremor rating scale

Level	Description
1	Head and neck tremor only
2	Whole body tremor on moving
3	Whole body tremor leading to abnormal gait on moving
4	Continuous tremor with convulsive episodes
5	Severe convulsions leading to death

push–pull cannulae (Plastic Products) were implanted in the right lateral ventricle of four rats under halothane-N_2O anaesthesia. One week after surgery the ventricle of each rat was superfused for the first time with sterile saline containing 1.8 mM Ca^{2+}, at a rate of 15 μl/min, using a peristaltic pump. The superfusion was continued for 30 min and the superfusates discarded. During the second and third weeks after surgery, 90 min superfusions were performed on each rat and nine 10 min samples of the superfusates collected in tubes containing 10 μl 1N HCl and 0.5 nmole norleucine, the internal standard for the amino acid analysis. During the first 5 min of the collection of the fourth sample verruculogen (200 μg) or the vehicle alone (2% v/v Mulgofen) was added to the superfusion solution. Two rats received the toxin first and two rats received vehicle first.

During the superfusion the rats were scored on the tremor rating scale at the beginning of each 10 min period. The time of the first appearance of any tremor signs was also noted.

Superfusion of the cortical surface. Cortical cannulae were implanted above the somatosensory cortical surface of four rats under halothane-N_2O anaesthesia as described by Dodd *et al.* [12]. Superfusion at 15 μl/min with sterile saline containing 1.8 mM Ca^{2+} was continuous from the time of implantation of the cannula. At 21 hr after the surgery a series of nine 10 min samples were collected in tubes containing 1 N HCl and 0.5 nmoles norleucine. During the first 5 min of the fourth sample, 2% (v/v) Mulgofen was added to the superfusion solution. Three hours later a second group of 9 samples was collected and a 5 min superfusion with 100 μg verruculogen in 2% (v/v) Mulgofen was included at the start of the fourth sample. The tremorgenic effects were assessed as with the ventricular superfusions.

Amino acid analysis. The superfusion samples were evaporated to dryness and treated with 0.5 ml absolute methanol to leave behind the salts and protein. The methanol was evaporated and the residue dissolved in 0.025N HCl for analysis using the automated amino acid analyser described by Bradford and Thomas [13]. The amount of aspartate, glutamate, alanine and leucine were determined by comparison of peak heights with the norleucine.

[^{14}C]*Verruculogen uptake by the brain.* To determine the amount of verruculogen absorbed from the superfusion solution by the brain, [^{14}C]verruculogen was added to the solutions superfusing two rats with cortical cannulae and two rats with ventricular cannulae.

Amounts of [^{14}C]verruculogen superfused during the 5 min period at the start of the fourth sample were the same as in the previous experiments. The specific activity of the [^{14}C]verruculogen was 0.6 mCi/mmole, and was made by the method described previously [14]. After 25 min of superfusion following the addition of the [^{14}C]verruculogen, the rats were killed and the brains removed. Tissue from 5 brain regions were oxidized and the $^{14}CO_2$ formed measured by scintillation counting. The superfusates collected were also counted to determine the amount of [^{14}C]verruculogen that had washed through the cannula and therefore had not been taken up by the brain.

RESULTS

Tremorgenic potency. Rats given a single dose of either mycelium of *P. simplicissimum* or verruculogen tremored with increasing severity until a maximal level of response was reached, usually within 10–15 min after the injection. Some diminution of the response occurred after 30–60 min, but there was nearly always some degree of tremor at the end of the 90 min observation session.

Table 1 sets out the full range of tremor responses induced in the animals. At all dosage levels there was a decrease in spontaneous movement by the animals during the initial few minutes. With the larger dose (100 mg mycelium/kg) there was progression to marked tremor and convulsive episodes. Only at the levels 3–5 was a resting tremor detectable. Levels 1 and 2 were characterized by the

Table 2. Tremorgenic potency of verruculogen after various routes of administration

Preparation	Dose	Route	Number	Mean response* (range)
Mycelium	50 mg/kg	i.p.	18	2.4 (2–3)
Mycelium	100 mg/kg	i.p.	10	4.0 (2–5)
Verruculogen	0.670 mg/kg	i.p.	6	2.7 (2–3)
Verruculogen	0.200 mg (0.098 mg)†	i.vent.	6	2.0 (1–3)
Verruculogen	0.100 mg (0.056 mg)†	Cortical	6	2.0 (1–3)

* See Table 1: the full ranges of tremor responses exhibited are shown in parantheses.

† Dose absorbed from superfusion determined by [^{14}C]verruculogen study: total dose per animal.

absence of tremor unless the animals were forced to move.

The addition of verruculogen to the solutions superfusing the cortex or lateral ventricular space resulted in tremor that was indistinguishable from that produced by i.p. administration. Superfusion of the lateral ventricle with 200 μg verruculogen or the cortex with 100 μg verruculogen resulted in a similar degree of tremor to that produced by i.p. verruculogen at 0.67 mg/kg (Table 2). First signs of tremor occurred at 5–10 min after the toxin and developed with a time course similar to that of the peripherally adminstered toxin. There were no signs of unilateral tremor despite the unilateral sites of cortical or ventricular superfusions.

The percentages of verruculogen absorbed from the superfusion solutions were 56–57% for the cortical superfusions and 44–54% for the ventricular superfusions, as determined by the ^{14}C studies. The brains of these ^{14}C superfused rats contained less than 1% of the ^{14}C absorbed when assayed 25 min later. This ^{14}C was evenly distributed throughout the ventricular-superfused brains but was detectable only in the cortex of the cortical-superfused brains.

Amino acid release. All the amino acids assayed in superfusate samples during the administration of Mulgofen alone were slightly, but non-significantly increased (Figs 1 and 2). In the absence of any additions, the amino acids in the cortical superfusions did not vary from sample to sample, while the ventricular superfusates showed decreasing amino acid levels over the shorter total superfusion time of this method.

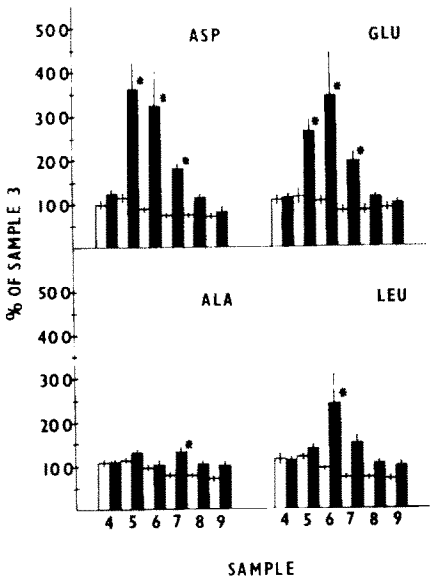

Fig. 1. Ventricular superfusate amino acid content. Each of 4 rats were superfused twice as described in Materials and Methods, once with 2% (v/v) Mulgofen vehicle alone (open bars) and once with the vehicle containing 5.2×10^{-6} M verruculogen (closed bars) during the first half of the fourth sample. The aspartate (ASP), glutamate (GLU), alanine (ALA) and leucine (LEU) content of samples 4–9 were calculated as a percentage of their content in sample 3, and the mean percentages for the 4 rats are presented. *Significant difference between verruculogen and Mulgofen vehicle, paired *t*-test, $P < 0.05$.

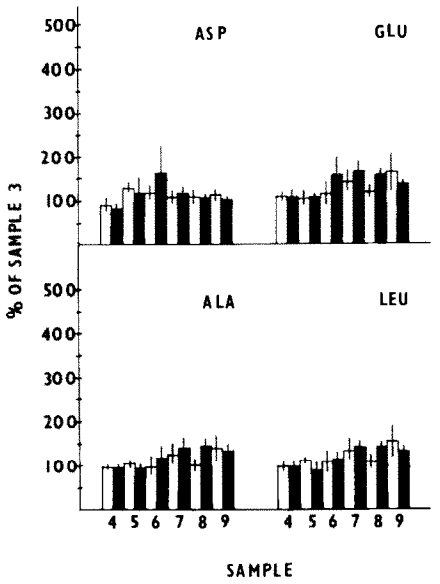

Fig. 2. Cortical superfusate amino acid content. Each of 4 rats were superfused twice as described in Materials and Methods, once with 2% (v/v) Mulgofen vehicle alone (open bars) and once with the vehicle containing 2.6×10^{-6} M verruculogen (closed bars) during the first half of sample 4. Data presentation is the same as described for Fig. 1.

The addition of verruculogen to the ventricular superfusion solution caused an immediate increase in aspartate and glutamate levels (Fig. 1). Allowing for the 10 min delay in collecting these samples, the 250% increase in aspartate and 175% increase in glutamate occurred within the 5 min of the toxin administration and at the same time as the first signs of tremor. These transmitter amino acids remained significantly elevated (paired *t*-test, $P < 0.05$) for a further 20 min (samples 6 and 7). Non-transmitter amino acids, alanine and leucine, were each significantly increased during a single 10 min sample.

In contrast, the addition of verruculogen to cortical superfusions produced no significant changes in any of the tested amino acids. The mean degree of tremor produced by this treatment was the same as that which was associated with amino acid release following toxin administration to the lateral ventricle (Table 2). However, in one animal which showed severe tremor at level 3 of the rating scale, an increase in aspartate release of 263% and in glutamate of 179% above control levels was seen.

DISCUSSION

The i.p. injection of verruculogen produced tremor identical to that produced by *P. simplicissimum* mycelium. Comparison of the degree of tremor with verruculogen and the mycelium of known toxin content indicates that the primary tremorgenic activity of this fungus resides in the verruculogen.

When verruculogen was placed directly into the brain via either cannula system, a comparable tremor effect was produced, with a time course similar to that caused by i.p. administration of toxin. Comparison of the degree of tremor produced by these routes of administration showed only a 2 to 3-fold

greater efficiency for the brain routes. The unilateral administration to the brain did not produce unilateral responses as has been reported for the cortical superfusion of scorpion toxin [15]. These observations indicate that the primary site of the tremorgenic activity of verruculogen is not the cortical surface or an area immediately adjacent to the lateral ventricle. The additional observation that little [^{14}C]-verruculogen remained in the brain after superfusion suggests that it is absorbed from the cannula sites and distributed in much the same way as after i.p. administration. Therefore the sites of action of verruculogen, even when administered to these limited areas of the brain, are not localized to these regions and could be anywhere in the central or in the peripheral nervous system.

In vitro amino acid studies of cortical synaptosomes from verruculogen treated rats have shown increased release of glutamate and aspartate [6]. This change was seen after a dose of *P. simplicissimum* mycelium (400 mg/kg, i.p.) that caused a more severe tremor than that produced by the superfused toxin in this study which was comparable to 50 mg mycelium/kg, i.p. At this lower tremor level a regional selectivity of amino acid release was seen. Levels of excitatory amino acids were increased in the lateral ventricle while they were unchanged in the cortex. Therefore, release of glutamate and aspartate in the subcortical area is associated with the pure tremoring symptoms (levels 1–3), while cortical involvement may only be seen when more severe symptoms of incoordination and convulsion are present. Supporting this proposal is the observation that the most severely affected cortical superfused rat showed a three-fold increase in aspartate and a two-fold increase in glutamate release.

If a subcortical site of amino acid release is the primary location of the tremorgenic action of verruculogen and related mycotoxins, its relationship to tremoring mechanisms must be further examined. Clearly biochemical lesions in the basal ganglia could lead to tremor of the type caused by imbalance of the various neurotransmitters in this region. Indeed the condition induced in rats at the higher dosages (100 mg mycelium/kg) and also in sheep [4], included a resting tremor of the type associated with the basal ganglia malfunction. Glutamate is thought to be a major neurotransmitter in the cortical–striate pathway [16].

Changes in glutamate release could reflect a direct effect of the toxin on these terminals or a disturbance due to an action on another transmitter system. Studies with synaptosomes from this region showed no action of a related tremorgen on dopamine terminals whilst a clear effect on the amino acid systems was seen, indicating preferential action on the latter [6].

A cerebellar site of action of the toxin may also be a component of the observed responses, since tremor was only detectable during enforced locomotion of the animals after toxin administration at the lower doses (50 mg mycelium/kg or 0.67 mg verruculogen/kg). Amino acids, including glutamate, are known to exert a profound effect on cerebellar function. Thus, glutamate is the proposed transmitter of the parallel fibre input to the granular and Purkinje cells, and aspartate is the likely transmitter of the climbing fibres which synapse directly on the Purkinje cells [17]. Thus, changes in these amino acid transmitters could be expected to lead to abnormal cerebellar function. This paper does not present data which proves a clear-cut causal relationship where an action on the amino acid transmitter system leads to tremor rather than vice versa. However, the fact that the actions of the tremorgen given *in vivo* persist in the synaptosomes subsequently isolated [6] strongly suggests that the agent is acting on the nerve-terminals themselves, and in this situation (i.e. *in vitro*) tremor itself is absent. It is difficult to conceive a mechanism by which a secondary action of tremor initiated *in vivo* would be manifest as a neurochemical response seen subsequently *in vitro*.

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