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The Tremorigen Aflatrem is a Positive Allosteric Modulator of the γ-Aminobutyric Acid$_A$ Receptor Channel Expressed in *Xenopus* oocytes

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SUMMARY

Aflatrem, a mycotoxin from *Aspergillus flavus*, potentiates the γ-aminobutyric acid (GABA)-induced chloride current. This positive allosteric regulatory action of aflatrem was quantitatively studied on the GABA$_A$ receptor channel expressed in *Xenopus* oocytes after injection with chick brain mRNA under voltage-clamp conditions. In this model system, aflatrem potentiates the current induced by 5 μM GABA in a concentration-dependent manner. Half-maximal potentiation was obtained with 2.4 μM aflatrem and maximal stimulation of the GABA (5 μM) response was more than 10-fold. The potentiation was not associated with a change of the reversal potential of the GABA-induced current. In the presence of 2 μM aflatrem, the GABA dose-response curve shifted to lower concentrations, with the K_a decreasing from 28 to 7 μM and the Hill coefficient, n, from 1.5 to 0.8, as measured at a membrane potential of −100 mV. At saturating concentration of GABA (250 μM), aflatrem (10 μM) was still able to enhance the current by about 21%. Further experiments suggest that the site of action of aflatrem on the GABA$_A$ receptor channel complex is different from that of benzodiazepines, pentobarbital, and picrotoxin. Aflatrem (10 μM) had no significant effect on the coexpressed voltage-dependent sodium and calcium channels and on the kainate channel. The potentiating action of aflatrem on the GABA$_A$ receptor channel may explain the initial symptoms of intoxication caused by aflatrem *in vivo*, i.e., diminished activity or immobility of the affected animal.

The first tremorgenic fungal toxin was an extract from several strains of *Aspergillus flavus* (1). The tremorgenic compound was later named aflatrem and its molecular structure (Fig. 1, *inset*) was elucidated (2). It is remarkably similar to some other tremorgenic mycotoxins found later (for review see Ref. 3). The signs of intoxication of mice given aflatrem orally or by intrapertioneal injection include diminished activity and immobility, uncoordinated movement, hyperactivity and hypersensitivity to sound and tactile stimuli, whole body tremor, and convulsions (1, 3–5). One recent report on effects of tremorgens including aflatrem showed that they inhibited $^{36}Cl^-$ influx induced by GABA and [^{35}S] *t*-butylbicyclophosphorothionate binding in rat brain membrane microsacs (6). This prompted us to study quantitatively the effect of aflatrem on the GABA-gated receptor channel using electrophysiological techniques.

Our study was carried out on GABA$_A$ receptor channels expressed in *Xenopus* oocytes after injection with mRNA from chick brain. GABA-gated channels expressed in *Xenopus* oocytes retain properties similar to those in the brain (7–12). This preparation allows accurate current measurements under voltage-clamp conditions and precise control of drug concentrations with the exclusion of presynaptic influences (11–13). It is, therefore, suited for quantitative studies of drug action.

Contrary to the former report (6), we found that aflatrem reproducibly potentiated the GABA-induced chloride current.

Materials and Methods

Expression of the GABA$_A$ receptor in *Xenopus* oocytes. Total mRNA was prepared from forebrains of 2-day-old chicks, following the procedures of Cathala *et al.* (14) with the modifications described earlier (15). Isolation and maintenance of follicles and oocytes, injection of mRNA, and removal of the surrounding cellular layers and connective tissue of follicles to obtain denuded oocytes have been described elsewhere (15).

Current measurements. Single denuded oocytes were placed in a small chamber with a bath volume of 0.4 ml and held by a nylon grid. The bath medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES-NaOH (pH 7.4). Drugs were diluted into the medium as described below and applied by bath perfusion at a rate of 6 ml/min. Membrane currents of the denuded oocytes were measured using the two-electrode voltage-clamp technique. The membrane potential was always held at −100 mV. All measurements were made at 20°, except those for GABA dose-response curves made at 6–8°, in

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide.

order to reduce desensitization. Current amplitudes were corrected by back-extrapolation of the desensitizing current trace to the time when the current reached half the peak value. Dose-response curves were computer fitted with the equation given in the text using a nonlinear least squares method.

Drugs. Aflatrem was a kind gift of Dr R. J. Cole (National Peanut Research Laboratory, Dawson, GA). It was dissolved in absolute ethanol or DMSO at a concentration of 2 mM as stock solution and stored at −20°. Aflatrem has no appreciable solubility in aqueous solution. It precipitated quite rapidly when diluted in the perfusion medium with a final ethanol or DMSO concentration of 0.5% (v/v). The aflatrem stock solution was, hence, diluted into the perfusion medium only 30 sec before its bath application. The freshly prepared drug solution was found to be essential in order to obtain reproducible results. Due to these problems with the solubility of aflatrem, all concentrations of aflatrem given below should be regarded as maximum estimates. Aflatrem dissolved in ethanol or DMSO yielded similar results. The current induced by application of 5 μM GABA was found to be enhanced by 8 ± 3% ($n = 6$) in the presence of 0.5% (v/v) ethanol. Therefore, all drug solutions, including the media containing GABA only, were adjusted to contain a final ethanol concentration of 0.5% (v/v) to correct for this marginal stimulatory effect of ethanol.

GABA, picrotoxin, and pentobarbital were purchased from Sigma. Ro 15-1788 (Anexate) was from Hoffmann-La Roche (Basel). Clorazepate was a gift from Boehringer-Ingelheim (U. K.).

Results

Xenopus oocytes injected with chick brain mRNA expressed several functional ion channels in their plasma membrane, including voltage-gated Na and Ca channels and ligand-gated GABA and kainate channels. Application by bath perfusion of GABA to mRNA-injected oocytes voltage-clamped at −100 mV induced an inward chloride current. The current decayed progressively after reaching a peak. The course of this desensitization was faster at higher concentrations of GABA. Recordings were taken from the fifth day onwards after injection of the oocytes with mRNA. At this time, typical current amplitudes induced by 100 μM GABA were between 1 and 3 μA. This newly expressed $GABA_A$ receptor channel has previously been found to be modulated by barbiturates (11), benzodiazepine receptor ligands, picrotoxin, and a novel modulator, avermectin B_{1a} (12, 13).

Stimulatory effect of aflatrem on the GABA-induced current response. A typical recording of the stimulatory effect of aflatrem on the GABA-induced inward chloride current is shown in Fig. 1. The current induced by 5 μM GABA was relatively small and displayed little desensitization and, hence, was chosen as control to study the stimulatory effect of aflatrem. After the microelectrodes were impaled into the oocytes, the GABA-induced current decreased initially, probably due to a leak of ions into the cell. GABA (5 μM) was, therefore, repeatedly applied at intervals of 4 min until a stable current response was reached. Subsequently, 5 μM GABA together with 1 μM aflatrem was applied. The action of aflatrem on the GABA current was fast, so the oocyte did not need to be preequilibrated with the drug. This was shown in experiments in which the perfusion medium was switched for 1 min to a medium containing 5 μM GABA and subsequently to a medium containing both 5 μM GABA and 1 μM aflatrem. In the presence of 1 μM aflatrem, the inward current amplitude induced by 5 μM GABA was increased more than 3-fold (Fig. 1). After 30 min of washout, 5 μM GABA elicited a current about 20% larger than that before application of 1 μM aflatrem (Fig. 1). This indicates that

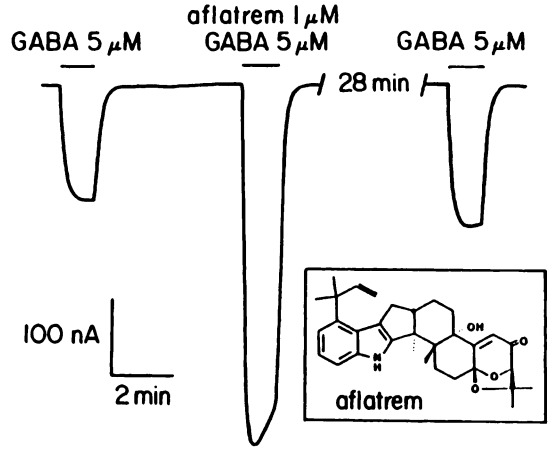

Fig. 1. Stimulation of the GABA-induced current by aflatrem. The membrane potential was clamped at −100 mV. The oocyte was continuously superfused with the bathing medium. A control application of 5 μM GABA was followed by a combined application of 5 μM GABA and 1 μM aflatrem. After 30 min of wash-out, another application of 5 μM GABA followed. The time periods of bath application of the drugs are indicated by the *bars*. The final ethanol concentration was adjusted to 0.5% (v/v) both during control applications of GABA and during the drug application. The *inset* shows the molecular structure of aflatrem (2).

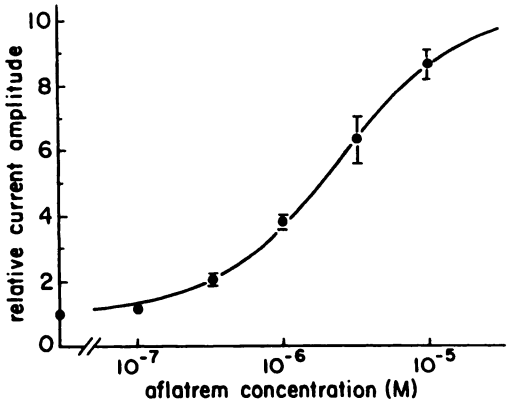

Fig. 2. Concentration dependence of the stimulatory effect of aflatrem on GABA-induced current. The current records were standardized (= 1) to the current amplitude induced by 5 μM GABA in control applications. Each *data point* (mean ± standard deviation) was obtained from at least four recordings in different oocytes. The *solid line* shows the best fit using the equation described in the text.

aflatrem could not readily be removed. Therefore, oocytes were exposed only once to aflatrem in all recordings to determine its stimulatory effect.

Aflatrem applied at 1 μM in the absence of GABA did not elicit any measurable membrane current, whereas 10 μM aflatrem induced only a very small inward current of less than 5 nA in oocytes displaying maximal GABA responses of 1.5–7 μA.

The stimulation of the GABA response by aflatrem is concentration dependent (Fig. 2). All current responses induced by 5 μM GABA in combination with aflatrem were standardized to the current induced by 5 μM GABA in control applications. Current responses were measured in at least four oocytes at each concentration of aflatrem. The membrane potential was held at −100 mV. The averaged data were fitted by the equation $I(c) - 1 = (I_{max} - 1)\, c^n/(c^n + K_a^n)$, where I_{max} is the maximal relative current induced by 5 μM GABA in the presence of aflatrem, c is the aflatrem concentration, n is the Hill coeffi-

cient, and K_a is the aflatrem concentration that elicits half-maximal current in combination with 5 μM GABA. The best fit was obtained with I_{max} = 10.4, n = 1.0, and K_a = 2.4 μM. This apparent Hill coefficient indicates that a single bound aflatrem molecule is sufficient to potentiate the response of a single receptor channel complex. The highest concentration of aflatrem applied was 10 μM. Recordings at higher concentrations of aflatrem were not made because of the problems with drug precipitation.

Effect of aflatrem on the reversal potential of the GABA response. The reversal potential of the GABA-induced current response did not change in the presence of 1 μM aflatrem (Fig. 3). The voltage-dependent Na current was blocked in these measurements by adding 0.5 μM tetrodotoxin to the medium. The reversal potential of the current induced by GABA (5 μM) was −29 mV, which is close to the reversal potential of about −25 mV for chloride ions in *Xenopus* oocytes (16–18). This result indicates that aflatrem does not alter the ionic selectivity of the GABA-gated chloride channels.

Although the reversal potential remained unaffected by aflatrem, its stimulatory effect was higher at low than at high membrane potentials. As shown in Fig. 3, the GABA response is increased about 2.7-fold at −120 mV and only 0.7-fold at +30 mV. We limited further studies on the effects of aflatrem to a membrane potential of −100 mV.

Effect of aflatrem on the GABA dose-response curve. GABA dose-response curves of the inward current obtained in the absence and presence of 2 μM aflatrem are shown in Fig. 4. The data points were fitted using the equation $I(c) = I_{max}\, c^n/(c^n + K_a^n)$, where I_{max} is the maximal current amplitude, c is the GABA concentration, n is the Hill number, and K_a is the GABA concentration that elicits half-maximal current response. All measurements were made at 6–8° to slow desensitization of the current response.

To obtain dose-response curves for GABA in the absence of aflatrem, single oocytes were repeatedly exposed to GABA starting at low concentrations. After each addition, GABA was washed out for long enough to allow full recovery of the response from desensitization. Thus, data obtained from each oocyte generated one complete dose-response curve. All current amplitudes measured from a single oocyte were standardized to the maximal current response (I_{max} = 100%) predicted by the best fit of the dose-response curve. Each point in the final curve represents the average of the relative current amplitudes determined from four oocytes. The average values were fitted again to yield the final dose-response curve as shown in Fig. 4.

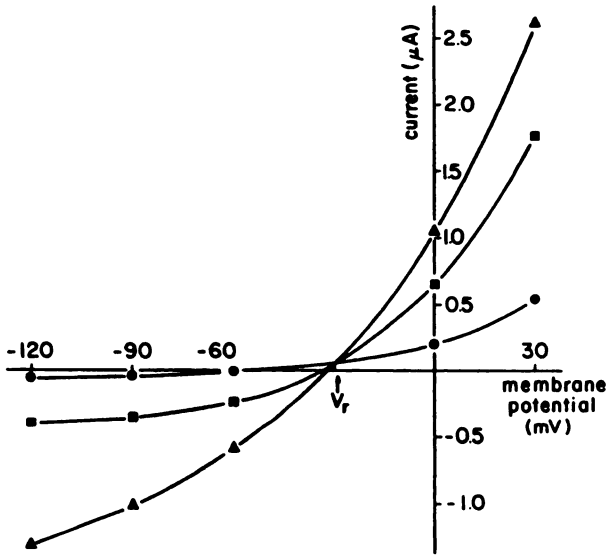

Fig. 3. Current-voltage relationships. Data were obtained from an experiment of the type shown in Fig. 1. A discontinuous voltage ramp was applied starting at −120 mV with increasing steps of 30 mV amplitude and 100 msec duration. Current amplitudes determined at the end of each voltage step are plotted against the membrane potential. ●, Control condition; ■, 5 μM GABA; ▲, 5 μM GABA and 1 μM aflatrem. V_r, reversal potential. The resting potential under control condition was −56 mV.

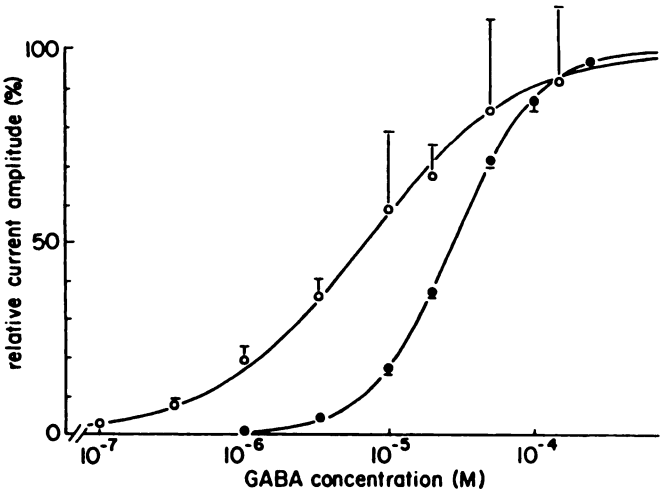

Fig. 4. Effect of 2 μM aflatrem on the concentration dependence of the GABA response. Dose-response curves of GABA were determined in the absence of aflatrem (●) and in the presence of 2 μM aflatrem (○). The recordings were performed at 6–8°. The *solid lines* represent the best fit of the data. I_{max} for both curves are standardized to 100%.

To obtain the dose-response curve for GABA in the presence of 2 μM aflatrem, oocytes were used only once and all current responses were standardized to the respective control current induced by 5 μM GABA. For each concentration of GABA, the relative current responses obtained from three oocytes were averaged. Subsequently, the averaged data points were fitted with the equation above and normalized with I_{max} = 100% (Fig. 4). Best fits of the dose-response curves indicated that, in the presence of 2 μM aflatrem, K_a was reduced 4-fold, from 28 μM in the absence of aflatrem to 7 μM, and the Hill coefficient n decreased from 1.5 to 0.8. The shift in the Hill coefficient suggests that the cooperativity between GABA binding sites is abolished or at least reduced in the presence of 2 μM aflatrem. This analysis of the data, however, does not show effects of aflatrem on the maximal current induced by GABA.

Effect of aflatrem on the maximal GABA current. The maximal current evoked by GABA could be further enhanced by aflatrem (Fig. 5). In the presence of 10 μM aflatrem, the current elicted by 250 μM GABA was increased by 25 ± 15% (mean ± SD, n = 4). The time course of desensitization was slightly faster, indicating that the current enhancement is not due to inhibition of the desensitization. According to our measurements, 250 μM GABA induces 97 ± 1% (n = 4) of its maximal current (I_{max}). Therefore, 10 μM aflatrem results in a true stimulation of I_{max} of about 21%. Assuming that aflatrem does not change single channel conductance or channel availability, it can be calculated that in the absence of aflatrem the open channel probability at I_{max} is less than 0.83.

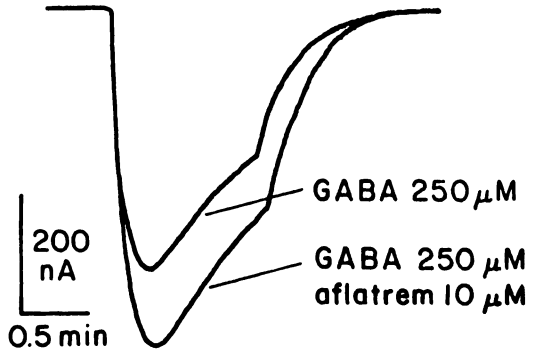

Fig. 5. Effect of aflatrem on the maximal GABA current response. GABA at 250 μM was applied as control and, subsequently, in combination with 10 μM aflatrem after an interval of 15 min to allow recovery of the current response from desensitization. The periods of bath application of the drugs were about 1 min. *Current traces* are shown superimposed.

TABLE 1

Interaction of aflatrem with other drugs acting on the GABA receptor channel complex

All drug concentrations are given in μM. All drug solutions contained a final concentration of 0.5% (v/v) ethanol. All data were standardized to the current amplitude elicited by 5 μM GABA (containing 0.5%, v/v, ethanol) in the same oocyte, in order to correct for differential expression in different oocytes. This amplitude was arbitrarily assigned the value of 1.0. Relative current amplitudes are given as mean ± standard deviation from at least three different experiments carried out using different oocytes.

Drugs perfused	Relative current amplitude
μM	
5 GABA + 1 aflatrem	3.32 ± 0.59
5 GABA + 1 Ro 15-1788	1.08 ± 0.07
5 GABA + 1 Ro 15-1788 + 1 aflatrem	3.20 ± 0.62
5 GABA + 5 picrotoxin[a]	0.10 ± 0.03
5 GABA + 10 aflatrem	7.84 ± 0.68
5 GABA + 10 aflatrem + 5 picrotoxin[a]	1.35 ± 0.17
0.5 GABA + 300 pentobarbital	5.09 ± 1.39
0.5 GABA + 10 aflatrem	1.58 ± 0.74
0.5 GABA + 10 aflatrem + 300 pentobarbital	11.63 ± 2.55

[a] 5 min before these measurements, the oocytes were exposed for 1 min to 5 μM GABA in combination with 5 μM picrotoxin in order to allow open channel block to occur.

Interaction of aflatrem with Ro 15-1788, picrotoxin, and pentobarbital. Table 1 summarizes the experiments carried out to investigate a possible interaction of aflatrem with known sites of action for other drugs acting on the GABA receptor channel.

The compound Ro 15-1788 is known to selectively bind to the central type of benzodiazepine binding sites with a K_D of about 1 nM (19). Therefore, about 99.9% of the benzodiazepine binding sites will be occupied in the presence of 1 μM Ro 15-1788. In control experiments, the stimulatory effect of 5 μM concentration of the benzodiazepine clorazepate (K_a = 0.39 μM) (12) on the response to 5 μM GABA was completely inhibited by 1 μM Ro 15-1788. By contrast, the potentiating effect of 1 μM aflatrem (K_a = 2.4 μM; this study) was not significantly influenced in the presence of 1 μM Ro 15-1788 (Table 1). This result indicates that the sites of action for aflatrem and benzodiazepine are different.

The GABA channel inhibitor picrotoxin (K_i = 0.6 μM) (12) at a concentration of 5 μM caused about 90% inhibition of the GABA (5 μM) response (Table 1). In the presence of 10 μM aflatrem, the same concentration of picrotoxin inhibited the aflatrem (K_a = 2.4 μM)-stimulated current by about 83% (Table 1). From the relative drug concentrations used in the experiment, their respective K_a and K_i values, and the maximal potentiation of the GABA (5 μM) current by aflatrem, it may be calculated that the inhibition should amount to 90% for a noncompetitive action and 59% for a competitive action of picrotoxin and aflatrem on the GABA receptor channel. The experimentally found value of 83% is in better agreement with a noncompetitive action of the two drugs, but an allosteric interaction between the two sites can not strictly be excluded.

The action of pentobarbital on the GABA receptor channel expressed in *Xenopus* oocytes has been analyzed quantitatively in another study (11). Currents elicited by 0.5 μM GABA in combination with near-maximal stimulatory concentrations of either aflatrem (10 μM) or pentobarbital (300 μM) could still be considerably enhanced by coapplication of the two stimulatory drugs (Table 1). These observations suggest different sites of action for pentobarbital and aflatrem.

Specificity of the action of aflatrem. In view of the rather high concentrations (up to 10 μM) of aflatrem used in this study, we were interested to see whether the activity of other channels coexpressed in the oocyte were affected by aflatrem. We did not find any significant effect of 10 μM aflatrem on the current response elicited by 20 μM kainate (three experiments). We also found no significant effect of 10 μM aflatrem on the amplitude, the voltage-dependent activation, and inactivation properties of the Na^+ and the Ca^{2+} channel (three experiments, each).

Discussion

Xenopus oocytes injected with exogenous mRNA have been shown to express efficiently corresponding functional proteins (20, 21). Specifically, $GABA_A$ receptors expressed in *Xenopus* oocytes displayed properties identical to vertebrate neuronal $GABA_A$ receptors, including effects of benzodiazepines, picrotoxin, barbiturates, etc. (7–13). This study demonstrates that aflatrem acts as a positive allosteric modulator of the $GABA_A$ receptor channel in the *Xenopus* oocyte system. The effects of aflatrem on the artificially expressed $GABA_A$ receptor channels in oocytes presumably correspond to those on the native receptors in neurons.

The effects of aflatrem on the GABA-induced current described in this study include 1) a concentration-dependent potentiation by aflatrem of the current elicited by 5 μM GABA, which is characterized by a K_a of 2.4 μM and a Hill coefficient n of 1.0; 2) a shift in the concentration dependence of the GABA response, with K_a decreasing from 28 to 7 μM and n from 1.5 to 0.8 in the presence of 2 μM aflatrem; and 3) an enhancement of the maximal GABA current response by about 21%. These data were collected at a membrane potential of −100 mV. Although the reversal potential of the current was unaffected by aflatrem, the potentiating effect by aflatrem decreased gradually at less negative membrane potentials. Further experiments indicated that aflatrem does not interact with any of the sites of action for benzodiazepines, pentobarbital, or picrotoxin.

The stimulatory effects of aflatrem on the GABA current are unexpected in view of the well known tremorgenic effect of aflatrem *in vivo*. However, the signs of intoxication by aflatrem are not merely tremors and convulsions but are more complex, initially displaying diminished activity and immobility (1, 3, 4). The potentiation by aflatrem of $GABA_A$ receptor channel-mediated responses may explain this initial poisoning symp-

tom. Other positive modulators of the $GABA_A$ receptor, such as benzodiazepines and barbiturates, exert anxiolytic, hypnotic, and anesthetic effects (22).

The tremorgenic mechanism of aflatrem cannot be explained by the effects of aflatrem on the $GABA_A$ receptor channel. Because aflatrem and other indole-derived tremorgenic mycotoxins (like penitrem A, verruculogen, etc.) share a similar molecular structure (3) and cause similar syndromes (23), the mechanism underlying tremor and convulsion may be common. Penitrem A and verruculugen administered *in vivo* have been shown to significantly increase spontaneous release of endogenous glutamate and aspartate (24, 25). Verruculogen has further been shown to cause a substantial increase in the miniature end-plate potential frequency at the locust neuromuscular junction, which uses glutamic acid as neurotransmitter (24). The excitatory effect resulting from the profound enhancement of glutamate and asparate release could counteract and surmount the potentiated inhibitory effect mediated by $GABA_A$ receptors. The dualistic mode of action of aflatrem is of interest because it may be possible to separate the two effects by modification of the molecular structure of the drug.

The reasons for the contradiction between our results and a former study (6) of aflatrem effects are not clear. In the latter, a different preparation, different recording methods, and different drug concentrations were used. Our results were obtained at apparent concentrations of aflatrem ranging from 0.1 to 10 μM, which were 30-fold lower than those in the former study. It would be interesting to see whether, at much higher concentrations, aflatrem can increase desensitization. As mentioned earlier, such experiments were impeded by the poor solubility of the drug under our experimental conditions.

Aflatrem is an indole-derived metabolite but has some structural similarity to steroids. It is interesting to note that steroid compounds, the short-acting general anesthetics alphaxolone and the metabolite pregnenolone have recently been reported to potentiate currents mediated by the $GABA_A$ receptor channel (26–29). From our data, it is not clear whether aflatrem interacts with the steroid site of action.

It is interesting to compare the effects of aflatrem on the GABA response with those of avermectin B_{1a}, another new positive allosteric modulator of the $GABA_A$ receptor (13). The stimulatory effects of aflatrem and avermectin differ in that 1) aflatrem displays a faster onset of stimulation than avermectin B_{1a}, 2) aflatrem appears not to suppress desensitization of GABA current as avermectin does, 3) aflatrem increases the maximal GABA current response in contrast to avermectin B_{1a}, and 4) whereas avermectin B_{1a} inhibits currents stimulated by high concentrations of pentobarbital, aflatrem stimulates these currents.

In conclusion, aflatrem has been found in this study to potentiate the current response mediated by $GABA_A$ receptor channels by an allosteric mechanism.

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