Convulsant actions of calycanthine

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Abstract

The principal alkaloid of the family Calycanthaceae, calycanthine has long been recognized as a central convulsant. The alkaloid inhibited the potassium-stimulated release of [3H]GABA from slices of rat hippocampus with an ED50 of 21 μM. This effect appeared to be moderately selective since calycanthine at 100 μM had only a weak effect on the potassium-stimulated release of [3H]acetylcholine (15%) and no significant effects on the release of [3H]D-aspartate from hippocampal and cerebellar slices or the release of [3H]glycine from spinal cord slices. Calycanthine blocked the L-type calcium currents with an IC50 of 42 μM and also weakly inhibited the N-type calcium currents (IC50 >100 μM) from neuroblastoma X glioma cells, suggesting voltage-dependent calcium channel blockade as a possible mechanism for its inhibition of GABA and ACh release. Calycanthine was also found to directly inhibit GABA-mediated currents (K135 μM) from human γ2L GABA receptors expressed in Xenopus laevis oocytes but had no effect at 100 μM on human ρ1 GABA receptors. The results indicated that calycanthine may mediate its convulsant action predominantly by inhibiting the release of the inhibitory neurotransmitter GABA as a result of interactions with L-type Ca2+ channels and by inhibiting GABA-mediated chloride currents at GABA receptors.

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As the major inhibitory neurotransmitter in the CNS, GABA is essential for the overall balance between neuronal excitation and inhibition. Compounds that block the synthesis, synaptic release, or postsynaptic action of GABA induce convulsions. GABA mediates fast synaptic inhibition by opening the Cl− channels intrinsic to GABA A and GABA C receptors (Chebib and Johnston, 2000).

Calycanthine (Fig. 1) is the principal alkaloid of the family Calycanthaceae and has been recognized as a centrally acting convulsant for a considerable period (Cushney, 1905; Chen et al., 1942). The convulsant effect of calycanthine is mediated, at least in part, by blocking the postsynaptic action of GABA as indicated by its inhibitory effect on the binding of the radiolabeled cage convulsant, [35S]butylbicyclopophosphorothionate ([35S]TBPS) (Squires et al., 1983; Squires and Saederup, 1997). TBPS (Fig. 1) mediates its effect through a convulsant site on the postsynaptic GABA A receptor, commonly referred to as the picrotoxinin site after the well known and extensively studied plant convulsant picrotoxinin (Fig. 1). Picrotoxinin and TBPS are convulsants that exert their actions by decreasing GABA-mediated Cl− currents (Van Renterghem et al., 1987; MacDonnald and Olsen, 1994), resulting in overexcitation of the neurons.

It has been shown that [35S]TBPS binding potencies and 50% lethal doses (LD50) of cage convulsants and picrotoxinin form a linear relationship (Palmer and Casida, 1988). Applying this relationship to the [35S]TBPS binding potency of calycanthine (IC50 = 10–12 μM) (Squires et al., 1983; Squires and Saederup, 1997) gave 50% lethal doses closer to 300 mg/kg, considerably less potent than the reported LD50 values of calycanthine (44, 17, and 8 mg/kg ip for mice, rats, and rabbits, respectively) (Chen et al., 1942). The discrepancy between the calculated and the experimen-
tal LD_{50} values implies that calycanthine exerts other actions in addition to its postsynaptic action at GABA_A receptors.

In order to gain a better insight into the convulsant action of calycanthine, the alkaloid was examined for its effects on chloride currents from GABA receptor channels and on transmitter release. Calycanthine was also examined for its effects on currents from voltage-dependent Ca^2+ channels since transmitter release is initiated by influx of extracellular calcium ions via these channels into the presynaptic nerve terminus (Tareilus and Breer, 1995).

This paper reports the effects of calycanthine on the release of GABA from hippocampal, cerebellar, and cortex slices, ACh and aspartate from hippocampal slices, and glycine from spinal cord slices using a superfusion technique. It also describes the effects of calycanthine on calcium currents from neuroblastoma X glioma cells (NG-108 cells) and GABA-mediated chloride currents from human α_{1}β_{2}γ_{2L} GABA_A and ρ_1 GABA_C receptors expressed in Xenopus laevis oocytes.

**Materials and methods**

Calycanthine and nimodipine were purchased from the Sigma Chemical Co. (St Louis, MO). ω-Conotoxin GVIA was purchased from Auspep Pty. Ltd. (Parkville, Victoria, Australia). Calycanthine was converted into its hydrochloride salt by saturating a 0.5% solution of calycanthine in water with dry HCl gas. The ^1H- and ^13C-NMR spectra of calycanthine hydrochloride were consistent with those of the free base reported previously (Duke et al., 1995). In addition to [^3H]GABA (75 Ci/mmol; NEN Dupont, Boston, MA), the following radiolabeled compounds were used in release studies: [^3H]o-aspartic acid (0.16 μM, 12.8 Ci/mmol; NEN Dupont), [^3H]choline chloride (0.05 μM, 81 Ci/m mole; NEN Dupont), and [^3H]glycine (0.11 μM, 19 Ci/mmol; Amersham International, Amersham, UK). Human ρ_1 cDNA subcloned in pcDNA 1.1 vector (Invitrogen, San Diego, CA) was a gift from Dr. George Uhl (National Institute for Drug Abuse, Baltimore, MD). Human α_{1}, β_{2}, and γ_{2L} cDNAs subcloned in pcDM8 vector (Stratogene, La Jolla, CA) were kindly provided by Dr. Paul Whiting (Merck Sharp and Dohme Research Laboratories, Harlow, Essex, UK). The restriction enzymes XbaI and NOTI were purchased from GeneWorks Pty. Ltd., (Adelaide, South Australia, Australia). All animal procedures were approved by the Animal Ethics Committee of the University of Sydney.

**Neurotransmitter release.** The potassium-stimulated release of [^3H]GABA, [^3H]o-aspartate (a nonmetabolizable substrate for high-affinity excitatory amino acid transport systems), and [^3H]glycine from slices of rat CNS tissues was studied using the methodology developed by Davies and Johnston (1976). The potassium-stimulated release of [^3H]ACh from slices of rat hippocampus prelabeled with [^3H]choline was studied using the method described by Lodge and Johnston (1985). Male Sprague–Dawley rats (weighing 300–350 g) were sacrificed by stunning followed by decapitation. The brain and spinal cord were rapidly removed into ice-cold Krebs buffer. Krebs buffer consisting of (in mm) 125 NaCl, 3 KCl, 1 NaH_2PO_4, 1.2 MgSO_4, 2.4 CaCl_2, 22 NaHCO_3, and 10 glucose oxygenated with 95% O_2 and 5% CO_2 was used as the standard medium. The contents of Krebs buffer used in the depolarizing medium were almost identical to those in the standard medium except for the concentrations of KCl, which were increased to 15 mM in [^3H]GABA and 35 mM in [^3H]o-aspartate, [^3H]glycine, and [^3H]choline experiments. KCl replaced an equal amount of NaCl to maintain osmolarity concentrations. In [^3H]GABA experiments, the standard and depolarizing media also contained 50 mM aminooxyacetic acid, which was used to inhibit GABA:2-oxoglutarate aminotransferase in order to prevent metabolism of GABA.

The hippocampi, cerebellar, and whole cerebral cortexes were dissected free on ice and minislices (250 × 250 μm) were cut (first in a sagittal direction and then in a transverse direction) using a McIlwain tissue chopper. Experiments using spinal cord (lumbar enlargement where only 250-μm slices were used) followed similar procedures. The slices were allowed to recover in oxygenated Krebs buffer (10 ml) for 30 min at 37°C and were then incubated with 0.02 μM [^3H]GABA (15 μL) for a further 30 min at 37°C. The slices were layered onto a presoaked Millipore AP prefilter (Catalog No. AP15 022 00; 0.5 ml Krebs) using moderate vacuum filtration and then were covered with another pre-soaked Millipore prefilter before loading onto a set of parallel superfusion chambers preincubated at 37°C.

Superfusion was started with the standard medium at a rate of 0.6 ml/min. After 38 min of superfusion using standard medium (T = 0), 10 fractions were collected at 2-min intervals, during which time, at T = 4, the system was depolarized for 2 min with 15 mM KCl (for [^3H]GABA experiments) and 35 mM KCl (for [^3H]o-aspartate, [^3H]glycine, and [^3H]choline experiments). The system was washed for 8 min before being superfused with the standard medium containing a fixed concentration of calycanthine (0.5–100 μM). Following 12 min equilibration (T = 40), an additional 10 fractions were collected at 2-min intervals and, at T = 44, the system was again depolarized with 15 mM KCl (for [^3H]GABA experiments) and 35 mM

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![Fig. 1. Structure of calycanthine. TBPS, and picrotoxinin.](image-url)
KCl (for [3H]o-aspartate, [3H]glycine, and [3H]choline experiments) containing the same concentration of calycanthine (0.5–100 μM) for 2 min. The slices were collected and placed in scintillation vials containing 1 ml of water.

The depolarization-evoked release (S1) was estimated by adding the percentage release of fractions 6 and 7 followed by subtracting twice the average percentage of basal release (baseline release), that was taken from fractions 1–5 (R1). This was repeated for the second depolarization-evoked release (S2). The percentage release of fractions 16 and 17 were added followed by subtracting twice the average percentage of spontaneous release that was taken from fractions 11–15 (R2). The effect of the calycanthine was evaluated as the ratio of the depolarization-evoked release calculated in the presence of the calycanthine (S2calycanthine/S1calycanthine) and compared to that obtained under control conditions (S2control/S1control) and determined as a percentage of the control. The unpaired, two-tailed Student t test using StatView SE + Graphics (Abacus Concepts, Berkeley, CA) was used to evaluate the significance of the results (\(^{*}p < 0.05\)).

Calycanthine inhibits the potassium-stimulated [3H]GABA release from hippocampal slices. The percent inhibition was analyzed using PRISM 2.0a. Each data point represents varying concentrations of calycanthine as the mean ± SEM of three independent experiments each performed in duplicate. The IC\(_{50}\) (concentration that inhibits 50% of the maximum potassium-stimulated GABA release) produced by calycanthine was determined using least squares analysis fitting:

\[
\text{% inhibition} = \frac{\text{% maximal inhibition} \times [A]^n}{(IC_{50}^m + [A]^m)}
\]

where \([A]\) is the concentration of calycanthine and \(n_H\) is the Hill coefficient.

**NG108-15 neuroblastoma X glioma cells and electro physiology.** Culture of and electrophysiological recording from NG108-15 neuroblastoma X glioma cells were carried out as described in Fletcher et al. (1997). For recordings, NG108-15 cells were cultured for at least 4 days in Dulbecco modified Eagle medium supplemented with 100 μM hypoxanthine, 10 μM aminopterin, 16 μM thymidine, 10 μM PGE\(_2\), 50 μM 3-isobutyl-1-methylxanthine (IBMX), and 1 % fetal bovine serum. Recordings of currents through calcium channels (\(I_{Ca}\)) were made using standard whole-cell recording techniques (Hamill et al., 1981). Cells were perfused with a solution comprising (in mM) 140 TEACl, 4 BaCl\(_2\), 2.5 CsCl, 10 Hepes, 10 glucose, and 20 sucrose, pH 7.3. Recordings were made with firepolished borosilicate pipettes of resistance 2–4 MΩ when filled with a solution comprising (in mM) 130 CsCl, 10 EGTA, 10 Hepes, 5 MgATP, and 0.2 NaGTP at pH 7.3. Cells were exposed to drugs and toxins via a series of flow pipes positioned above the cells. Calcium channel currents were evoked by stepping the membrane potential of the cells repetitively from a holding potential of −90 mV to a test potential of −10 or 0 mV.

N- and L-type channels were isolated as described by Connor and Henderson (1997). Briefly, to isolate the L-type \(I_{Ca}\), cells were preincubated with the irreversible N-type \(I_{Ca}\) inhibitor o-conotoxin GVIA (1 μM, 15 min). The L-type current recorded ranged from −85 to −870 pA (average −375 ± 75 pA). To isolate the N-type \(I_{Ca}\), cells were continuously perfused with buffer containing the L-type \(I_{Ca}\) inhibitor nimodipine (3 μM). The N-type current recorded ranged from −80 to −860 pA (average −379 ± 145 pA). Nimodipine (3 μM) inhibited the total current by 47 ± 8% (\(n = 9\)), indicating that about half the current was L-type and the rest was N-type.

**Expression of GABA receptors and electrophysiology.** *X. laevis* were anesthetized with 0.17% ethyl 3-aminobenzoate and a lobe of the ovaries was removed. The lobe of the ovary was rinsed with OR2 (containing in mM 82.5 NaCl, 2 KCl, 1 MgCl\(_2\), and 5 Hepes, pH 7.5) and treated with Collagenase A (2 mg/ml in OR2, Bohringer Mannheim) for 2 h. Released oocytes were then rinsed in frog Ringer solution (containing in mM 96 NaCl, 2 KCl, 1 MgCl\(_2\), 1.8 CaCl\(_2\), and 5 Hepes, pH 7.5) supplemented with 2.5 mM pyruvate, 0.5 mM theophylline, and 50 μg/ml gentamycin, and stage V–VI oocytes were collected.

Human α\(_1\), β\(_2\), and γ\(_{2L}\) subcloned in pcDM8 were linearized using the restriction enzyme NOTI and human p\(_1\) cDNA subcloned in pcDNA1.1 Xba1. Capped RNAs were synthesized from linearized plasmid containing p\(_1\), α\(_1\), β\(_2\), and γ\(_{2L}\) cDNAs using the “mMESSAGE mMACHINE” kit from Ambion Inc. (Austin, TX). p\(_1\) cRNA (10 ng/50 nl) and 10 ng/50 nl of a 1:1:1 mixture of α\(_1\), β\(_2\), and γ\(_{2L}\) cRNAs were injected into defolliculated stage V–VI *Xenopus* oocytes. Oocytes containing p\(_1\) cRNAs and the α\(_1\)β\(_2\)γ\(_{2L}\) cRNAs were kept at 18°C. Two to 8 days later, receptor activity was measured by two-electrode voltage-clamp re-
cordings using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA), a MacLab 2e recorder (AD Instruments, Sydney, New South Wales, Australia), and Chart version 3.5 program. Oocytes injected with \(/H92671\) and the \(/H92511\)/\(/H92522\)/\(/H92532L\) cRNAs were voltage clamped at \(/H1100260\) mV and continuously superfused with frog Ringer solution. For receptor activation measurements, the indicated concentrations of drug were added to the buffer solution.

**Analysis of kinetic data.** Current \((I)\) as a function of agonist concentration \((A)\) was fitted by least squares to

\[
I = I_{\text{max}} \frac{[A]^m}{[EC_{50}^m + [A]^m)},
\]

where \(I_{\text{max}}\) is the maximum current, \(EC_{50}\) is the effective concentration that activates 50% of the maximum current produced by a given drug, and \(n_H\) is the Hill coefficient. \(EC_{50}\) values are expressed as mean ± SEM \((n = 4–6\) oocytes) and are determined by fitting data from individual oocytes using Kaleidograph 3.0.

Estimated \(K_B\) value is the inhibitory constant for the antagonist and was determined using the following equation:

\[
K_B = \frac{[Ant]/(A)/(A^*) - 1},
\]

where \(A\) is the \(EC_{50}\) of GABA in the presence of a known antagonist concentration, \(A^*\) is the \(EC_{50}\) of GABA in the absence of the antagonist, and \([Ant]\) is the concentration of the antagonist.

**Results**

**Effect of calycanthine on neurotransmitter release**

Calycanthine (100 μM) inhibited the potassium-stimulated release of \([^{[H]}GABA]\) from hippocampal and cerebellar slices by 65 ± 3% (Fig. 2A) and 55 ± 5% (Fig. 2B), respectively, but had no effect on cerebral cortical slices (Fig. 2C). Calycanthine (100 μM) inhibited the potassium-stimulated release of \([^{[H]}ACh]\) by 15 ± 2% (Fig. 2D). The potassium-stimulated release of
Aspartate from slices of rat hippocampus (Fig. 2E) and glycine from slices of rat spinal cord slices (Fig. 2F) was unaffected by 100 μM calycanthine. Fig. 3 shows the inhibitory dose–response curve of calycanthine on the release of [3H]GABA from hippocampal slices. The concentration of calycanthine required for inhibiting 50% [3H]GABA release (IC50 value) was calculated to be 21.4 ± 2.5 μM.

Increasing the potassium ion concentration to 15 mM resulted in a twofold greater increase than the spontaneous release in the efflux of [3H]GABA from slices of rat hippocampus and cerebellum. Increasing the potassium ion concentration to 35 mM resulted in a threefold increase in the observed efflux of [3H]-aspartate and [3H]ACh from slices of rat hippocampus and a twofold increase in the efflux of [3H]glycine from slices of rat spinal cord.

Effect of calycanthine on Ca2+ channel currents (ICa)

To determine whether voltage-sensitive calcium channels were involved in the inhibitory action of calycanthine on neurotransmitter release, the alkaloid was examined for its effect on the currents from NG-108 cells. NG-108 cells express predominantly N- and L-type ICa (Docherty, 1988; Kasai and Neher, 1992; Connor and Henderson, 1997). L-type calcium channel currents were recorded from the cells treated with ω-conotoxin GVIA to block N-type calcium currents, while N-type calcium currents were recorded in the presence of nimodipine to block L-type calcium currents.

Following pretreatment of the cells with ω-conotoxin GVIA, calycanthine inhibited the remaining calcium channels with an IC50 of 42 μM (pEC50 4.4 ± 0.2, Fig. 4A). Calycanthine inhibited ICa recorded in the presence of nimodipine less potently (IC50 > 100 μM, Fig. 4A). Fig. 4B shows L-type calcium channel currents from NG-108 neuroblastoma X glioma hybrid cell pretreated with the irreversible inhibitor of N-type calcium channels, ω-conotoxin GVIA, in the presence of increasing concentrations of calycanthine.

Effect of calycanthine on human α1β2γ2L GABA<sub>A</sub> and ρ1 GABA<sub>C</sub> receptors

Expression of the human α1β2γ2L and ρ1 cRNAs in X. laevis oocytes generated GABA-gated ion channels with pharmacological profiles similar to those previously described for α1β2γ2L GABA<sub>A</sub> receptors (Mihic et al., 1994) and ρ1 GABA<sub>C</sub> receptors (Kusama et al., 1993). The amplitude of the whole-cell currents recorded from human α1β2γ2L GABA<sub>A</sub> receptors and from human ρ1 GABA<sub>C</sub> receptors ranged from 200 to 3000 nA and from 200 to 2000 nA, respectively, when the oocytes were held at −60 mV. Calycanthine was first screened at 100 μM to determine antagonist activity by blocking the activation of the α1β2γ2L.
The inhibitory dose–response curve of GABA indicated by filled bar was coapplied with GABA (1 μM; duration indicated by filled bar), the GABA response was not significantly changed.}

**Discussion**

The convulsant effect of calycanthine may be mediated presynaptically by inhibiting synaptically evoked release of GABA in the hippocampus and postsynaptically by blocking the action of GABA at GABA<sub>A</sub> receptors. The action of calycanthine on GABA release was regionally specific as it was most efficacious for potassium-stimulated GABA release in hippocampal slices, slightly less potent in cerebellar slices, and ineffective in slices of cerebral cortex. This action appears to be moderately selective for GABA in that calycanthine had no effect on the release of n-aspartate as a marker for release of the excitatory neurotransmitter glutamate from hippocampal slices or the release of the inhibitory neurotransmitter glycine from spinal cord slices and only a weak effect on the release of the excitatory neurotransmitter ACh from hippocampal slices.

Calycanthine was found to be moderately potent as an inhibitor of the L-type and weakly potent as an inhibitor of the N-type Ca<sup>2+</sup> channel currents from NG-108 cells, suggesting that its inhibition of GABA and ACh release may involve voltage-dependent Ca<sup>2+</sup> channels. The N-type voltage-sensitive Ca<sup>2+</sup> channels play a major role in coupling neuronal excitation with GABA and ACh release (Poncer et al., 1997; Dolezal and Tucek, 1999) whereas the L-type Ca<sup>2+</sup> channels are involved in the presynaptic accumulation of calcium ions (Jensen et al., 1999). Blockade of the L-type Ca<sup>2+</sup> channels in cultured hippocampal GABAergic neurons has been shown to depress the accumulation of calcium ions, which, in turn, causes a reduction in the probability of GABA-vesicular release (Jensen et al., 1999). Moreover, the L-type Ca<sup>2+</sup> channels on cell soma also contribute to maintaining the depolarization of the cell, thus promoting action potentials going to the nerve terminals that are required in transmitter release (Tareilus and Breer, 1995).

Both N- and L-type Ca<sup>2+</sup> channels have also been shown to participate in the potassium-stimulated release of GABA from cerebellar slices (Momiyama and Takahashi, 1994), cultured cerebellar and striatal neurons (Philibert and Dutton, 1989; Pin and Bockeart, 1990), and ACh from rat hippocampal slices (Clos et al., 1994). Calycanthine inhibiting both potassium-stimulated releases of GABA and ACh is probably due to its ability to block both the N- and the L-type Ca<sup>2+</sup> channels.

Ca<sup>2+</sup> channel subtypes in various parts of the brain are differentially localized and this may also account for calycanthine having no effect on GABA release from rat cortical slices. Not only is the evoked GABA release in the rat frontal cortex not mediated by N-type Ca<sup>2+</sup> channels (Martinez-Martos et al., 1997) but also the Ca<sup>2+</sup> channels that predominate in cultured mouse neocortical neurons are of the Q-type (Timmerman et al., 2001). The action of calycanthine on P/Q-type calcium channels is presently unknown. P/Q-type calcium channels have been shown to be essential for mediating neurotransmitter release in many parts of the CNS. Thus, differential expression of P/Q-type channels on nerve terminals may contribute to the regional selectivity of calycanthine.

Although calycanthine was shown to be a moderately
potent noncompetitive antagonist (IC_{50} 10–12 μM) of GABA_A receptors in rat brain synaptosomes as assessed by TBPS binding (Squires et al., 1983; Squires and Saederup, 1997), in functional studies we found it to be a relatively weak antagonist (K_B ~135 μM) of human recombinant α1β2γ2L GABA_A receptors. The result was unexpected as this receptor combination represents the major receptor populations in human brain. In addition, calycanthine appears to exert complex antagonism at α1βγ2L GABA_A receptors. The GABA dose–response curve in the presence of calycanthine exhibited a nonparallel shift but not a reduction in the maximal response of GABA.

In summary, calycanthine exhibits multiple mechanisms of action. Calycanthine may mediate its convulsant action predominantly by inhibiting the release of the inhibitory neurotransmitter GABA in certain brain regions as a result of interactions with L-type Ca^{2+} channels and by inhibiting GABA-mediated chloride currents at GABA_A receptors.

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References


