(+)-Hydrastine, a potent competitive antagonist at mammalian GABA_A receptors

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1 (+)-Hydrastine is a phthalide isoquinoline alkaloid, isolated from *Corydalis stricta*. It has the same 1S,9R configuration as the competitive GABA_A receptor antagonist bicuculline and is the enantiomer of the commercially available (–)-hydrastine.

2 (+)-Hydrastine (CD₅₀ 0.16 mg kg^{–1}, i.v.) was twice as potent as bicuculline (CD₅₀ 0.32 mg kg^{–1}, i.v.) as a convulsant in mice. This action was stereoselective in that (+)-hydrastine was 180 times as potent as (–)-hydrastine.

3 (+)-Hydrastine was a selective antagonist at bicuculline-sensitive GABA_A receptors in the guinea-pig isolated ileum. It did not influence phaclofen-sensitive GABA_B receptors or acetylcholine receptors in this tissue. (+)-Hydrastine was a competitive antagonist of GABA_A responses (pA₂ 6.5) more potent than bicuculline (pA₂ 6.1).

4 When tested against the binding of [³H]-muscimol to high affinity GABA_A binding sites in rat brain membranes, (+)-hydrastine (IC₅₀ 2.37 μM) was 8 times more potent than bicuculline (IC₅₀ 19.7 μM).

5 As an antagonist of the activation of low affinity GABA_A receptors as measured by the stimulation by GABA of [³H]-diazepam binding to rat brain membranes, (+)-hydrastine (IC₅₀ 0.4 μM) was more potent than bicuculline (IC₅₀ 2.3 μM).

6 (+)-Hydrastine, 10 nM to 1 mM, did not inhibit the binding of [³H]-(–)-baclofen to GABA_B binding sites in rat brain membranes.

Introduction

The discovery of bicuculline as an antagonist of certain inhibitory actions of 4-aminobutanoic acid (GABA) on central neurones provided vital pharmacological evidence for the role of GABA as an inhibitory neurotransmitter in the CNS (Curtis *et al.*, 1970). Mammalian GABA receptors are currently classified into at least two pharmacological classes: GABA_A receptors are antagonized by bicuculline and insensitive to baclofen, whereas GABA_B receptors are activated by baclofen, antagonized by phaclofen and insensitive to bicuculline (Hill & Bowery, 1981; Johnston, 1986; Kerr *et al.*, 1987). Bicuculline is a phthalide isoquinoline alkaloid first isolated from *Dicentra cucullaria* (Manske, 1932) and subsequently from a variety of species of *Corydalis*, *Dicentra* and *Adlumia* (Kametani, 1969). Its convulsant action was reported by Welch & Henderson (1934). Many isoquinoline alkaloids produce convulsions on systemic administration to mammals, but GABA antagonism appears to be restricted to the phthalide isoquinoline alkaloids that have the 1S,9R configuration, i.e. bicuculline and corlumine (Figure 1, Curtis & Johnston, 1974). Thus (–)-bicuculline (1R,9S), (–)-hydrastine (1R,9S), (–)-adlumine (1R,9R) and (+)-adlumine (1S,9S) are inactive as GABA antagonists (Curtis & Johnston, 1974).

Bicuculline is more potent than the closely structurally related corlumine (Figure 1) as a convulsant and as a selective GABA antagonist (Rice, 1938; Johnston *et al.*, 1972). The present study concerns (+)-hydrastine, a 1S,9R, configured phthalide isoquinoline alkaloid structurally related to bicuculline and corlumine, which has been isolated from *Corydalis stricta* (Fang *et al.*, 1981) and is the enantiomer of the commercially available (–)-hydrastine (Figure 1). (+)-Hydrastine appears to be more potent than bicuculline in 4 test systems using 3 animal species.

Methods

Convulsant potencies

Convulsant potencies were assessed on injection of aqueous solutions of the alkaloids as hydrochlorides into the tail veins of male mice weighing between 18 and 25 g. The CD₅₀ value was defined as the dose causing convulsive seizures in 50% of mice within 1 min of injection.

Figure 1 Structures of the phthalide isoquinoline alkaloids bicuculline, corlumine, (+)-hydrastine and (–)-hydrastine, showing the numbering system and indicating the absolute configurations about carbons 1 and 9.

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Guinea-pig isolated ileum preparation

Guinea-pigs of either sex, weighing between 200–400 g, were stunned by a blow on the head, segments of the distal ileum 3–4 cm in length were quickly removed, emptied of their contents and mounted vertically in a 20 ml organ bath containing modified Krebs solution of following composition (mM): Na^+ 151.0, K^+ 4.6, Mg^{2+} 0.6, Ca^{2+} 2.8, Cl^- 134.9, HCO_3^- 24.9, H_2PO_4^- 1.3, SO_4^{2-} 0.6, glucose 7.7 (pH 7.4 at 37°C). The Krebs solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2 . The tissues were initially placed under a resting tension of 1 g and were allowed to equilibrate for 60 min in the bath. Isometric contractions of the longitudinal muscle were measured with a Grass FT03 force transducer and recorded on a Grass polygraph. Antagonists were added at least 2 min before agonists were tested at 8–15 min intervals, depending on the recovery of the tissue responses to base line level. Added drug volumes never exceeded 2% of the bath volume (Krantis & Kerr, 1981; Ong *et al.*, 1988).

Ligand binding studies — [^3H]-muscimol, [^3H]-diazepam, and [^3H]-(-)-baclofen

Whole rat brain was homogenized in 1:10 (w/v) volumes of cold 0.32 M sucrose. The homogenate was centrifuged at 1000 g for 10 min. The sucrose supernatant was centrifuged at 12,000 g for 20 min to yield a crude mitochondrial pellet which was subsequently processed in different ways for the different ligand binding assays.

[^3H]-diazepam binding was studied by a filtration assay essentially as described by Skerritt *et al.* (1982a): the well-washed crude mitochondrial pellet was incubated for 20 min at 4°C in 1 ml containing 530 μg membrane protein, 50 mM Tris HCl buffer, 560 pM [^3H]-diazepam and test compounds. Nonspecific binding was determined by the addition of 100 μM unlabelled diazepam.

[^3H]-muscimol binding was studied in a similar manner using membranes extracted with 0.05% Triton X-100 Tris citrate buffer at 37°C for 30 min. After thorough washing the Triton treated membranes were incubated for 60 min at 4°C in a volume of 1 ml containing 690 μg membrane protein, 50 mM Tris HCl buffer (pH 7.4), 1.47 nM [^3H]-muscimol and test compounds. Nonspecific binding was determined by the addition of 100 μM unlabelled GABA.

[^3H]-(-)-baclofen binding was studied in a centrifugation assay as described by Drew *et al.* (1984).

Protein for all of the receptor binding assays was measured by the method of Lowry *et al.* (1951).

Materials

(+)-Hydrastine, isolated from *Corydalis stricta*, was the gift of Professor Fang Qi-Cheng in the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing. Bicuculline methochloride and phaclofen were synthesized in the Department of Pharmacology at the University of Sydney by Ms Christine Apostopoulos and Dr Ken Mewett respectively. Other substances were obtained from the following sources: (-)-hydrastine, bicuculline, GABA, acetylcholine (Sigma), (-)-baclofen (Ciba Geigy), diazepam (Roche). Radioligands: [^3H]-muscimol (New England Nuclear), [^3H]-diazepam (Amersham International), and [^3H]-(-)-baclofen (New England Nuclear). (+)-Hydrastine, bicuculline, (-)-hydrastine were dissolved in 1 N HCl and neutralised with NaOH to pH 3–4.

Data analyses

Binding data were analysed by the computer programmes EBDA and LIGAND (McPherson, 1983) to obtain K_d (apparent dissociation constant), B_{max} (maximal number of binding sites) and IC_{50} (concentration of inhibitor producing 50% inhibition) values. pA_2 values (a measure of the affinity of

a competitive antagonist for its receptor) were calculated from Clark plots (see Krantis & Kerr, 1981) and CD_{50} values (dose producing convulsions in 50% of animals) from log-probit analyses as described by Tallarida & Murray (1981). Values are expressed as means $\pm$ standard error (s.e.).

Results

Convulsant potencies

Bicuculline and (+)-hydrastine produced similar convulsions in mice on intravenous injection. (+)-Hydrastine was more potent than bicuculline on the basis of relative CD_{50} values: $0.16 \pm 0.01 \text{ mg kg}^{-1}$ and $0.32 \pm 0.01 \text{ mg kg}^{-1}$ respectively (means $\pm$ s.e., $n = 10$). The action of (+)-hydrastine was stereoselective in that (-)-hydrastine was some 180 times less potent ($\text{CD}_{50} 29.8 \text{ mg kg}^{-1}$) than (+)-hydrastine.

Guinea-pig ileal responses to GABA and acetylcholine

When GABA was applied to the guinea-pig isolated ileum, it produced either contractions or biphasic responses (transient contractions followed immediately by relaxation). The GABA-induced contractions, which are known to be mediated by bicuculline-sensitive GABA_A receptors, were antagonized in a dose-dependent manner by (+)-hydrastine, which had no effect upon tissue activity when added to the bath alone. (+)-Hydrastine at doses of 1 and 5 μM elicited a parallel shift to the right in the dose-response curve for GABA-induced contractions (Figure 2). Clark analysis indicated the pA_2 value for (+)-hydrastine was 6.5 ± 0.2 (0.31 μM) while the pA_2 value for bicuculline was 6.12 ± 0.14 (0.75 μM) (Krantis & Kerr, 1981). The pA_2 values showed that (+)-hydrastine is 2.4 times stronger than bicuculline in inhibiting GABA-induced contractile responses in guinea-pig ileum.

(+)-Hydrastine at 1 μM did not show any effect on acetylcholine-induced contractile responses in guinea-pig ileum or on relaxations mediated by GABA_B receptors.

Muscimol binding to high affinity GABA_A binding sites on rat brain membranes

Equilibrium binding assays revealed that (+)-hydrastine and bicuculline inhibited [^3H]-muscimol binding to rat brain membranes, whereas (-)-hydrastine was inactive (Figure 3). The following IC_{50} values were determined by computer analyses: (+)-hydrastine $2.37 \pm 0.45 \mu\text{M}$; bicuculline $19.7 \pm 5.2 \mu\text{M}$. Scatchard analysis of the results of [^3H]-muscimol binding studies indicated binding to a single population of high affinity sites with a K_d of $2.91 \pm 0.86 \text{ nM}$ and a B_{max} of $1.09 \pm 0.18 \text{ nmol mg}^{-1}$ protein.

Figure 2 Log dose-response curves of the effects of (+)-hydrastine on GABA-induced contractile responses in the guinea-pig isolated ileum: (●) control response; (○) 1 μM (+)-hydrastine; (□) 5 μM (+)-hydrastine.

Figure 3 Displacement of [^3H]-muscimol binding to rat brain membranes by (+)-hydrastine (■—■), (–)-hydrastine (▲–▲) and bicuculline (●---●).

GABA-activated diazepam binding to rat brain membranes

GABA at concentrations of 0.1 and 0.2 μM showed no activating effect on [^3H]-diazepam binding, but at higher concentrations of 1 μM , especially of 2 μM and 10 μM , GABA had a large activating effect on [^3H]-diazepam binding (Figure 4). This action of GABA is known to be mediated via bicuculline-sensitive low affinity GABA receptors. (+)-Hydrastine at a concentration of 10 μM had a strong inhibitory effect on GABA-activated [^3H]-diazepam binding. Bicuculline at the same concentration as (+)-hydrastine exhibited a weaker effect on GABA-activated [^3H]-diazepam binding. The respective IC_{50} values were (+)-hydrastine 0.4 μM , and bicuculline 2.3 μM , indicating that in this action (+)-hydrastine was some 6 times more potent than bicuculline.

(–)-Baclofen binding to rat brain membranes

(+)-Hydrastine, 10 nM to 1 mM, did not inhibit the binding of [^3H](–)-baclofen to GABA $_B$ binding sites in rat brain membranes.

Figure 4 Effects of 10 μM (+)-hydrastine and bicuculline on GABA-stimulated binding of [^3H]-diazepam binding to rat brain membranes at various concentrations of GABA: open columns, control; stippled columns, bicuculline; hatched columns, (+)-hydrastine.

Stability of (+)-hydrastine hydrochloride

Olsen *et al.* (1975) found, when they studied several types of assay, that aqueous solutions of bicuculline salts stored at neutral pH values for several hours were less active than fresh solutions due to opening of the lactone ring to give bicucine, which is inactive as a GABA antagonist. A fresh Tris-HCl solution of (+)-hydrastine hydrochloride at pH 7.4 gave an IC_{50} value of 2.4 μM in the muscimol binding assay. The same solution after 3 months storage at 4°C gave IC_{50} value of 5.3 μM in the muscimol binding assay.

Discussion

(+)-Hydrastine is clearly a more potent competitive antagonist at GABA $_A$ receptors than bicuculline. The structural difference between the two alkaloids is that (+)-hydrastine has methoxy substitutions at carbons 3' and 4' whereas bicuculline has a methylenedioxy bridge at these carbons. Binding studies suggest that bicuculline and GABA are able to bind to some common binding sites on the GABA $_A$ receptor complex, since bicuculline is a competitive inhibitor of GABA binding and GABA is a competitive antagonist of bicuculline binding (Andrews & Johnston, 1979). The common binding sites are considered to be the N2-C1-C9-O10-C11-O12 atoms in bicuculline which match the N-C-C-C-C-O sequence of atoms in GABA (Curtis *et al.*, 1970; Andrews & Johnston, 1979). The present studies indicate that the substituents on C4' and C3' on the phthalide system in bicuculline and (+)-hydrastine are important to the GABA antagonist actions of these alkaloids.

GABA binds to 2 kinetically and pharmacologically distinct classes of bicuculline-sensitive GABA $_A$ binding sites on rat brain membranes, with the low affinity binding site being that linked to benzodiazepine receptors (Skerritt *et al.*, 1982b; Johnston, 1986; Allan *et al.*, 1986). The present study indicates that both bicuculline and (+)-hydrastine are more potent antagonists of the low affinity GABA $_A$ binding sites than of the high affinity sites.

Since the discovery of bicuculline as a selective GABA antagonist (Curtis *et al.*, 1970), a number of other competitive GABA antagonists have been described. These include the amidine steroid analogue RU5135 (Hunt & Clements-Jewery, 1981), pirtazepin (Gähwiler *et al.*, 1984), the pyridazinyl-GABA derivative SR95531 (Heaulme *et al.*, 1986) and the alkaloid securinine (Beutler *et al.*, 1985). Studies on the ability of these various classes of GABA $_A$ antagonists to reverse the inhibitory effects of GABA on the binding of [^3S]-TBPS (*t*-butylbutyrophosphorothionate) to rat brain membranes suggest that the rank order of potency is RU5135 > pirtazepin > SR95531 > bicuculline > securinine which is similar to that found in studies on antagonism of GABA-induced inhibition of neuronal activity (Squires & Saederup, 1987). The present studies would place (+)-hydrastine as approximately equipotent with SR95531 (2-(carboxy-3'-propyl)-3-amino-6-*p*-methoxyphenylpyridazinium chloride). (+)-Hydrastine may offer some advantages over bicuculline in terms of solubility and stability in aqueous solution. The structures of these GABA $_A$ receptor antagonists may aid in the design of further antagonists which may show some selectivity for possible subclasses of GABA $_A$ receptor sites as was proposed by Andrews & Johnston (1979) on the basis of studies of conformationally restricted GABA $_A$ agonists and has been supported by recent cDNA studies indicating a molecular heterogeneity of GABA $_A$ receptor subunit proteins (Ymer *et al.*, 1989).

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