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Identification of 5-HT_3 recognition sites in the ferret area postrema

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Abstract—The 5-HT_3 receptor antagonist [^{3}H]zacopride was used to identify 5-HT_3 recognition sites in the ferret area postrema. Specific binding was determined by the inclusion of the 5-HT_3 receptor antagonist BRL 43694 in the incubation media, and was shown to be much higher in the area postrema than in other brain regions. The increased binding in the area postrema may reflect either a greater number of binding sites, a higher affinity for such sites, or both. The results indicate that 5-HT_3 recognition sites are present within the area postrema and may afford an antiemetic site of action for zacopride and other 5-HT_3 receptor antagonists.

5-Hydroxytryptamine (5-HT) receptor antagonists of the 5-HT_3 subtype have been shown in both detailed laboratory studies and preliminary clinical investigations to inhibit the emesis caused by cancer chemotherapy (Alphin et al 1986; Andrews et al 1986; Miner & Sanger 1986; Miner et al 1986; Costall et al 1986; 1987; Cunningham et al 1987; Leibundgut & Lancranjan 1987). The data are indicative that emesis may be related to an enhanced 5-HT function and nausea and vomiting have been shown to occur in patients with the carcinoid syndrome and those administered 5-hydroxytryptophan or 5-HT reuptake inhibitors (Engelmann et al 1967; Greenwood et al 1975; Saletw et al 1986; Lader et al 1986).

The site of interaction between 5-HT and the 5-HT_3 receptor antagonists to moderate emesis has not been established and may involve a peripheral and/or central effect. 5-HT_3 receptors are located on the vagus nerve and abdominal vagal afferents from the gut are known to be important in evoking emesis following gastric irritation (Borison & Wang 1953). Miner & Sanger (1986) hypothesized that an agent such as cisplatin may cause a release of 5-HT which stimulates the vagal 5-HT_3 receptors, and Gunning et al (1987) have reported that cisplatin elevates 5-HT/5-hydroxyindoleacetic acid levels in the intestine. The ability of abdominal vagotomy to antagonize the initial phase of retching and vomiting in the ferret caused by whole body irradiation may also be indicative of a peripheral site of action (Andrews & Hawthorn 1987). However, in the latter study, vagotomy failed to antagonize a later phase of emesis, indicating a further site of emetic action. This may be central since emesis follows the intracerebroventricular injection of cisplatin into the cat brain, a response which is antagonized by the injection of the 5-HT_3 receptor antagonist zacopride into the same system (Smith et al 1988). Whilst within the brain the locus of emetic and antiemetic action is uncertain, the area postrema is considered to possess specialized chemoreceptive mechanisms and to be involved in a wide variety of functions including behavioural, metabolic, cardiovascular, endocrine and autonomic effects (see reviews by Borison 1984; Borison et al 1984; Barnes et al 1984 and also Ossenkopp et al 1986). In particular, the area postrema is considered a major component in the emetic reflex and contains high concentrations of 5-HT (see reviews by Borison 1984; Borison et al 1984; Pickel & Armstrong 1984; also Bhargava et al 1981; Carpenter et al 1983; Rabin et al 1986; Beleslin & Krstic 1987; Beleslin & Strbac 1987; Barnes et al 1988a). Furthermore, agents such as *p*-chlorophenylalanine and fenfluramine which deplete 5-HT within the area postrema will antagonize cisplatin-induced emesis in the ferret (Barnes et al 1988a).

It was hypothesized that a raised 5-HT function within the area postrema may cause emesis, the effects being mediated via 5-HT_3 receptors to afford a site of action for the 5-HT_3 receptor antagonists. The recent introduction of ligands for the 5-HT_3 receptors, [^{3}H]ICS 205-930 (Hoyer & Neijt 1987), [^{3}H]GR65630 (Kilpatrick et al 1987) and [^{3}H]zacopride (Barnes et al 1988b) has provided important tools to identify 5-HT_3 receptors. In the present study we use [^{3}H]zacopride to investigate the presence of 5-HT_3 recognition sites in the area postrema of the ferret brain.

Methods

Albino or Fitch female ferrets (1·0–1·5 kg) bred at the University of Bradford were anaesthetized with halothane and exsanguinated. The brains were rapidly removed and tissue from the entorhinal cortex (350 mg), frontal cortex (120 mg) and caudate nucleus (80 mg) dissected out on ice. Tissue was also taken from the cerebellum (230 mg) which was removed to expose the area postrema, lying as a bilateral wing-shaped protuberance in the caudal margins of the floor of the IVth ventricle. The area postrema, viewed through a binocular microscope was removed using a pair of fine forceps, care being taken to exclude adjacent tissue; the area postrema weighed approximately 2 mg.

Fresh tissues from four animals were pooled for each experiment and homogenized (Polytron, setting 7 for 10 s) in 20 volumes of 50 mM Hepes buffer containing all the constituents of Krebs (NaCl 118·0, KCl 4·75, KH_2PO_4 1·2, $MgSO_4$ 1·2, $CaCl_2$ 2·5, $NaHCO_3$ 25·0, glucose 11·0) with a final pH of 7·4. The homogenate was centrifuged at 48 000 × *g* for 10 min at 4°C and the pellet resuspended and again centrifuged before final resuspension in the Hepes/Krebs buffer.

50 μL of displacing drug (BRL 43694, 10 μM) or buffer (Hepes/Krebs) was added to the assay tubes followed by 50 μL [^{3}H]zacopride (54·9 Ci mmol^{-1}) in Hepes/Krebs; 500 μL of the brain tissue homogenate was added to initiate binding. The assay tubes were incubated for 20 min at 37°C and the incubation terminated by rapid filtration through pre-wet Skatron glass fibre filters which were immediately washed with 3·5 mL of ice cold Hepes/Krebs buffer. Each assay was completed within 30 min. The filter discs were placed in 4 mL of Instagel scintillant, left for 6 h for dark adaptation and radioactivity assayed by liquid scintillation counting. Assays were carried out in replicates of at least 3 and results are the means ± s.e.m. of 4 separate experiments. Protein estimation was performed using the Bio-Rad Coomassie blue method using bovine serum albumin as standard (Bradford 1976); assay tubes using tissue from the entorhinal cortex, frontal cortex, cerebellum and caudate nucleus contained 0·23–0·4 mg protein and from the area postrema 0·05 mg protein.

Drugs. [^{3}H]Zacopride.HCl (NEN), zacopride (4-amino-*N*-(1-azabicyclo[2.2.2.]oct-3yl)-5-chloro-2-methoxybenzamide(E)-2-butenedioate) (A. H. Robins) and BRL 43694 (endo-*N*-(9-

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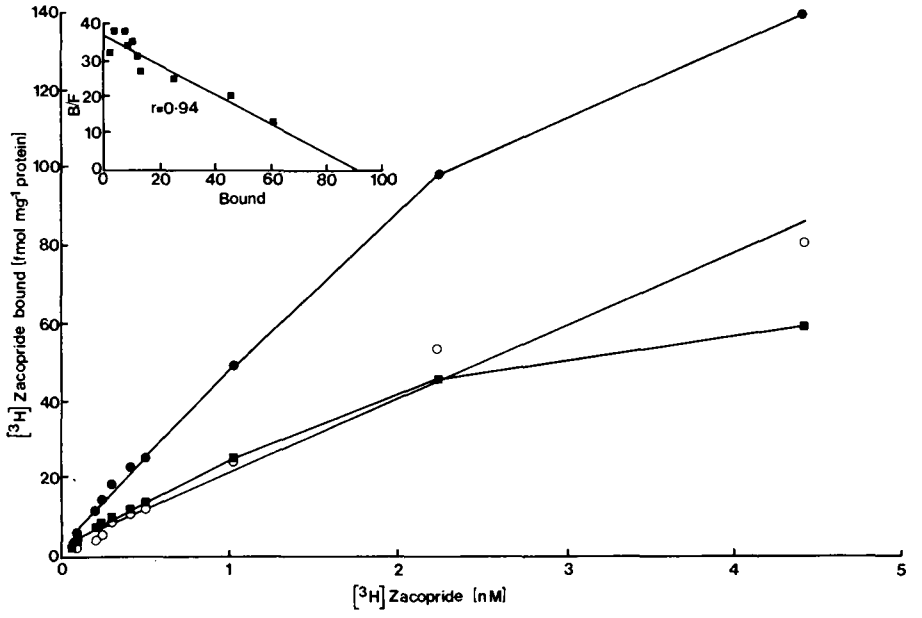

FIG. 1. The binding of [^{3}H]zacopride (0·07–4·41 nM) in the absence (total binding, ●—●) and presence (non-specific binding, ○—○) of BRL 43694 (10 μM) to homogenates of ferret entorhinal cortex. The specific binding (■—■) is subject (inset) to a Scatchard transformation ($K_D = 2{\cdot}5$ nM, $B_{max} = 92$ fmol (mg protein)$^{-1}$). Typical results are presented from a single experiment where each value is the mean of 3 determinations.

methyl-9-azabicyclo[3,3,1]non-3-yl)-1-methyl-indazole-3-carboxamide) (Glaxo) solutions were prepared immediately before use in Hepes/Krebs buffer.

Results and discussion

Initial experiments used homogenates prepared from the entorhinal cortex to allow a comparison of the binding of [^{3}H]zacopride in ferret brain to that previously obtained in the rat (Barnes et al 1988b). The amount of entorhinal tissue was sufficient to allow the use of a range of concentrations of [^{3}H]zacopride (0·07–4·41 nM) and saturable binding was determined by the use of BRL 43694 (10 μM), selected as a potent and selective 5-HT$_3$ receptor antagonist and chemically dissimilar to zacopride (Fake et al 1987) (Fig. 1). From three experiments, Scatchard transformation of the data revealed a single high affinity site ($K_D = 2{\cdot}42 \pm 0{\cdot}17$ nM, $B_{max} = 67{\cdot}0 \pm 15{\cdot}2$ fmol (mg protein)$^{-1}$; mean ± s.e.m.).

The very small amounts of tissue obtained from the area postrema (2 mg per animal) precluded attempts to determine K_D and B_{max} values for [^{3}H]zacopride, or to assess the selectivity and specificity of the binding; it is calculated that such studies would require over one thousand animals. Experiments were restricted to the use of a single concentration of 0·5 nM [^{3}H]zacopride, the binding to 5-HT$_3$ recognition sites again being determined by the use of BRL 43694 (10 μM). Comparison of the binding of [^{3}H]zacopride to other brain regions was assessed using the same procedure. BRL 43694 displaced 94% of the total binding in the area postrema, 46–49% of the total binding in the entorhinal cortex and caudate nucleus and 30–31% of that in the frontal cortex and cerebellum. Non-specific binding in the area postrema, entorhinal cortex, caudate nucleus, frontal cortex and cerebellum was respectively 638 ± 361, 1283 ± 292, 1641 ± 291, 1708 ± 479 and 776 ± 116 dpm/mg protein. Low specific binding was detected in the cerebellum ($2{\cdot}8 \pm 0{\cdot}4$ fmol (mg protein)$^{-1}$) with increasing amounts in the frontal cortex ($5{\cdot}8 \pm 1{\cdot}2$ fmol (mg protein)$^{-1}$) the entorhinal cortex ($10{\cdot}1 \pm 1{\cdot}2$ fmol (mg protein)$^{-1}$) and the caudate nucleus ($12{\cdot}0 \pm 2{\cdot}1$ fmol (mg protein)$^{-1}$). However, such levels were modest compared with the high level of binding in the area postrema ($82{\cdot}5 \pm 11{\cdot}4$ fmol (mg protein)$^{-1}$) (Fig. 2).

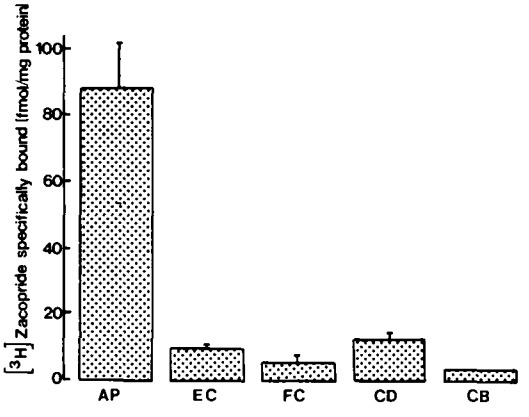

FIG. 2. The specific binding of [^{3}H]zacopride (0·5 nM, determined from its displacement by BRL 43694, 10 μM) from the area postrema (AP), entorhinal cortex (EC), frontal cortex (FC), caudate nucleus (CD) and cerebellum (CB). Each value is the mean ± s.e.m. determined from 3 experiments.

Thus [^{3}H]zacopride has been used to identify 5-HT$_3$ recognition sites in the entorhinal cortex of rat brain (Barnes et al 1988b) and in the present studies 5-HT$_3$ binding sites were also detected in the entorhinal cortex of the ferret brain. In both species [^{3}H]zacopride identified a single high affinity site and a comparable number of binding sites. The important finding of the present work is the identification of 5-HT$_3$ recognition sites in the area postrema. Due to the small size of the area postrema, the ligand was used at only a single concentration, selected as causing a binding to approximately 50% of the sites in the entorhinal cortex. Although this must necessarily limit the interpretation of the findings, it is noteworthy that the binding was eight fold greater in the area postrema than in the entorhinal cortex, frontal cortex and caudate nucleus. Whilst the increased binding may reflect either or both a greater number of binding

sites or a higher affinity for the 5-HT_3 recognition sites, the present data would confirm the hypothesis that 5-HT_3 binding sites are to be found within the area postrema. Since the area postrema also contains high levels of 5-HT and 5-hydroxyindoleacetic acid (Amin et al 1954; Armstrong et al 1981; Dew et al 1973; Barnes et al 1988b), [^{3}H]5-HT uptake sites (Pickel & Armstrong 1984) and perhaps nerve cells of the 5-HT type (Fuxe & Owman 1965), it is concluded that 5-HT systems within the circumventricular organ may be involved in the emetic reflex and that 5-HT_3 receptors may afford a site of antiemetic action for zacopride and other 5-HT_3 receptor antagonists.

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