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Effects of *p*-chlorophenylalanine on the sensitivity of rat intestine to agonists and on intestinal 5-hydroxytryptamine levels during *Nippostrongylus brasiliensis* infection

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1 Infection of rats with the nematode *N. brasiliensis* caused non-specific increases in maximum response of isolated intestine to acetylcholine and 5-hydroxytryptamine (5-HT), and a specific subsensitivity to 5-HT.

2 Intestinal levels of 5-HT, measured fluorimetrically, increased approximately 2 fold during infection.

3 Treatment of infected rats with parachlorophenylalanine (PCPA) depleted the gut of 5-HT, and prevented the specific subsensitivity to the amine but not the increases in maximum response.

4 Depletion of intestinal 5-HT did not prevent the immune expulsion of the parasites.

5 It is concluded that the specific subsensitivity of the gut is due to the elevated levels of 5-HT during infection, but that the increased maximum responses are due to some other factor. Further, the lack of effect of PCPA on parasite rejection casts doubt on the proposed role of 5-HT in this process.

Introduction

During infection with the nematode *Nippostrongylus brasiliensis* changes occur in the responsiveness of rat intestinal smooth muscle to agonists. First, there occur non-specific increases in the maximum responses of isolated small intestine to acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) (Farmer, 1981; Farmer *et al.*, 1983). Secondly, the gut exhibits specific subsensitivity to 5-HT, the pD_2 value decreasing during the infection. Moreover, although control maximum responses to ACh and 5-HT are the same, the maximum response to 5-HT is not increased to the same degree as that to ACh during infection (Farmer, 1981).

This infection has been extensively employed as a model in the study of immunity to helminth parasites (reviewed by Ogilvie & Love, 1974). After about day 10 of infection, *N. brasiliensis* is expelled from the rat intestine by an incompletely understood process involving antibodies and immune cells (Ogilvie & Love, 1974), and perhaps also by local intestinal changes (Barth *et al.*, 1966; Murray *et al.*, 1971a). Intestinal 5-HT levels are markedly elevated during infection (Murray *et al.*, 1971a; Wingren *et al.*, 1983) and it is a widely held, though controversial hypothesis that mucosal mast cell degranulation and subsequent 5-HT release are important during the expulsion of the parasites (Boreham & Wright, 1976).

Prolonged exposure to 5-HT renders rat intestine unresponsive to the contractile activity of this amine (Gillan & Pollock, 1980), and it is well known that intestinal tachyphylaxis to 5-HT readily develops (Schild, 1973). We considered therefore, the possibility that the specific subsensitivity of infected intestine to 5-HT may be due to the abnormal levels of this substance in the infected gut. The primary objective of the present study therefore, was to determine the effect of treating *Nippostrongylus*-infected rats with *p*-chlorophenylalanine (PCPA), which depletes tissue 5-HT (Koe & Weissman, 1966), on intestinal sensitivity. Secondly, it was of interest to determine the effect of PCPA treatment on the number of parasites remaining in the intestine of infected rats.

A preliminary account of some of the results described in this paper was presented to the British Pharmacological Society (Farmer & Laniyonu, 1983).

Methods

Parasitological techniques

N. brasiliensis was maintained in a colony of rats by the method of Jennings *et al.* (1963). Male PVG rats (150–200 g) were lightly anaesthetized with halothane and injected (s.c.) with a suspension of *N. brasiliensis* larvae (1 ml 0.9% w/v NaCl solution (saline) containing approximately 5000 larvae per rat). The technique of worm recovery from the small intestine was that described by Mulligan *et al.*, (1965). Total worm counts were carried out on day 14 of infection.

Drug pretreatment

On day 3 post-infection rats were injected (i.p.) with 200 mg kg^{-1} PCPA, and on days 5, 7, 9, 11 and 13 with 100 mg kg^{-1} PCPA. Uninfected rats also received the drug, and groups of uninfected and infected rats were injected with saline, which was the vehicle for PCPA, on corresponding days.

Recording of intestinal responses

On day 14 of the experiment, rats were killed by a blow to the head, and the small intestine excised. A segment of 5 cm in length was removed from the region approximately 20 cm distal to the pylorus, where the parasites are normally localized. Each tissue was dissected free of mesentery and bisected, half being immediately weighed and frozen in liquid N_2 before estimation of 5-HT content (see later). The other half of the intestinal segment was suspended in a 40 ml organ bath containing Krebs-bicarbonate solution (composition mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.0, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, glucose 11.1), maintained at 37°C and gassed continuously with 95% O_2 and 5% CO_2. Before the application of tension each tissue was incubated in Krebs solution for 1 h, with washes every 15 min, before exposure to drugs.

The initial tension on each tissue was adjusted to 5g and after about 15 min, when the tissue had stretched to give a resting tension of 2–3g, responses to ACh and 5-HT were obtained. Tension changes were recorded isometrically and displayed on a Linseis LS4 two-channel pen recorder. The agonists, dissolved freshly each day in Krebs solution, were added to the bath in volumes not exceeding 0.4 ml and removed by washing. Responses were measured as the peak rise in tension to any agonist concentration, and subsequent doses were not added until the tissue had returned to its resting tension following removal of the drug. Not less than 10 min elapsed between successive doses of either agonist.

Dose-response curves were constructed and pD_2 values were calculated and maximum responses determined. The mean pD_2 values and maximum responses in the different groups were compared statistically by a multiple range test (Duncan, 1955) and probability values of less than 0.05 were considered significant.

Assay for 5-HT

The 5-HT content of small intestine was assayed fluorimetrically using *o*-phthalaldehyde (OPT), which forms a fluorescent complex with 5-HT (Curzon *et al.*, 1981).

Each segment of small intestine was homogenized in 5 ml of acidified butanol (850 μl HCl in 1 l butanol, 0°C) in a glass tube by a motor-driven pestle. The volume of each homogenate was adjusted to 25 ml and these homogenates were centrifuged (3000 *g*, 10 min, 4°C). The 5-HT content of each supernatant was determined by a slightly modified version of the method of Curzon & Green (1970). This modification was necessary because preliminary studies had shown that tissue blanks, containing tissue extract but no OPT gave fluorescence values twice those of reagent blanks, which contained OPT but no tissue extract. In the present study the addition of potassium ferricyanide (200 μg in 20 μl) to each tissue blank reduced the fluorescence produced to the level obtained in the reagent blanks.

A 2 ml aliquot of each supernatant was transferred to a tube containing 5 ml of *n*-heptane and 600 μl of an acid solution of cysteine (0.1% w/v in 0.1 M HCl). the contents of each tube were mixed for 2 min and centrifuged (3000 *g*, 5 min, 4°C). From each tube the upper, organic phase together with the disc at the organic/aqueous interphase were removed by aspiration and discarded. A 200 μl aliquot of the lower, aqueous phase was incubated for 15 min at 77°C with 20 μl 1% cysteine and 800 μl conc. HCl containing 0.004% OPT. The fluorescence that developed was measured, when the tubes had cooled to room temperature, in an Aminco-Bowman spectrophotofluorimeter at an activation wavelength of 370 nm and an emission wavelength of 480 nm.

Standards were prepared by dissolving 5-HT in distilled water so that 200 μl volumes containing between 50 and 200 ng 5-HT could be added to tissue extracts to serve as internal standards, which were carried out through the entire assay procedure. Recoveries obtained in these internal standards were 80–100%.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), halothane (Fluothane, I.C.I.), 5-

hydroxytryptamine creatinine sulphate (Sigma), *p*-chlorophenylalanine methyl ester (Sigma).

Results

Rats tolerated PCPA treatment or infection well. All survived, exhibiting no obvious abnormal symptoms such as weight loss, diarrhoea or behavioural disturbances, although the latter were not studied specifically. In contrast, four of the ten infected rats treated with PCPA died on days 6 or 7 post-infection. Conversely, the six surviving PCPA-treated, infected animals at no time exhibited any adverse symptoms during drug treatment.

Intestinal responses during infection

On day 14 of infection the maximum response of isolated small intestine to ACh was significantly increased ($P<0.001$) to more than 9 g as compared to the control maximum response of less than 3 g (Figure 1). Infection with *N. brasiliensis* did not affect intestinal sensitivity to ACh. This was reflected in the fact that the control pD_2 value was not significantly different from the pD_2 value in infected gut (Table 1).

Responses of infected intestine to 5-HT were varied and tachyphylaxis readily developed. There was considerable variation in the maximum responses of infected gut to 5-HT, ranging from as little as 1.6 g to as much as 9.2 g (mean, 4.72 ± 0.94 g). This mean maximum response, although apparently greater than control (2.75 ± 0.17 g, Figure 1), was not significantly so. Frequently, the dose-response curve to 5-HT in infected gut was of a complex nature in that a higher concentration of 5-HT often produced a smaller response than the previously applied lower concentration (Figure 2). Upon application of even higher concentrations of agonist the response usually

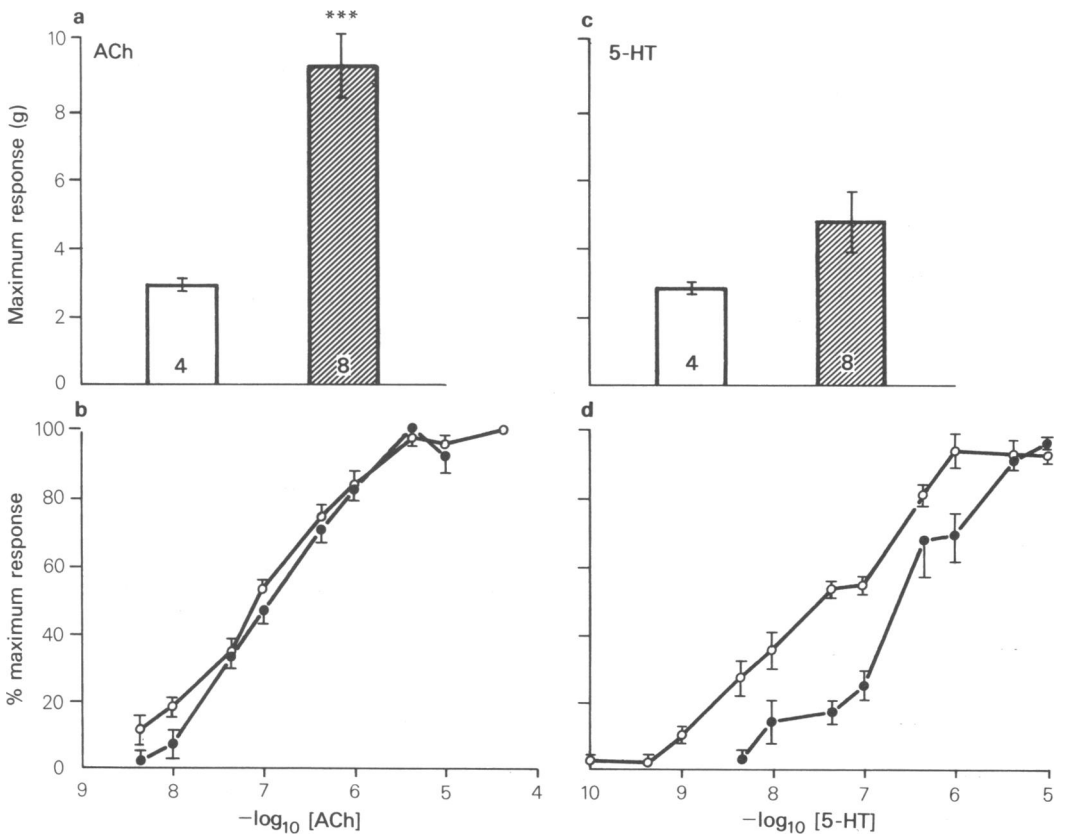

Figure 1 Maximum responses (a and c) and concentration-response curves (b and d) to acetylcholine (ACh, a and b) and 5-hydroxytryptamine (5-HT, c and d) of isolated small intestine from control rats (○ and open columns) and from rats at day 14 post-infection with *N. brasiliensis* (● and shaded columns). Data are presented as mean with vertical lines showing s.e.mean. The figures in the columns represent the number of observations; ***$P<0.001$.

Table 1 Effects of *p*-chlorophenylalanine (PCPA) pretreatment and/or *N. brasiliensis* infection on pD_2 values and maximum responses of rat small intestine to agonists

	Acetylcholine			*5-Hydroxytryptamine*	
Treatment	*Maximum* (g)	pD_2	n	*Maximum* (g)	pD_2
Control	2.85 ± 0.14^a	6.99 ± 0.07^a	4	2.75 ± 0.17^a	7.45 ± 0.03^a
PCPA	3.34 ± 0.12^a	6.79 ± 0.11^a	7	2.99 ± 0.08^a	7.83 ± 0.04^b
Infected	8.14 ± 0.30^b	6.95 ± 0.07^a	8	4.72 ± 0.94^{ab}	6.70 ± 0.10^c
Infected and PCPA	9.35 ± 2.27^b	6.77 ± 0.05^a	6	6.78 ± 0.84^b	7.13 ± 0.11^a

Values are given as mean ± s.e.mean; n = no. of observations. Within columns, values bearing different superscripts are significantly different ($P < 0.05$).

increased again with succesively greater concentrations until the maximum response was achieved. Sometimes however, the response at the peak of the dose-response curve 'foot' was the maximum tension the infected tissue developed to 5-HT, even though the maximum response to ACh was much larger.

Infected intestine was subsensitive to 5-HT, the dose-response curve being shifted to the right (Figure 1). The pD_2 value for 5-HT in infected preparations was significantly decreased ($P < 0.001$) as compared with control (Table 1), infected gut being of the order of six times less sensitive to 5-HT than uninfected intestine.

The effect of PCPA on intestinal sensitivity

PCPA treatment did not alter the sensitivity or the maximum response of intestine from control rats to ACh (Table 1). Neither did treatment with the drug affect the sensitivity or increased maximum response of infected gut to ACh (Table 1).

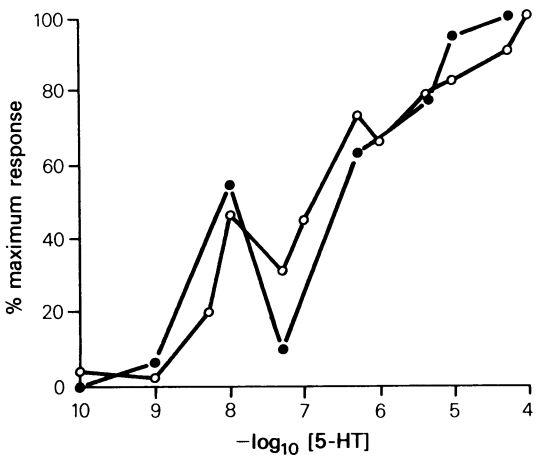

Figure 2 Two examples of the unusual dose-response curves to 5-hydroxytryptamine (5-HT) in small intestine from *N. brasiliensis*-infected rats.

Conversely, intestinal preparations from PCPA-treated rats were supersensitive to 5-HT (Table 1), the pD_2 value increasing, though the drug caused no alteration in the maximum response. On the other hand, the maximum response to 5-HT of gut from PCPA-treated infected rats was significantly greater ($P < 0.01$) than the maximum response of control gut to 5-HT (Table 1). Moreover, the responses of these tissues to 5-HT were not as variable as those of infected gut from rats which received no drug treatment. The sensitivity of intestine from PCPA-treated, infected rats to 5-HT was not significantly different from that of control intestine, since the pD_2 value was unchanged (Table 1).

Intestinal 5-HT levels

Table 2 gives the amounts of 5-HT found in intestinal tissues from the four experimental groups. The 5-HT level in infected gut was significantly greater ($P < 0.01$) than the level in controls. PCPA treatment depleted intestinal 5-HT by 73%. Further, PCPA not only prevented the increase in intestinal 5-HT during infection, but also depleted infected gut of its 5-HT content (by approximately 90%).

Table 2 Effect of *N. brasiliensis* infection and/or *p*-chlorophenylalanine (PCPA) treatment on rat intestinal 5-HT levels

Treatment	*Amount of 5-HT* ($\mu g\,g^{-1}$)	n	*No. worms per rat*
Control	2.73 ± 0.21^a	5	n.a.
PCPA	0.73 ± 0.27^b	6	n.a.
Infected	4.74 ± 0.54^c	5	130 ± 36
Infected & PCPA	0.49 ± 0.13^b	5	142 ± 58

Values expressed as mean ± s.e.mean; n = no. of observations; n.a. = not applicable. Values bearing different superscripts are significantly different ($P < 0.05$; multiple range test).

From Table 2 it is also evident that PCPA treatment of infected animals did not affect the numbers of parasites remaining in the intestine at day 14.

Discussion

The observation that infection with *N. brasiliensis* causes non-specific increases in the maximum responses of rat isolated intestine to ACh and 5-HT, and a specific subsensitivity to 5-HT confirms earlier results (Farmer, 1981; Farmer *et al.*, 1983). We have also verified previous reports (Murray *et al.*, 1971a; Wingren *et al.*, 1983) that intestinal 5-HT levels increase during this infection. Depletion of intestinal 5-HT by treating infected rats with PCPA prevented the specific subsensitivity to the amine. This subsensitivity therefore, is probably due to the abnormal levels of 5-HT in infected gut. Moreover, the marked variations in maximum response of infected gut to 5-HT (in contrast to the relatively consistent responses to ACh) and the atypical nature of dose-response curves to 5-HT are probably due to elevated intestinal levels of the amine, and may be related to the subsensitivity. This is supported by the fact that gut from PCPA-treated infected animals, unlike that from untreated infected rats, exhibited relatively consistent maximum responses to 5-HT which were significantly greater than control maximum responses and did not develop tachyphylaxis to 5-HT as readily as did tissues from infected rats which received no PCPA. However, since the increase in maximum response of infected tissue to ACh was unaffected by PCPA, it is unlikely that this phenomenon is due to elevated intestinal 5-HT.

The mechanism whereby chronic exposure of intestinal smooth muscle to 5-HT brings about subsensitivity to this agonist is not known, though the relative ease by which tachyphylaxis to 5-HT can be brought about in this tissue is well documented (Schild, 1973; Crossland, 1980; Gillan & Pollock, 1980). Since the subsensitivity is specific it may be due to some alteration in 5-HT receptors in infected gut smooth muscle. Moreover, this intestinal subsensitivity may be an expression of some process whereby the smooth muscle cells adapt to chronic overstimulation by the abnormal amounts of 5-HT in infected gut.

Small intestine from uninfected rats which had been treated with PCPA was not only depleted of its 5-HT, but exhibited a more than twofold increase in sensitivity to 5-HT. Further, like the subsensitivity, this supersensitivity was also specific for 5-HT since no change in intestinal sensitivity to ACh was evident. Postjunctional supersensitivity can be brought about by several procedures such as denervation or receptor blockade, which chronically interfere with interactions between excitatory agents and smooth muscle cells (Westfall, 1981). Because postjunctional supersensitivity, referred to by Westfall (1981) as 'non-deviation supersensitivity', is usually non-specific it has been suggested that it is not caused by an increase in either the affinity or density of receptors (Fleming, 1981). Conversely, most examples of this type of supersensitivity are due to a 'chronic decrease in the normal contact between an excitatory neurotransmitter and its effector cells' (Westfall, 1981). In the present study PCPA treatment clearly decreased intestinal 5-HT levels and probably, therefore, reduced the contact between the amine and its receptors. Thus, the supersensitivity to 5-HT is probably of the postjunctional type since sensitivity of the smooth muscle to this agonist is increased. As it is a specific supersensitivity this suggests it is also due to an alteration in 5-HT receptors. The specific supersensitivity and subsensitivity may be expressions of the same homeostatic mechanism in the smooth muscle cells which compensates for under- and over-stimulation by 5-HT (Fleming, 1981). Nevertheless, this is unlikely to be the only explanation of the changes in intestinal sensitivity. Although PCPA treatment prevented the development of intestinal subsensitivity to 5-HT during *N. brasiliensis* infection, these tissues were, rather surprisingly, not rendered supersensitive to the amine, even though PCPA depleted infected gut by 90% of its 5-HT.

An interesting observation in the present study was the fact that several of the PCPA-treated rats died during infection. The reason for this is unknown. Possibly 5-HT plays a role in protecting the animals from some pathological effects of the parasite disease, in a way similar to the beneficial effect of the amine on *E. coli* endotoxin mortality in mice (Gordon & Lipton, 1957).

It was also interesting that intestinal 5-HT depletion did not prevent the expulsion of the parasites. *N. brasiliensis* is expelled from the rat, beginning at around day 10 of infection, by an incompletely understood process involving an immune response (reviewed by Ogilvie & Love, 1974). At the time of expulsion intestinal mastocytosis occurs, with a subsequent elevation in 5-HT levels, and it has been suggested (Murray *et al.*, 1971a; Wingren *et al.*, 1983) that this temporal relationship implies a causal association between increased intestinal 5-HT and parasite rejection. Other studies using various 5-HT antagonists or compound 48/80, a mast cell degranulator, have produced conflicting results as to the role of 5-HT (and indeed histamine) in parasite expulsion (Murray *et al.*, 1971b; Keller & Ogilvie, 1972; Kelly *et al.*, 1974). Moreover, treatment of infected rats with reserpine inhibits worm expulsion (Sharp & Jarrett, 1968) and these authors attributed this to the ability of reserpine to prevent the storage

of 5-HT and histamine in mast cells. However, it has been established that rat mucosal mast cells are totally resistant to 48/80 (Befus *et al.*, 1982), and it is known that rat mast cells are hardly affected by reserpine (Carlsson, 1966). Indeed, the 5-HT content of rat colon is not reduced by reserpine (Laniyonu, unpublished observation). It has also been shown (Farmer, 1982) that treatment of infected rats with betamethasone, which inhibits both worm expulsion and the increase in intestinal maximum responses, does not prevent the subsensitivity to 5-HT (and probably therefore, the increase in gut 5-HT). These results, together with the present finding that 5-HT depletion with PCPA does not prevent worm expulsion, cast doubt on the role, if indeed there is one, of 5-HT in this process.

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