The effects of 5-HT and m-chlorophenylpiperazine (m-CPP) on the efflux of [³H]-5-HT from human perfused platelets

JANET G. CARVER, DAVID G. GRAHAME-SMITH, E. STEWART JOHNSON & ZAHRA MADGWICK
MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE

- 1 m-Chlorophenylpiperazine (m-CPP), a 5-HT_{1c}-receptor agonist, induces migraine-like headaches when taken orally by migraine sufferers. The present study was undertaken to see what effects m-CPP had on 5-HT function in platelets.
- 2 Platelets from healthy male volunteers were loaded with [³H]-5-HT and continuously perfused *in vitro* with carboxygenated Krebs solution at 37° C. After 30 min washout the effects of m-CPP, thrombin, 5-HT and ADP on the efflux of [³H]-5-HT were recorded.
- 3 m-CPP (0.5–500 µM) did not evoke an increase in the efflux of [³H]-5-HT over that occurring spontaneously whereas thrombin, unlabelled 5-HT and ADP did. The effects of 5-HT were potentiated by ADP. The results were identical whether or not the 5-HT reuptake blocker paroxetine (1 µM) was present.
- 4 m-CPP inhibited the increase in the efflux of [³H]-5-HT evoked by different concentrations of unlabelled 5-HT in the presence of ADP (2.5 µM) and displaced the 5-HT log concentration response curve to the right. A similar result was obtained with the 5-HT₂-receptor antagonist ketanserin.
- 5 We conclude that m-CPP is a 5-HT₂-receptor antagonist on human platelets, which is unlikely to account for its headache-inducing property, as many drugs effective in migraine prophylaxis have this action.

Keywords 5-HT m-CPP [³H]-5-HT efflux perfused platelets

Introduction

Fozard & Gray (1989) suggested that 5-HT_{1c}-receptor stimulation might be an important step in the pathogenesis of migraine. They based their suggestion on a number of observations, including the finding of Brewerton *et al.* (1988) that m-chlorophenylpiperazine (m-CPP), a potent 5-HT_{1c}-receptor agonist (see Hoyer, 1989), induced a migraine-like headache when taken orally in a dose of 0.5 mg kg⁻¹ by patients who were also migraine sufferers. Brown *et al.* (1991) pointed out that, as anti-migraine drugs such as ergotamine and dihydroergotamine were also 5-HT_{1c}-receptor full agonists, yet on acute use did not commonly induce migraine-like headaches, m-CPP might have triggered headache by another means, such as its reported ability to release 5-HT, as observed in hypothalamic slices *in vitro* (Pettibone & Williams, 1984).

Alternatively, the possibility remained that the headache caused by m-CPP might be associated with the release of 5-HT from platelets. Platelet 5-HT release has been said to occur in migraine (Lance, 1990).

We have therefore studied the efflux of 5-HT from

platelets preloaded with [³H]-5-HT and perfused with Krebs solution containing different concentrations of 5-HT receptor agonists, antagonists and/or uptake inhibitors. The method used was a modification of that described by Andersson & Vinge (1991).

Methods

The study protocol was approved by the Central Oxford Research Ethics Committee and written consent was obtained from all subjects.

Preparation of platelets

Venous blood (18 ml) was taken from eight healthy male volunteers, who had taken no medication for at least 2 weeks.

The blood was mixed with 3 ml acid-citrate-dextrose

(ACD) solution of the following composition: 15 g citric acid; 25 g trisodium citrate; 20 g dextrose in 1 l of distilled water.

Platelet rich plasma (PRP) was obtained by centrifugation of the blood-ACD mixture for 15 min at 180 g at 10° C. The PRP was then transferred to a separate tube and its volume measured. The blood residue was spun for 10 min at 1700 g to give platelet poor plasma (PPP). Platelets in PRP were counted in a Coulter counter (Coulter Electronics) and the count was adjusted by the addition of PPP to give a final count of 2.5×10^8 cells ml^{-1} .

The platelets were incubated with [^3H]-5-HT at 37° C ($0.1 \mu\text{M}$, 100 kBq ml^{-1}) for 30 min. The platelet suspension was then centrifuged at 1000 g for 10 min and the fluid was removed from above the platelet plug. The platelets were resuspended in 1:6 ACD-Krebs, previously bubbled with 5% CO_2 in O_2 and the platelets were counted again in the Coulter counter. Platelets (2.5×10^7) were added to a Swinnex® polypropylene 0.25 ml perfusion chamber containing a micropore filter of pore size $0.22 \mu\text{m}$, and the platelets were then continuously perfused at 0.5 ml min^{-1} with Krebs solution at 37° C, bubbled with 5% CO_2 in O_2 . Three $10 \mu\text{l}$ aliquots of incubation fluid and three samples of 2.5×10^7 platelets were counted for tritium.

During the first 30 min perfusion the cells were allowed to equilibrate and then the Krebs was changed to Krebs containing different concentrations of thrombin, 5-HT, m-CPP, ADP, or mixtures of agonists for a contact time of 4 min, after which the perfusing fluid was changed back to agonist-free Krebs solution. Perfusion was then continued for a further 36 min, at which time a second agonist (usually thrombin) treatment was administered for 4 min in order to confirm platelet viability. Perfusion was halted after a total of 90–110 min. When antagonists or uptake inhibitors were used, these were present in the Krebs solution throughout the experiment.

The perfusate was collected in scintillation vials at 2 min intervals, except during agonist dosing when 10 collections were made at intervals of 1 min.

At the end of the experiment the platelets on each filter were dissolved in 4 ml Aquasafe 500 (Zinsser) scintillation fluid.

The radioactivity in the platelets, perfusates, and incubation fluid was determined by liquid scintillation counting in a Beckman LS 6000SE counter.

Increases in the efflux of radioactivity were estimated by measuring the area under the curve (AUC) over the basal level calculated from a line interpolated from the d min^{-1} efflux value at $t = 30$ min to the value at $t = 60$ min. The second agonist responses were likewise calculated as the AUC above the interpolated basal d min^{-1} values between $t = 70$ min and $t = 90$ (or 110) min. The AUC was then expressed as a percentage of the tissue radioactivity present just before the drug was applied. Other agonist response end-points that were occasionally used for the purpose of constructing log concentration-response curves were changes in the fractional loss of radioactivity from the tissue per min at the start of each collection, the maximal rate of [^3H]-5-HT efflux (V_{max} , measured as the percentage fractional loss per min (units = $\% \text{ min}^{-2}$), or the peak fractional loss resulting from the application of the agonist.

Fractional loss (efflux rate) was obtained by dividing the number of counts lost from the platelets over the collection interval by the total number of counts in the platelets at the beginning of each time interval.

The data have been presented as mean ($\pm$ s.d., n = the number of platelet batches from different individuals; N = the number of filters). The AUC was determined on the Apple Macintosh IIfx computer using the Flexitrace software.

Buffer and drugs

Krebs solution was composed of the following (mM): NaCl 119, KCl 4.6, CaCl_2 1.5, NaH_2PO_4 1.2, MgCl_2 1.2, NaHCO_3 15, and glucose 11. The drugs used were [^3H]-5-HT creatinine sulphate (New England Nuclear), adenosine diphosphate, 5-HT creatinine sulphate, human thrombin, 1-(m-chlorophenyl) piperazine dihydrochloride (Sigma), and paroxetine hydrochloride (SmithKline Beecham).

Results

The spontaneous efflux of [^3H]-5-HT from perfused platelets

The initial washout of [^3H]-5-HT was exponential and this was followed after 20 to 25 min by a relatively steady rate of efflux (measured as fractional efflux $\%$). Thus at 30 min after commencement of washout, the spontaneous efflux in drug-free Krebs was $0.78 (\pm 0.23) \% \text{ min}^{-1}$ ($n = 5$; $N = 20$). In Krebs containing paroxetine ($1 \mu\text{M}$), the efflux at 30 min was $0.59 (\pm 0.19) \% \text{ min}^{-1}$ ($n = 5$; $N = 20$), which was not statistically significantly different from that in drug-free Krebs. This suggests that there was no appreciable re-uptake of released [^3H]-5-HT by the perfused platelets.

The effects of thrombin, ADP, 5-HT and m-CPP, alone, or in combination, on the efflux of [^3H]-5-HT

Thrombin Thrombin caused a concentration-related increase in [^3H]-5-HT efflux when added for 4 min at $t = 30$ min or $t = 70$ min (see Figure 1). The increase in efflux was clearly evident with 0.0156 u ml^{-1} and rose to a maximum with $0.5\text{--}1.0 \text{ u ml}^{-1}$.

m-CPP, 5-HT and ADP The addition of m-CPP in final concentrations of $0.5\text{--}500 \mu\text{M}$ at $t = 30$ min for a contact time of 4 min caused no increase in [^3H]-5-HT efflux. This was also true when paroxetine ($1 \mu\text{M}$) was present in the Krebs solution. When thrombin ($0.25\text{--}1 \text{ u ml}^{-1}$) was added to these same platelets at $t = 60$ or 70 min there was always a massive release of [^3H]-5-HT (Figure 2) in the region of 70% of the total amount of [^3H]-5-HT available for release.

5-HT caused only very small increases in [^3H]-5-HT efflux and the peak response was far less than that evoked by thrombin. The efflux in response to 5-HT was potentiated by a submaximal concentration of ADP ($2.5 \mu\text{M}$; Figure 3) in that the resultant efflux of [^3H]-5-HT was greater than expected from summation of the two responses.

Figure 1 The increase in efflux of [³H]-5-HT induced by different concentrations of thrombin ($n = 4$) added to the Krebs solution for 4 min at $t = 30$ or $t = 70$ min. The efflux was calculated as the fractional release of [³H]-5-HT. ▽ control, ◆ 0.0156 u ml⁻¹, ▼ 0.0313 u ml⁻¹, ◇ 0.0625 u ml⁻¹, ■ 0.125 u ml⁻¹, □ 0.25 u ml⁻¹, ▲ 0.5 u ml⁻¹, △ 1.0 u ml⁻¹, ○ 2.0 u ml⁻¹ thrombin.

Inset: The log thrombin concentration/[³H]-5-HT efflux curve ($n = 4$). The [³H]-5-HT efflux evoked by thrombin was measured as the AUC (% tissue radioactivity at time of administration).

Figure 2 The efflux of [³H]-5-HT from platelets induced by *m*-CPP (500 μM) added to the Krebs solution for 4 min at $t = 30$ min (M) and by thrombin (0.5 u ml⁻¹) added to the Krebs solution for 4 min at $t = 70$ min (T). Each point is the mean of 5 experiments ($\pm$ s.d.) in the absence (squares) and presence (solid circles) of paroxetine (1 μM) throughout the experiment.

m-CPP did not evoke an excess release of [³H]-5-HT in the presence of ADP (2.5 μM), confirming the absence of an agonist action of this drug on platelets (Figure 3). However, in a concentration of 50 μM, *m*-CPP prevented 5-HT from potentiating the ADP-induced release of [³H]-5-HT, suggesting that *m*-CPP has 5-HT-receptor antagonist activity (Figure 3). When *m*-CPP (1 μM) was included in the Krebs solution throughout the experiment

the 5-HT-concentration-response curve (responses to 5-HT in the presence of ADP minus the response to ADP alone) was displaced to the right (Figure 4), as was that in the presence of the 5-HT₂-receptor antagonist ketanserin (1 μM; not shown). Assuming parallel displacement of the curves the mean pKB values for *m*-CPP and ketanserin were respectively 5.18 (range 3.43–7.32) and 6.11 (range 4.26–7.71).

Figure 3 a) A typical experiment showing the efflux of [^3H]-5-HT evoked by 5-HT (1 μM , ■), ADP (2.5 μM , ●), 5-HT (1 μM) + ADP (2.5 μM) (Δ), m-CPP (50 μM , ◇), ADP (2.5 μM) + m-CPP (50 μM) (○), 5-HT (1 μM) + ADP (2.5 μM) + m-CPP (50 μM) ($\blacktriangle$) and no treatment control ($\square$).

b) Data from 6 experiments showing the mean AUCs ($\pm$ s.d.) for the efflux of [^3H]-5-HT induced by each drug/combination. Note: 1. The potentiation of the 5-HT response by ADP. 2. That m-CPP did not evoke an efflux of [^3H]-5-HT over that occurring spontaneously in the absence or presence of ADP, but it reversed the increased efflux to 5-HT potentiated by ADP to that of ADP alone (** P < 0.01; *** P < 0.001).

Discussion

m-CPP has both agonist and antagonist actions in different areas of the rat brain. Thus, Conn & Sanders-Bush (1987) showed that m-CPP was an agonist at 5-HT $_1$ c-receptors in the rat choroid plexus but an antagonist of rat cortical 5-HT $_2$ -receptors. It was also an antagonist at 5-HT $_2$ -receptors in rat jugular venous smooth muscle (Cohen & Fuller, 1983). m-CPP had agonist actions on the 5-HT $_1$ -like autoreceptor which modulates 5-HT

release from rat cortical slices (Baumann & Waldmeier, 1981) and rat hypothalamic synaptosomes (Martin & Sanders-Bush, 1982).

Furthermore, Pettibone & Williams (1984) showed that m-CPP and m-trifluoromethylphenylpiperazine (m-TFMPP) could release 5-HT from hypothalamic slices *in vitro*. They provided evidence that this action required the neuronal uptake of m-CPP and m-TFMPP as it was prevented by fluoxetine. m-CPP presumably displaced 5-HT from neuronal stores, as the induced

Figure 4 The effect of m-CPP (1 μM) administered throughout the experiment on the efflux of [^3H]-5-HT evoked by 5-HT ($n = 5$). The AUC is the response to 5-HT in the presence of ADP minus the response to ADP alone. The open symbols represent the control responses to 5-HT, the closed symbols represent the responses to 5-HT in the presence of m-CPP (1 μM). The mean responses to the two doses common to both concentration-response lines were statistically significantly different (** $P < 0.01$; *** $P < 0.001$).

5-HT efflux was not affected by the removal of calcium from the incubation medium, thereby excluding depolarization-induced exocytosis.

If m-CPP were also to induce 5-HT release from human neurones or platelets, it might provide a possible explanation for the migraine-like headaches caused by the oral administration of m-CPP to patients (Brewerton *et al.*, 1988; Brown *et al.*, 1991).

The observation that the concentrations of m-CPP which caused 5-HT to be released from rat hypothalamic slices *in vitro* were 5–50 times the peak values in plasma (0.18 μM) of the patients of Brewerton *et al.* (1988) should not be taken as evidence that neuronal release is unlikely to be important to the headache-inducing mechanisms in man, as Caccia *et al.* (1981) showed that m-CPP, given orally to rats, accumulated in the brain, reaching concentrations several times those in plasma (25 times when measured as AUCs). It would be interesting to challenge the neuronal release hypothesis by seeing whether doses of fluoxetine required to block 5-HT uptake would prevent m-CPP-induced migrainous headaches in patients, such as those of Brewerton *et al.* (1988).

The alternative mechanism, that m-CPP might release 5-HT from platelets, was examined in our study. The perfusion method we used allowed us to study the con-

tinuous efflux of [^3H]-5-HT and the effects of different drugs for varying periods of time. After being flushed on to the filter membrane the platelets remained functional, as judged by their responsiveness to thrombin, for at least 2 h. The platelets were extremely sensitive to thrombin, [^3H]-5-HT release being stimulated by as little as 0.016 u ml^{-1} . The maximal peak increase in fractional release, V_{max} and % AUC occurred with 1 u ml^{-1} .

The platelets were much less sensitive to 5-HT and ADP than to thrombin, although ADP clearly potentiated the efflux of [^3H]-5-HT evoked by unlabelled 5-HT, just as it potentiates 5-HT-induced aggregation in conventional aggregation studies (Kinlough-Rathbone & Mustard, 1986; Mustard *et al.*, 1989).

m-CPP had no effect on [^3H]-5-HT release in concentrations up to 500 μM in the presence or absence of a supramaximal uptake-inhibitory concentration of paroxetine (1 μM ; K_i paroxetine is 1.1 nM in rat brain synaptosomes, Thomas *et al.*, 1987; K_d of paroxetine binding to human platelet membranes is 0.1 nM (Galzin *et al.*, 1988) to 0.43 nM (Cotrim *et al.*, 1990)). This suggests that m-CPP did not displace 5-HT from dense granules, and that it did not act as an agonist at 5-HT₂-receptors, a conclusion confirmed by the failure of m-CPP to enhance the efflux of [^3H]-5-HT evoked by ADP. However, in common with ketanserin, m-CPP inhibited the release of [^3H]-5-HT evoked by unlabelled 5-HT, suggesting that m-CPP is a 5-HT₂-receptor antagonist. These findings are in accord with those of Conn & Sanders-Bush (1987) and Cohen & Fuller (1983), who found that m-CPP was an antagonist at 5-HT₂-receptors in the brain and vascular smooth muscle of rats.

Blockade of 5-HT₂-receptors is unlikely to be a mechanism contributing to the migraine-like headache following the oral administration of m-CPP to patients, as many of the drugs effective in preventing migraine, such as pizotifen, mianserin, cyproheptadine and methysergide, are 5-HT₂ antagonists (Fozard & Gray, 1989). This leaves 5-HT_{1c}-receptor activation (Fozard & Gray, 1989) or neuronal 5-HT release (Brown *et al.*, 1991) as the most likely explanation of m-CPP's headache-inducing property (Brewerton *et al.*, 1988). The possible cephalalgic action of other 5-HT_{1c}-receptor agonists, observed when agents such as ergotamine and dihydroergotamine are used chronically (Tfelt-Hansen, 1988), might be offset by their potent 5-HT₁-like constrictor agonist activity on large dilated cerebral arteries (Brown *et al.*, 1991) when used for the acute treatment of migraine.

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