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INCREASES IN AGGREGATION BY AND UPTAKE OF 5-HYDROXYTRYPTAMINE WITH PLATELETS FROM RABBITS TREATED WITH CHLORPROMAZINE

M. BALDACCI, MARA BALDACCI[1], T.D. BERGEL, G.V.R. BORN[2] & MARGARET HICKMAN

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD

1 Citrated platelet-rich plasma was prepared from New Zealand white rabbits before, during and after administration of chlorpromazine (2 mg/kg) intramuscularly once daily for 3 to 4 weeks.

2 In these plasmas, the velocity of platelet aggregation by 5-hydroxytryptamine (5-HT) added at 1, 3 and 10 μM increased greatly, beginning 3 to 4 days after the start of chlorpromazine injections and lasting for a similar period after they were terminated. The increase had two maxima, the first after 6 to 10 days and the second after 17 to 24 days. Chlorpromazine treatment did not affect aggregation by adenosine diphosphate (ADP).

3 The uptake of 5-HT by rabbit platelets was very fast and linear for less than 10 s. In platelets from untreated rabbits the uptake had a K_m of 0.35 ± 0.08 μM and a V_{max} of 39.8 ± 6.1 pmol 10^{-8} platelets 10^{-1} ($n = 5$).

4 In platelets from rabbits injected with chlorpromazine (see (2) above) both kinetic constants increased significantly, the K_m to 0.88 ± 0.08 μM and the V_{max} to 67.8 ± 5.5 pmol.10^{-8} platelets.10^{-1} s ($n = 9$).

Introduction

After patients have been treated with chlorpromazine for more than about a week, the effectiveness of 5-hydroxytryptamine (5-HT) in causing aggregation of their platelets is greatly increased (Boullin, Gelder, Grahame-Smith, Grimes, Kolakowska, Wiles & Woods, 1975). This effect is specific in as much as there is no concomitant change in the response of the platelets towards the aggregating action of adenosine disphosphate (ADP). Furthermore, the effect is reversible, in that about a week after administration of chlorpromazine is stopped, the sensitivity of the patients' platelets to 5-HT decreases to what it was before chlorpromazine was administered. Finally, the effect is repeatable in the same patients.

In order to analyse the mechanism of this drug-induced change in the response of platelets to 5-HT, it seemed useful to find out whether a similar effect could be demonstrated in another species. Experiments described in this paper establish that platelets from rabbits treated with chlorpromazine are also aggregated more strongly by 5-HT and that, furthermore, its uptake by these platelets is accelerated.

[1] Present address: Laboratori Baldacci SpA, via S Michele degli Scalzi 73, 56100 Pisa, Italy.

[2] Present address: Department of Pharmacology, King's College, Strand, London WC2R 2LS.

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Methods

Preparation of platelet-rich plasma

New Zealand white rabbits of either sex, weighing 3 to 7 kg, were maintained on standard diet and unlimited water. During control periods each rabbit was bled twice a week from a scalpel incision into the marginal vein of a shaved ear. The first 5 drops of blood were discarded and subsequent drops were collected into polypropylene measuring cylinders containing 3.8% (w/v) trisodium citrate as anticoagulant, until the ratio of blood to citrate solution was ten to one. Each blood sample was centrifuged at 400 *g* for 10 min. The supernatant platelet-rich plasma was separated and left at room temperature of about 20°C for 1 h while the concentration of platelets was determined: this varied in various plasmas from 5×10^8 to 2×10^9 platelets/ml.

Drug treatment

Chlorpromazine hydrochloride (Largactil, obtained as a 2% w/v aqueous solution from May & Baker Ltd.) was administered at a dose of 2 mg/kg body weight by intramuscular injection into the gluteal region once daily in the late afternoon. From these rabbits, platelet-rich plasma was prepared as described above

at various times during treatment and after it was discontinued.

Methysergide maleate (Sandoz Ltd., Basle) as a specific receptor antagonist to 5-HT was injected into 2 rabbits at a dose of 62.5 μg/kg body weight.

Platelet aggregation

Platelet-rich plasma in samples of 0.8 ml was used for determining aggregation velocity photometrically (Born, 1962) in a Mark IV aggregometer (Michal & Born, 1971) at 37°C, in response to 5-HT at concentrations of 1, 3 and 10 μM and also to ADP at a concentration of 100 μM, in order to permit comparison of aggregation velocities produced by 5-HT with the maximal velocity attainable by each particular plasma.

Uptake of 5-hydroxytryptamine

The initial velocity of 5-HT uptake was measured by incubation of 0.5 ml samples of platelet-rich plasma with 0.05 to 0.5 μM [5-hydroxy-^{3}H]-tryptamine [^{3}H]-5-HT: specific activity 13.8 Ci/mmol obtained from The Radiochemical Centre, Amersham, Bucks). The platelet-rich plasma in microcentrifuge tubes was brought to 37°C in a water bath for 15 min. [^{3}H]-5-HT was added as a drop less than 10 μl in volume to the inner surface of the lid which was closed. The drop remained stable in this situation until uptake was initiated by inversion and shaking of the tube.

Uptake was stopped after 10 s by the addition of 0.5 ml of ice-cold iso-osmotic saline containing 1% w/v disodium edetate (EDTA-saline). After mixing, the contents of the tube were immediately filtered through a millipore filter, pore size 0.8 μm. The filter was then washed three times with 0.5 ml of EDTA-saline.

This procedure was repeated 6 times at each of 5 concentrations of 5-HT. In addition, for each concentration the procedure was repeated 3 times with platelet-free plasma to enable corrections to be made for any binding of 5-HT to the filter. To diminish such binding, each filter was pretreated by filtering through it, 1 ml of saline containing 1% w/v bovine serum albumin.

The filters were transferred to polythene scintillation vials. Control vials contained known amounts of [^{3}H]-5-HT. One ml 19 M formic acid, 4 ml ethoxyethanol and 10 ml PPO/POPOP scintillant fluid were added to each vial. Radioactivity was measured in a Packard model 3320 scintillation counter, converted to disintegration per minute and the amount of 5-HT taken up by 10^8 cells was calculated. K_m and V_{max} values were obtained by a non-linear regression method (Wilkinson, 1961). Calculations were made with a P.D.P-8e processor using programmes written in this department.

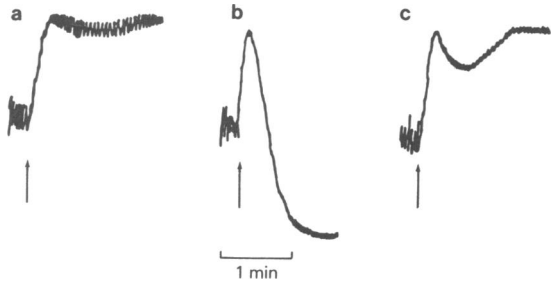

Figure 1 Aggregation of rabbit platelets by 5-hydroxytryptamine (5-HT), added at times indicated by the arrows. Aggregation was measured by changes in light transmission through citrated platelet-rich plasma as described under Methods. The initial upward movement is due to the rapid shape change induced by 10 μM 5-HT. Downwards movement after the shape change is due to aggregation. Platelet-rich plasma came from: (a) control rabbits; (b) rabbits treated with chlorpromazine for 6 days; (c) rabbits treated with chlorpromazine for 10 days.

Where reference to mean values of K_m and V_{max} is made, n gives the number of separate experiments. Multiple results obtained with the same platelet-rich plasma were pooled to give one experiment with lower errors.

Results

Aggregating potency of 5-hydroxytryptamine

As already known (Sinakos & Caen, 1966; Baumgartner & Born, 1969; Michal & Motamed, 1976) 5-HT has only a comparatively small aggregating effect on platelets from normal, untreated rabbits which may, indeed, be only just demonstrable photometrically and is rapidly reversed (Figure 1a). Even so, 5-HT produced its characteristic shape-changing effects on the platelets (Born, Dearnley, Foulks & Sharp, 1978) which was clearly demonstrable (Figure 1a).

After rabbits had been injected intramuscularly once a day with chlorpromazine (2 mg/kg) for 3 to 4 days, the aggregating effect of 5-HT increased greatly, and the aggregation did not reverse within 2 to 3 min (Figure 1b). Aggregation by 5-HT then became similar to that produced by ADP although maximal aggregation by ADP remained greater than that inducible by 5-HT.

The time course of the increased aggregating potency of 5-HT is shown in Figure 2, where velocity of aggregation is plotted against time in days before,

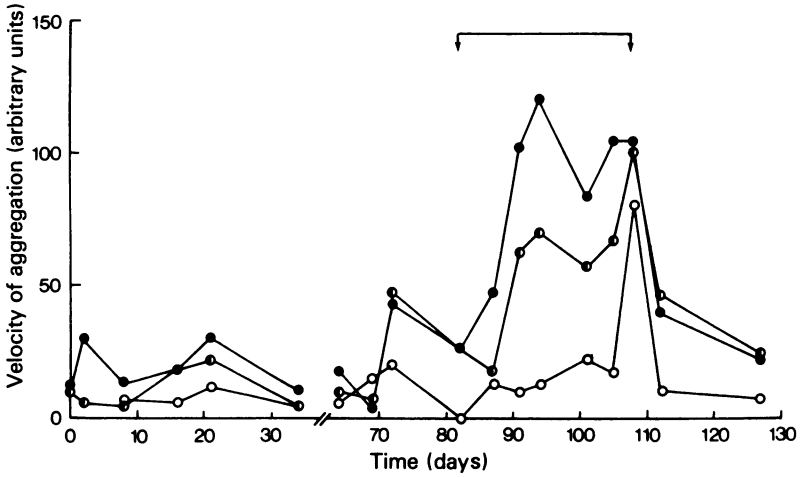

Figure 2 Velocity of platelet aggregation by 5-hydroxytryptamine (5-HT) at concentrations of 1 (○), 3 (◑) and 10 (●) μM before, during and after once daily injections of chlorpromazine (2 μg/kg i.m.) in apparently healthy rabbits. The injections were given from the first to the second arrow. Abscissa scale: time in days; ordinate scale: aggregation velocity in arbitrary units (maximal rate of change per unit time).

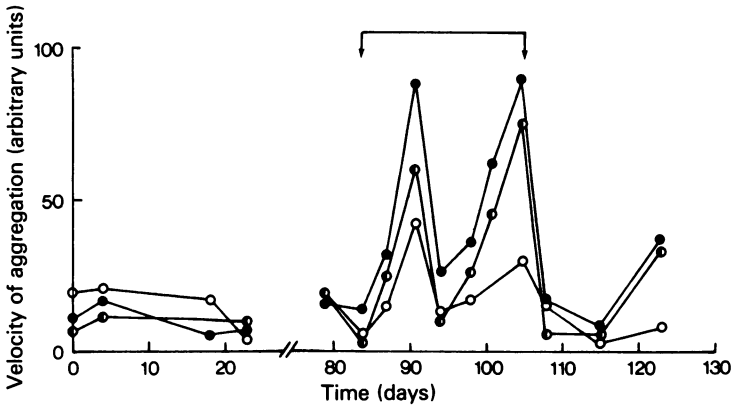

Figure 3 Velocity of platelet aggregation by 5-hydroxytryptamine (5-HT) at concentrations of 1 (○), 3 (◑) and 10 (●) μM before, during and after once daily injections of chlorpromazine (2 μg/kg i.m.) in apparently healthy rabbits. The injections were given from the first to the second arrow. Abscissa scale: time in days; ordinate scale: aggregation in velocity in arbitrary units (maximal rate of change per unit time).

during and after treatment with chlorpromazine. The increase occurred in each of six rabbits although it varied in magnitude. In 5 out of the 6 rabbits, the increased aggregation by 5-HT occurred in two consecutive phases, with a first maximum 6 to 10 days and a second maximum 17 to 24 days after the chlorpromazine injections began (Table 1). This curious feature can be seen in Figure 2 and is shown more strikingly in Figure 3. During the interval between the two maxima, 5-HT caused somewhat less aggregation which reversed quite rapidly (Figure 1c).

To quantify all the results, areas under the curves relating aggregation velocity to time were measured for equal periods during chlorpromazine treatment

Table 1 Interval from start of chlorpromazine injections to the first and second maxima in the increased velocity of platelet aggregation by 5-hydroxytryptamine

Rabbit No.	*Time (days) to first maximum*	*second maximum*
1	7	17
2	10	24
3	8	22
4	no early maximum	21
5	8	22
6	7	21

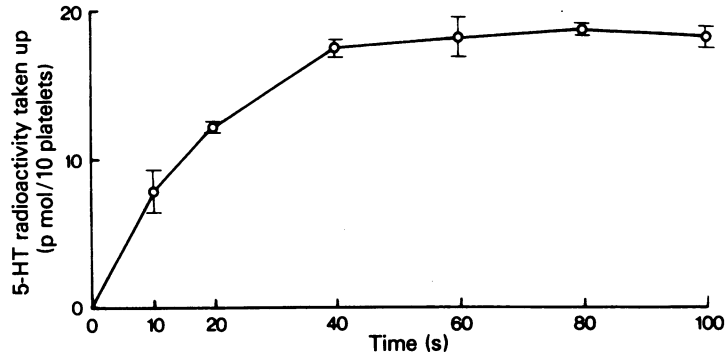

Figure 4 Rate of uptake of 5-hydroxytryptamine (5-HT) by rabbit platelets. Radioactive 5-HT was added to 3.5 ml platelet-rich plasma to give an initial concentration of 100 μM. At the times shown, 0.5 ml samples were taken and uptake stopped by addition of EDTA/saaline. The samples were then treated as described in Methods. Vertical bars indicate s.e. means of 3 determinations.

and before treatment as controls. As the effect of chlorpromazine did not begin for about 4 days and persisted for about the same time after the last injection, this was taken into account in determining areas representing the periods of treatment. Table 2 shows that there were rather similar increases of between 1.8 and 4.3 fold in area for 5-HT concentrations from 1 to 10 μM. Within this range, the higher the 5-HT concentration, the greater were the velocities during both the control and the chlorpromazine periods. Platelets obtained from these rabbits during control periods or at any time during chlorpromazine treatment showed no changes, qualitative or quantitative, in their responses to ADP. The chlorpromazine effect could not be accounted for by changes in platelet counts.

Uptake of 5-hydroxytryptamine

The uptake of 5-HT by platelets from untreated or treated rabbits was very fast (Figure 4). The linear portion of the uptake curve lasted for less than 10 s and uptake was complete after 60 s. Wilkinson (1961) analysis showed that the process was saturable. For platelets from untreated, control rabbits the K_m for 5-HT uptake was 0.35 ± 0.08 μM and the V_{max} was 39.8 ± 6.1 pmol.10^{-8} platelets.10^{-1} s (mean ± s.e., $n = 5$).

The effect of chlorpromazine treatment on 5-HT uptake by platelets was determined with platelets obtained from rabbits after 6, 8 to 10 and more than 14 days of chlorpromazine injections. In all three groups the 5-HT uptake was changed (Table 3). Analysis of variance revealed no significant differences in 5-HT uptake between the three groups; the K_m values showed the greatest heterogeneity which was, however, far short of being statistically significant. When the results from all chlorpromazine-treated rabbits were combined, the K_m was 0.88 ± 0.08 μM and the V_{max} was 67.5 ± 5.7 pmol.10^{-8} platelets. 10^{-1} s (mean ± s.e., $n = 9$). Student's t test showed that the differences between these values and those for the control group of rabbits were significant ($P < 0.05$).

Two rabbits were injected intramuscularly once daily with methysergide maleate (62.5 μg/kg) a potent

Table 2 Increased velocity of *in vitro* aggregation by 5-hydroxytryptamine (5-HT), as indicated by area under the velocity/time curve, of platelets from rabbits injected intramuscularly with chlorpromazine (2 mg/kg) once a day for 3 weeks

Rabbit No.	*Aggregation velocity ratio: chlorpromazine period/control period at the following concentrations of 5-HT (μM)*		
	1	3	10
1	3.6	3.7	3.5
2	2.2	2.4	3.4
3	2.4	3.2	2.4
4	2.3	2.2	1.8
5	4.3	3.3	2.6
6	3.3	3.4	3.0

Table 3 Effect of treating rabbits with chlorpromazine for 6 to 28 days on the kinetic constants of 5-hydroxytryptamine (5-HT) uptake by their platelets

Duration (days) of chlorpromazine treatment	*No. of rabbits*	K_m (μM) mean s.e. mean	V_{max} (pmol.10^{-8} platelets.10^{-1} s) mean s.e. mean
Nil, (controls)	5	0.36 ± 0.08	39.2 ± 6.1
6	4	0.86 ± 0.19	66.8 ± 11.7
8–10	3	1.04 ± 0.12	71.1 ± 10.0
14–28	2	0.69 ± 0.07	63.4 ± 5.5
Chlorpromazine values combined	9	0.88 ± 0.08	67.5 ± 5.7

blocking agent of 5-HT receptors on platelets (Born, Jungjaroen & Michal, 1972) and other tissues (see Born, 1970). Eighteen daily injections had no demonstrable effect on the velocity of platelet aggregation by 5-HT; an effect on its uptake was not looked for.

Discussion

First, the results confirm for rabbit platelets the discovery made with human platelets (Boullin, *et al.*, 1975) that their normally slight aggregability by 5-HT is greatly increased after chlorpromazine has been administered for more than a few days. The aggregability of rabbit platelets by ADP, like that of human platelets, was not affected, so that the chlorpromazine effect was to that extent specific for 5-HT. The increased aggregability of rabbit platelets by 5-HT during the administration of chlorpromazine occurred in two successive phases, with one maximum after 7 to 10 and another after 18 to 24 days. This particularly curious effect has, apparently, not been described for human platelets. Secondly, it has now been established, at least for rabbits, that their treatment with chlorpromazine also affects the high affinity uptake system for 5-HT in platelet membranes (Baumgartner & Born, 1969). Both the V_{max} and the K_m were increased, suggesting that the number of uptake sites is increased but that the average affinity of the sites for 5-HT is decreased.

Both effects, on aggregation and uptake, would be most simply explained by assuming that the administration of chlorpromazine for more than a few days induces increases in the number of platelet receptors that are specific for 5-HT (Born *et al.*, 1972; Born & Michal, 1975). As the effects took about 3 to 4 days to manifest themselves, and as this is also the mean survival time of circulating platelets in rabbits (Morgan, Keating & Reisner, 1955) it would seem likely that such an increase in 5-HT receptors occurred in the megakaryocytes from which platelets are derived. This would be consistent with an increase in the biosynthesis of receptor proteins of which platelets themselves are incapable.

By analogy with other drug-induced receptor modulations (see Raff, 1976) such an increased biosynthesis could be a consequence of the blocking *in vivo* of 5-HT receptors by chlorpromazine for which it has a high affinity (Graf & Pletscher, 1979). A diminution in the ability of cells which normally do so to react to 5-HT, would induce a mechanism for increasing their production of 5-HT receptors. This would show itself in the case of platelets as increases in aggregation by and uptake of 5-HT.

However, this explanation does not take into account the fact that added 5-HT induces a characteristic change in shape in normal rabbit platelets (Born, 1970; Michal & Born, 1971; Graf & Pletscher, 1979; and Figure 1a) indicating that they possess receptors for 5-HT with which it reacts very effectively. Furthermore the magnitude of the shape change, unlike that of aggregation, is not increased by chlorpromazine treatment. There is evidence (see Born, 1977) that platelets can aggregate without having undergone demonstrable changes in shape. It may be, therefore, that the effect of chlorpromazine treatment is to increase the number or the availability of only those platelet receptors which, together with fibrinogen and calcium (Born, 1969) are essential for aggregation.

Both shape change and aggregation are inhibited by methysergide in low concentrations (Born *et al.*, 1972). However, injection of methysergide into rabbits did not affect aggregation in the same way as did chlorpromazine. This may have been because the doses of methysergide were too small, or because the explanation of the chlorpromazine effect as a consequence of 5-HT receptor blockade is too simple.

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