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EVIDENCE FOR FUNCTIONAL 5-HT$_2$ RECEPTOR SITES ON HUMAN BLOOD PLATELETS

F. DE CLERCK, B. XHONNEUX, J. LEYSEN and P. A. J. JANSSEN

Department of Life Sciences, Laboratory of Haematology and Department of Biochemical Pharmacology, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium

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Abstract—The aggregation of normal human platelets by 5-hydroxytryptamine (5-HT) is the result of a specific interaction of the monoamine with a platelet receptor since it is not influenced by adrenergic receptor blockade, inhibition of fatty acid cyclo-oxygenase or ADP-scavenging. The 5-HT induced platelet reaction is inhibited in a concentration-dependent way by various serotonergic antagonists; the potency of these compounds in this respect correlates strongly with their potential to inhibit the specific binding of [^{3}H] ketanserin, a selective label for 5-HT$_2$ binding sites, to rat prefrontal cortex and striatum and to cat platelet membranes. This study thus provides evidence for a functional role as true receptor initiating a physiological response of the 5-HT$_2$ receptor on human platelets.

5-Hydroxytryptamine (5-HT) interacts with blood platelets to produce activation, expressed as shape change and aggregation, and to be taken up by an energy-consuming transport system, both processes being regulated by two distinct surface membrane receptors [1–3]. The inhibition of 5-HT-induced and -amplified human platelet aggregation by ketanserin, a selective 5-HT$_2$ receptor antagonist without activity on the 5-HT$_1$ binding sites [4–6], suggested the presence of functional receptors of the 5-HT$_2$ subtype on human platelets related to platelet activation [1, 7]. Subsequent studies enabled us to demonstrate such receptors on feline platelets [8, 9]. In the present study, we present further evidence for the functional significance of the 5-HT$_2$ receptor sites in the activation of human platelets induced by 5-HT, using a comparison of the binding affinities of various drugs for the 5-HT$_2$ sites labelled with [^{3}H] ketanserin with the inhibition by these drugs of the physiological platelet response to the monoamine.

MATERIALS AND METHODS

Platelet aggregation. Using a Chronolog Model 440 Dual Channel Automatic Aggregometer the turbidimetric measurement of platelet aggregation was performed. To 0.4 ml of citrated (0.313% F.C.) human platelet-rich plasma (P.R.P.; 350×10^3 platelets/μl) prepared from venous blood [7] and stored under CO_2 5%–O_2 95% [10] at 37°, 50 μl of NaCl 0.15 M, 50 μl of Tris–saline pH 7.8 and 1 μl of solvent (D.M.S.O. or ethanol 0.2%) or compound solution were added and further incubated (5 min at 37°) before the initiation of aggregation with 5 μl 5-HT 5×10^{-6} M final concentration (F.C.) or 1-epinephrine 2×10^{-5} M F.C. Percentage inhibition of aggregation produced by the compounds, and IC$_{50}$-values were calculated from the reduction of the initial slopes of the aggregation tracings [7].

Release of intraplatelet ATP. For the simultaneous measurement of aggregation and of ATP release, luciferase–luciferin (Chrono-lume No. 325), 4 mg/ml was incorporated in the P.R.P. sample. The total amount of ATP release was calculated from the luminescence, as measured with a Chronolog Model 400 Lumi-Aggregometer, induced by the addition of a standard concentration of ATP 1×10^{-6} M and is expressed as μM ATP/1.4×10^8 platelets [11].

Biosynthesis of thromboxane A$_2$. In the same experimental conditions as for the aggregation test, the 0.5 ml sample was supplemented 5 min after the initiation of the aggregation reaction with 0.1 ml of an inhibitory cocktail yielding E.D.T.A. 1.16 mg/ml F.C. and suprofen 8.3×10^{-5} M F.C. [7]; after mixing, the content of the tube was centrifuged (5 min × 10,000 *g*) to platelet-free plasma (P.F.P.). The P.F.P. was kept at −25° before the assessment of its content of thromboxane B$_2$ (TXB$_2$) by radioimmunoassay, using a specific antiserum and [^{3}H] TXB$_2$ (Radiochemical Centre, Amersham) as a tracer [12]. Results are expressed as pg TXB$_2$/100 μl.

Platelet malondialdehyde (MDA) production. MDA production by human platelets, as an indicator for the prostaglandin biosynthesis [13], was evaluated spectrofluometrically using a modification of the method of McMillan [14].

Briefly, 0.4 ml of P.R.P. (250×10^3 platelets/μl), in the presence of solvent (D.M.S.O. or ethanol 0.2%) or compound solution was pre-incubated for 10 min at 37° and then challenged with 50 μl of $CaCl_2$ 1×10^{-3} M F.C. and 50 μl of thrombin 20 N.I.H. U/ml F.C.; after 30 min at 37°, the reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid. After isolation of the supernatant by centrifugation (10 min × 320 *g*), the thiobarbituric acid reaction for MDA was performed using acid-hydrolyzed malondialdehydebis (dimethylacetal) (Merck) as a standard; the fluorescence of the samples at 553 nm after excitation at 510 nm was measured. Results are calculated as nM MDA/1×10^8 platelets/30 min and are expressed as a percentage inhibition produced by the compounds relative to their solvent.

Membrane ligand binding. In order to obtain in-

formation about the serotonergic receptor subtype involved in the functional response of human platelets to 5-HT in relation to brain tissue, the inhibition (log K_i-values in nM) by various drugs of the specific binding of [^{3}H] ketanserin, a selective label for 5-HT_2 receptor sites [5], to rat pre-frontal cortex, rat striatum and cat platelet membrane preparations, as recently reported [8], was compared to that (log IC_{50}-values in M) of the slope of the 5-HT-induced aggregation of human platelets.

For this comparison, the mentioned membrane preparations, rather than human platelets, were selected for radioligand experiments since previous data on platelet preparations from e.g. man revealed a much lower specific [^{3}H] ketanserin binding and a ratio between specific and total binding which was unfavourable for pharmacodissection [8]. Labelled [^{3}H] ketanserin rather than [^{3}H] 5-HT was used in radioligand binding experiments because of the low number of binding sites on platelet membranes for the latter compound [2, 8].

Chemicals. 5-HT creatinine sulphate, *l*-epinephrine, ATP (Serva), E.D.T.A., $CaCl_2$ (Merck), creatine phosphate (CP, phosphocreatine disodium salt hydrate) and creatine phosphokinase (CPK, E.C. 2.7.3.2, Type I, Sigma) and suprofen (Janssen Pharmaceutica) were dissolved in NaCl 0.15 M.

For platelet aggregation studies, prazosin, dazoxiben (Pfizer), phentolamine (Ciba), yohimbine (Kali-Chemie), clonidine (Boehringer), atenolol (I.C.I.), butoxamide (Burroughs Wellcome), practolol (Ayerst), pipamperone, suprofen, ketanserin, spiperone, domperidone, haloperidol, R 19 019 (Janssen Pharmaceutica), methysergide (Sandoz), cyproheptadine, indomethacin (M.S.D.), cinanserin (Squibb), amitryptiline (Labaz), clomipramine, desimipramine (Geigy), pyrilamine (Rhône-Poulenc), citalopram (Lundbeck), BW 755 C (Wellcome Research Labs), and esculetin (Sigma) were dissolved in dimethylsulfoxide (D.M.S.O.). Nordihydroguaiaretic acid (N.D.G.A., Fluka, A.G.) was freshly dissolved in ethanol. Tris–saline consisted of 9 parts NaCl 0.15 M and 1 part Tris 0.15 M pH 7.8. All drug concentrations given in the section on results are final concentrations (F.C.) after all additions to the platelet suspension.

Statistical evaluation. Results are expressed as mean ± S.E.M. of the stated number of experiments. IC_{50}-values and 95% confidence limits were computed by probit analysis [15] on an IBM 3031 computer. Correlations between the various parameters were calculated using the Spearman rank correlation coefficient r [16]. Statistical evaluation was performed using the Student's t-test, P-values ≤ 0.05 being considered as statistically significant [17].

RESULTS

Specificity of the 5-hydroxytryptamine-induced aggregation. Contrary to 1-epinephrine, 5-HT (5×10^{-6} M) induces a shape change and a reversible wave of aggregation in human P.R.P., which is not accompanied by a detectable release of intraplatelet ATP or a formation of TXB_2 (Table 1).

The initial slope of this aggregation reaction is not inhibited by specific α- or β-adrenergic receptor antagonists; the reaction is stimulated by the partial α_2-adrenergic agonist/antagonist clonidine (Table 2). The 5-HT-induced aggregation is not affected by the fatty acid cyclo-oxygenase inhibitors suprofen and indomethacin (1×10^{-5} M), the specific thromboxane A_2 synthetase inhibitors R 19 091 and dazoxiben (1×10^{-5} M) and the combined lipoxygenase-cyclo-oxygenase inhibitors BW 755 C, N.D.G.A. and esculetin at a concentration (1×10^{-4} M) reducing by 25–90% the formation of MDA by thrombin-stimulated human platelets (Table 3).

Pre-incubation of the platelet sample with suprofen (1×10^{-5} M) together with CP/CPK (8×10^{-3} M/80 U/ml)—a procedure which strongly (>90%) inhibits the ADP—(1×10^{-4} M)-induced aggregation—does not significantly affect the platelet reaction to 5-HT (results not shown).

Potency of various antagonists against 5-HT-induced aggregation. The slope of the aggregation induced by 5-HT is inhibited in a concentration-dependent way by various agonists, the order of potency, as judged by the IC_{50}-values, being pipamperone > spiperone > cyproheptadine > methysergide > ketanserin > cinanserin > amitriptiline > haloperidol > domperidone > clomipramine > desimipramine > pyrilamine > prazosin > citalopram (Fig. 1). When tested for their effect on platelet MDA production these compounds (1×10^{-5} M) produced less than 10% inhibition (results not shown).

Table 1. Aggregation, release of intraplatelet ATP and formation of TXB_2-induced by 5-hydroxytryptamine or 1-epinephrine in human platelets

Parameter*	Control	Agonist† 5-HT	Agonist† 1-Epinephrine
Slope aggregation (Δ % T/min)	0 ± 0	14.6 ± 2.6	11.5 ± 1.6
Maximum (Δ % T)	0 ± 0	5.7 ± 1.09	46.2 ± 2.16
Slope desaggregation (Δ % T)	0 ± 0	16.9 ± 8.4	0 ± 0
Release of ATP (μM/ 1.4×10^8 platelets)	0 ± 0	0 ± 0	2.23 ± 0.76
Plasma TXB_2 (pg/100 μl)	47.7 ± 3.48	59.6 ± 7.28	4400 ± 605.4

* Mean ± S.E.M., N = 4.

† Human platelets (1.4×10^8) pre-warmed to 37° and mixed for 5 min without agonist (control) or with 5-HT 5×10^{-6} M or 1-epinephrine 2×10^{-5} M.

Table 2. Effect of α- and β-adrenergic receptor antagonists on the human platelet aggregation induced by 5-hydroxytryptamine and 1-epinephrine

Compound*	Agonist: 1-epinephrine IC_{50}†	Agonist: 5-HT Effect at IC_{50}‡
Phentolamine	1.04×10^{-6}	−3.3 ± 5.9
Yohimbine	2.4×10^{-7}	−2.1 ± 7.1
Clonidine	3.82×10^{-6}	+27.1 ± 6.4§
Prazosin	$>10^{-5}$	−7 ± 3.1
Atenolol	$>10^{-5}$	−2.1 ± 5.5
Butoxamide	$>10^{-5}$	−2.7 ± 9.5
Practolol	$>10^{-5}$	−9.8 ± 9.2

* Compounds or solvent incubated for 5 min at 37° before the induction of aggregation with 1-epinephrine 2×10^{-5} M or 5-HT 5×10^{-6} M.

† IC_{50}-value in M for inhibition of the slope of the first wave of 1-epinephrine induced aggregation.

‡ Percentage inhibition (−) or increase (+) versus solvent of the slope of 5-HT-induced aggregation at the IC_{50}-concentration for 1-epinephrine, or at 1×10^{-5} M. Mean ± S.E.M., N = 4.

§ $P \leq 0.05$ vs solvent; Student's *t*-test two-tailed probability.

Table 3. Effect of manipulation of arachidonic acid metabolism on the human platelet aggregation induced by 5-hydroxytryptamine and the formation of malondialdehyde by thrombin-stimulated platelets

Compound*	Aggregation§	MDA‖
Suprofen†	−2.2 ± 3.7	−84.8 ± 0.79
Indomethacin†	+16.6 ± 4.3	−92.9 ± 1.09
R 19 091†	+3.6 ± 5.9	−94.1 ± 0.45
Dazoxiben†	+9.5 ± 7.9	−92.2 ± 2.84
BW 755 C‡	−16.5 ± 5.6	−78.4 ± 0.79
N.D.G.A.‡	−14.1 ± 12.2	−30.9 ± 2.35
Esculetin‡	+13 ± 12.4	−25.7 ± 1.65

* Compound or solvent incubated for 5 min (aggregation) or 10 min (MDA) at 37° before challenge with 5-HT 5×10^{-6} M (aggregation) or thrombin 20 N.I.H. U/ml (MDA).

† Final concentration 1×10^{-5} M.

‡ 1×10^{-4} M.

§, ‖ Percentage inhibition (−) or increase (+) versus solvent of the slope of 5-HT-induced aggregation (§) or the formation of MDA after thrombin stimulation (‖). Mean ± S.E.M., N = 4.

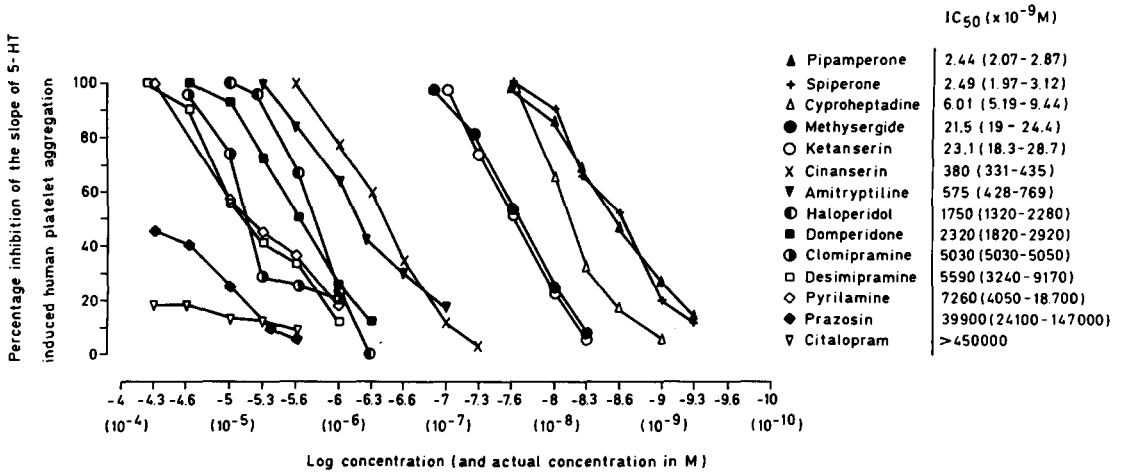

Fig. 1. Concentration-dependent inhibition by various compounds of the human platelet aggregation induced by 5-hydroxytryptamine. Human platelets (1.4×10^8) preincubated for 5 min at 37° with the stated compound concentration before stimulation with 5-HT 5×10^{-6} M. Percentage inhibition and IC_{50}-values calculated on the initial slope of aggregation. Mean of N = 4.

Table 4. Correlations between membrane ligand binding and physiological response data for various antagonists

Parameters	Correlation coefficient *r*†
5-HT-induced human platelet aggregation vs specific [^{3}H] K-binding cat platelet membrane*	0.92 ($P < 0.0001$)
5-HT-induced human platelet aggregation vs specific [^{3}H] K-binding rat prefrontal cortex*	0.90 ($P < 0.0001$)
5-HT-induced human platelet aggregation vs specific [^{3}H] K-binding rat striatum*	0.93 ($P < 0.0001$)

* Data obtained from [8].

† Spearman correlation coefficient for the inhibitory potency (IC_{50}) on human platelet aggregation versus that (K_i) of radioligand binding of 14 antagonists as listed in Fig. 1.

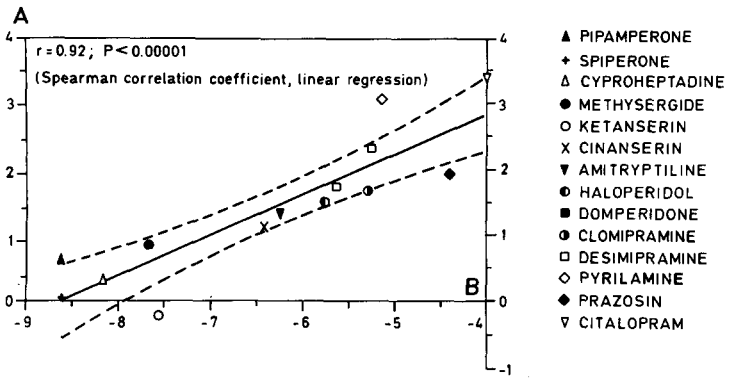

Fig. 2. Correlation between the inhibition of specific [^{3}H] ketanserin binding to cat platelet membrane preparations and the inhibition of 5-hydroxytryptamine-induced aggregation of human platelets. (A) log K_i (nM) for inhibition of specific [^{3}H] ketanserin binding to cat platelet membrane preparations (from [8]). (B) log IC_{50} (M) for inhibition of 5-HT (5×10^{-6} M)-induced aggregation (initial slope) of human platelets.

Correlation between membrane ligand binding- and physiological response data. Similar to the inhibition of the 5-HT-induced human platelet aggregation, the specific [^{3}H] ketanserin binding—as a selective label for 5-HT_2 receptor sites [5]—to rat prefrontal cortex-, rat striatum- and cat platelet membrane preparations are inhibited differentially by the various agonists (see [8]).

Linear regression analysis (Spearman correlation coefficient) reveals a strong positive correlation between the log IC_{50} for inhibition of 5-HT-induced human platelet aggregation and the log K_i for inhibition of specific [^{3}H] ketanserin binding to cat platelet membranes, to rat prefrontal cortex and to rat striatum membrane preparations respectively (Table 4 and Fig. 2).

DISCUSSION

Initially, platelet receptors involved in the activation of these cells by 5-HT were assumed to resemble the pharmacologically defined D-type receptor described by Gaddum [18] in intestinal smooth muscles, rather than the M-type [19]. These pharmacologically defined receptors were shown to be different from the 5-HT_1 and 5-HT_2 subtypes as defined by radioligand experiments in brain membrane preparations [4, 6]. Although an heterogeneity of serotonergic receptors involved in the activation of human platelets thus has already been suggested [1–3], attempts to characterize them in relation to the 5-HT_1 and 5-HT_2 types remained inconclusive: our initial studies with ketanserin, a selective 5-HT_2 receptor antagonist [5] inhibiting specifically 5-HT-induced platelet reactions, suggested the presence of functional receptors of the 5-HT_2 subtype on human platelets [1, 7] but such a contention has been challenged [20].

The present study provides further evidence for a functional role of the 5-HT_2 receptor sites in the activation of human platelets by 5-HT.

Our experiments show that 5-HT does not operate through the activation of adrenergic receptors, as claimed for dopamine [21] nor through the formation of arachidonic acid metabolites or the release of intracellular nucleotides. The enhanced response found with clonidine matches the reported pro-aggregating state induced with this partial α_2-agonist [22]. Therefore, the aggregation reaction we measure is the result of a specific interaction of the monoamine with a platelet receptor which initiates a sequence of alternative biochemical processes leading to the physiological response. This 5-HT-induced reaction is inhibited in a concentration-dependent way by various serotonergic antagonists. For the most potent antagonists, this inhibition is the result of a specific interaction with the platelet receptor and not the consequence of an effect on other biochemical processes involved in platelet activation since these drugs do not reduce the primary aggregation reaction to ADP [7, 9] and do not affect the prostaglandin biosynthesis by platelets (this study). The potency of the compounds to inhibit the 5-HT-induced human platelet aggregation correlates strongly with their potency to inhibit the specific binding of [^{3}H] ketanserin to rat prefrontal cortex and striatum membrane preparations. Moreover, a similar strong correlation is found with the inhibition of 5-HT-induced aggregation of cat platelets [9] where the 5-HT_2 binding sites were explicitly demonstrated with radiolabelled ketanserin [8]. Since [^{3}H] ketanserin specifically labels the 5-HT_2 receptor type [5, 6, 23], the high correlation between radioligand binding data and physiological response suggests that the receptors mediating the aggregation reaction of human platelets to 5-HT are equivalent to the 5-HT_2 receptor binding sites in brain tissues. Previous studies with human and feline platelets have shown that there is a large dissociation between the effective concentrations of 5-HT receptor antagonists including ketanserin, pipamperone, spiperone, methysergide and cyproheptadine against 5-HT-induced aggregation and against the active uptake of 5-HT by the platelet, the latter process being affected only at substantially higher concentrations [7, 9]. This finding corroborates the concept that functional activation and uptake of 5-HT by/of platelets operate through two distinct membrane receptor groups [1–3]. Previously, the vascular receptors responsible for the 5-HT-induced contraction have been shown to be also of the 5-HT_2 subtype in rat aorta, caudal artery and jugular vein [23–25]; the receptors

mediating 5-HT-induced aggregation of feline platelets were also characterized as 5-HT_2 sites [8, 9]. The present study provides further evidence for a functional role of the 5-HT_2 binding site, rather than the D receptor [19], as a true receptor mediating a physiological response in human platelets.

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