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SHORT COMMUNICATIONS

Serotonin binding in human platelets *

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Blood platelets are considered to be a model system for the investigation of the physiological effects of serotonin. Serotonin can induce platelet shape change and aggregation [1]. In other systems, serotonin is known to regulate such activities as neurotransmission [2] and contractility in intra- and extracranial arteries [3]. Platelets also share with the neuron the ability to rapidly take up and store serotonin [1]. There is pharmacological evidence for two sites for the interaction between serotonin and platelets [1]. Serotonin-induced platelet shape change and aggregation are readily inhibited by serotonin receptor antagonists. Serotonin uptake in platelets is blocked primarily by serotonin uptake inhibitors.

Serotonin binding to the platelet surface has been demonstrated only in rat platelets [4]. In our study we have demonstrated and characterized serotonin binding in human platelets. The effects of serotonin receptor antagonists and serotonin uptake inhibitors on serotonin binding in platelets have also been defined. The effects of these agents suggest that we have demonstrated the binding of serotonin to the uptake site on the surface of human platelets.

The following radiochemicals and reagents were used in the study: [^{3}H]hydroxytryptamine creatinine sulfate (15 Ci/m-mole), New England Nuclear Corp., Boston, MA, NET 498); [^{14}C]hydroxytryptamine creatinine sulfate (57 mCi/m-mole), Amersham/Searle Corp., Arlington Heights, IL., CFA 170); 5-hydroxytryptamine creatinine sulfate complex, H7752, imipramine–HCl, 17379, tryptamine–HCl, T9628, D,L-tryptophan, T0129, and 6-hydroxytryptamine creatinine sulfate complex, H3005 (Sigma Chemical Co., St. Louis, MO). The following drugs were kindly provided by pharmaceutical companies: fluoxetine–HCl (Lilly Research Labs, Indianopolis, IN), chlorimipramine–HCl (Ciba-Geigy, Summit, NJ), cinanserin–HCl (The Squibb Institute, Princeton, NJ), methysergide bimaleate (Sandoz, Inc., East Hanover, NJ), and cyproheptadine–HCl (Merck, Sharp & Dohme, West Point, PA).

Platelet-rich plasma was prepared from freshly donated citrated human blood, and platelets were counted as described previously [5].

Platelet serotonin binding was determined by incubating 2 ml volumes of platelet-rich plasma with varying concentrations of [^{3}H]serotonin (10^{-9} M to 5×10^{-6} M). The binding studies were carried out at 4° to inhibit serotonin uptake. Following incubation, platelets were sedimented and then washed twice in Tris-buffered saline as described previously [6]. Pellets were resuspended in 1 ml of buffered saline and prepared for liquid scintillation counting. Non-specific binding was determined in parallel experiments by the addition of unlabeled serotonin 100 times the concentration of the [^{3}H]serotonin to displace the reversibly bound ligand. Under these conditions, about 10 per cent of the bound serotonin remained in the pellet and was considered to represent non-specific binding. This amount was subtracted from total binding to obtain an estimate of specific serotonin receptor binding. Each data point shown in Fig. 1 represents specific binding which was calculated in this manner.

Several experiments were designed to determine whether dissociation of bound serotonin occurred during the washing

* Part of this work has already appeared in abstract form, *Blood* **52**, suppl. 1, abstr. 293, p. 172 (1978).

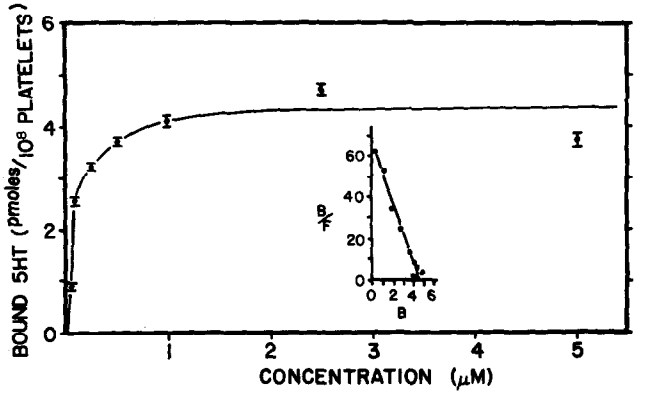

Fig. 1. Relationship between concentration of serotonin and serotonin binding. Two-ml volumes of platelet-rich plasma were placed in a 4° water bath. To assess total binding, at zero time, 1/40 volume of [^{3}H]serotonin was added to obtain the desired final concentration and the binding incubation was carried out for 30 min at 4° with occasional shaking. In parallel experiments to assess non-specific binding, after 5 min of incubation unlabeled serotonin 100 times the concentration of the labeled ligand or saline (1/40 volume) was added and incubation was carried out for 30 min. Following incubation, platelets were sedimented and then washed twice in Tris-buffered saline. The pellet was resuspended in 1 ml of buffered saline and prepared for liquid scintillation counting. Quench and efficiency corrections were made and dis./min used to determine binding. Specific binding in each data point was determined by subtracting non-specific binding from the total binding. Data for each concentration represents four experiments ± S.D. A Scatchard plot analysis of the data is also shown. Key: B, bound serotonin; and F, free serotonin. The K_D was 6.3×10^{-8} M.

Table 1. Inhibition of serotonin-induced platelet shape change and aggregation*

Agent	Platelet shape change (IC_{50})	Platelet aggregation (IC_{50})
Serotonin receptor antagonists		
Methysergide	10^{-7} M	10^{-8} M
Cyproheptadine	5×10^{-7} M	5×10^{-8} M
Cinanserin	5×10^{-6} M	5×10^{-6} M
Serotonin uptake inhibitors		
Imipramine	5×10^{-4} M	5×10^{-4} M
Chlorimipramine	5×10^{-5} M	5×10^{-5} M
Fluoxetine	5×10^{-5} M	5×10^{-5} M
Serotonin analogues		
Tryptamine	5×10^{-7} M	5×10^{-5} M
6-Hydroxytryptamine	5×10^{-4} M	5×10^{-4} M
Tryptophan	None	None

* Drugs (1/10 volume) were added to platelet-rich plasma in an aggregometer and the sample was stirred at 37° for 5 min. Next, serotonin (1/10 volume) was added and changes in O.D. were measured with a 1 mV recorder. Serotonin (10^{-6} M) was used in these experiments since it most consistently caused platelet shape change and aggregation. Data for each drug represent at least four experiments.

procedure. The washing procedure lasted for 30 min. Platelets were incubated with serotonin (10^{-7} M) and washed as described above. The washed platelets were kept at 4°, and at frequent intervals during a 120-min period the radioactivity in the pellet and in the supernatant fraction was determined. There was no evidence of dissociation of bound serotonin.

Platelet serotonin uptake studies were carried out by incubating platelet-rich plasma with [^{14}C]serotonin at 37° as described previously [5]. Platelet shape change and aggregation were determined in platelet-rich plasma using a Chron-o-log aggregometer [5]. A 1 mV recorder was used to augment the deflection of shape change and aggregation, which is small with serotonin as the aggregating agent.

The results demonstrate that platelet serotonin receptor binding was rapid, reversible, saturable, and specific. Binding reached equilibrium within 30 min when platelets were incubated with [^{3}H]serotonin (10^{-9} M to 10^{-8} M). Under these conditions, over 80 per cent of the serotonin bound to the platelets at equilibrium was bound at 5 min. Binding was reversible since unlabeled serotonin, 100 times the concentration of the labeled ligand, displaced about 90 per cent of bound [^{3}H]serotonin. In the displacement experiment, the effect of adding the unlabeled serotonin was similar whether it was added before or during the binding incubation. Figure 1 demonstrates specific binding of serotonin in platelets. The binding was saturable in relation to the concentration of serotonin and about 4.6 pmoles ligand can be bound by 10^{8} platelets. The apparent K_D is 6.3×10^{-8} M. In order to characterize further platelet serotonin binding, the relative potency of serotonin analogues in displacing bound serotonin

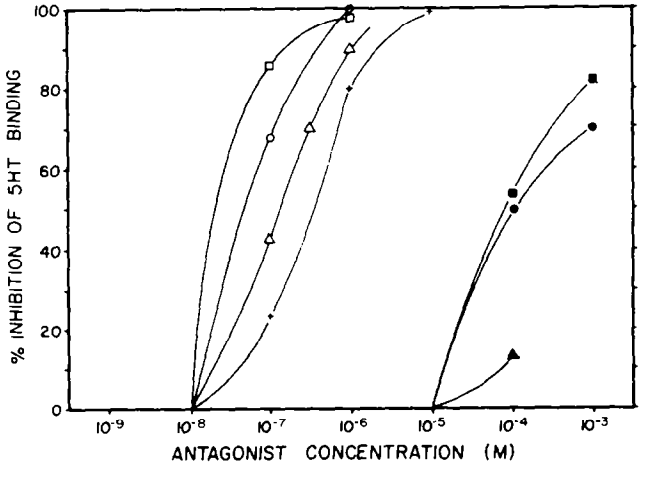

Fig. 2. Inhibition of serotonin receptor binding. Key: (○) chlorimipramine; (□) imipramine; (△) fluoxetine; (+) tryptamine; (■) cinanserin; (●) cyproheptadine; and (▲) methysergide. The procedure was the same as that described in Fig. 1 except that drugs were added in place of unlabeled serotonin. The concentration of [^{3}H]serotonin was 10^{-7} M. Data for each drug represent at least four experiments. Results are expressed as per cent inhibition of binding in control experiment (saline instead of drug).

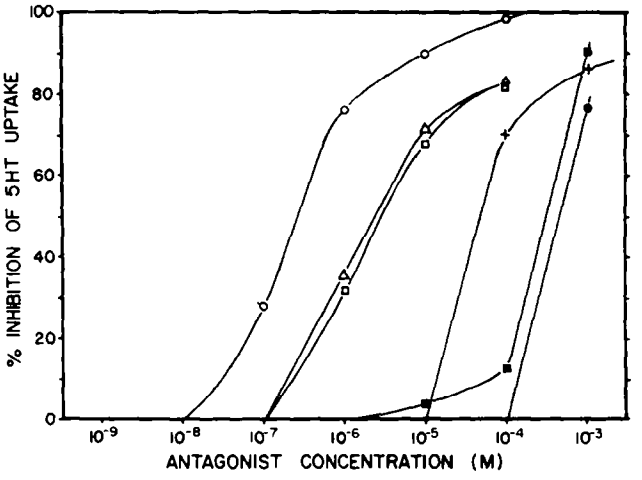

Fig. 3. Inhibition of serotonin uptake. Key: (○) chlorimipramine; (□) imipramine; (△) fluoxetine; (+) tryptamine; (■) cinanserin; and (●) cyproheptadine. [^{14}C]serotonin (1/100 volume) was added to 2 ml of platelet-rich plasma to achieve a final concentration of 10^{-7} M. In the control experiment, incubation was carried out at 37° for 30 min. In the inhibition experiment, drugs were added after 5 min and incubation was continued for the remainder of 30 min. Platelet serotonin uptake was terminated by rapid filtration as described previously [5]. Results are expressed as per cent inhibition of serotonin uptake in control experiments. Data for each drug represent at least four experiments.

was tested, it was found that 6-hydroxytryptamine was 100 times less effective than unlabeled serotonin and that tryptophan did not displace bound ligand. Tryptamine was as effective as unlabeled serotonin in displacing labeled serotonin.

Serotonin uptake inhibitors were more effective than serotonin receptor antagonists in displacing bound serotonin (10^{-7} M). The IC_{50} values of the uptake inhibitors, as shown in Fig. 2, were as follows: chlorimipramine, 6.3×10^{-8} M; imipramine, 3.1×10^{-8} M; and fluoxetine, 1.3×10^{-7} M. The IC_{50} values of the serotonin receptor antagonists were as follows: cinanserin, 9.0×10^{-5} M; and cyproheptadine, 1.0×10^{-4} M; methysergide was soluble only up to 10^{-4} M which inhibited 14 per cent of platelet serotonin binding. The IC_{50} of tryptamine, a serotonin analogue, was 5.0×10^{-7} M. Serotonin uptake inhibitors also were more effective than serotonin receptor antagonists when serotonin (10^{-8} M) was used.

Serotonin uptake inhibitors were more effective than serotonin receptor antagonists in blocking platelet serotonin (10^{-7} M). The IC_{50} values of uptake inhibitors, as shown in Fig. 3, were as follows: chlorimipramine, 3.8×10^{-7} M; imipramine, 4.4×10^{-6} M; and fluoxetine, 3.1×10^{-6} M. The IC_{50} of serotonin receptor antagonists were as follows: cinanserin, 5.0×10^{-4} M; and cyproheptadine, 6.2×10^{-4} M; methysergide was soluble only up to 10^{-4} M final concentration which did not inhibit platelet serotonin uptake. The IC_{50} of tryptamine was 6.9×10^{-5} M.

In contrast, serotonin receptor antagonists were more effective than serotonin uptake antagonists in inhibiting serotonin-induced platelet shape change and aggregation. The IC_{50} values of serotonin uptake and receptor antagonists on serotonin-induced platelet shape change and aggregation are listed in Table 1.

This study demonstrates a serotonin binding in human platelets which is saturable, rapid, reversible, and specific. Scatchard plot analysis revealed one class of binding sites with an apparent K_D of 6.3×10^{-8} M.

Platelet serotonin binding would have been difficult to assess if serotonin uptake had occurred in the experimental procedure. Serotonin uptake would have been extremely unlikely in our experiments since they were carried out at 4° which has been shown to reduce uptake drastically [7]. Most likely, serotonin would not have been displaced by low concentrations of uptake inhibitors if the amine had been taken up.

The binding demonstrated in this study appears to represent binding to the serotonin uptake site on the platelet surface rather than binding to the receptor site responsible for serotonin-induced shape change and aggregation. The evidence for this interpretation is that the uptake inhibitors were considerably more effective than the serotonin receptor antagonists in inhibiting binding. Also, the relative effectiveness of the various inhibitors was similar for both serotonin binding and uptake with the following relative ability to inhibit: chlorimipramine > imipramine > fluoxetine > cinanserin > cyproheptadine. Methysergide, considered to be a potent receptor antagonist, did not inhibit platelet serotonin uptake and also was far less effective than cinanserin or cyproheptadine in inhibiting platelet serotonin binding.

Our study confirms previous observations of two separate receptor sites for serotonin on the platelet surface, one for serotonin-induced shape change and aggregation and the other for serotonin uptake. This conclusion is based on pharmacological data which demonstrate that serotonin receptor antagonists are considerably more effective than serotonin uptake inhibitors in inhibiting platelet shape change and aggregation. There may be subtle differences in the serotonin-platelet interaction responsible for serotonin-induced shape change and aggregation since methysergide inhibited serotonin-induced aggregation more effectively than shape change. Also, tryptamine was a more effective inhibitor of shape change than of aggregation.

The investigation of serotonin binding in rat platelets suggested three binding sites [4]. The K_D of the highest affinity site in rat platelets was similar to that demonstrated in human platelets in our study. Cinanserin was more effective in displacing bound serotonin and inhibiting shape change in rat platelets [4] than it was in our study with human platelets. In the study using rat platelets, the relative effects of serotonin receptor antagonists and uptake inhibitors on binding were calculated by their ability to decrease cinanserin-sensitive binding [4]. In our study, the IC_{50} was calculated from the concentration of drug that inhibited either serotonin binding, shape change and aggregation, or uptake. Therefore, variance between the two studies in the effects of inhibition of serotonin-platelet interaction by drugs could be attributed to

differences in methodology. Also, serotonin binding in human platelets may differ from that in rat platelets.

There are several possibilities why this study did not demonstrate the binding of serotonin to the receptor site responsible for shape change and aggregation: (1) the specific activity of the ligand might not have been sufficient to detect extremely small numbers of receptors for shape change and aggregation; (2) leakage of minute amounts of endogenous serotonin may have occurred during the incubation conditions which diluted low concentrations of labeled serotonin or occupied serotonin receptor sites, and (3) the incubation of platelets at 4° altered the binding site for shape change. This possibility is not likely because serotonin receptor binding is easily identified in nerve tissue when incubated at 4°.

This study did identify binding to the serotonin uptake site which will enable us to identify the role of specific membrane components in platelet serotonin receptor binding.

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The effects of incorporation into microsomes of purified NADPH-cytochrome *c* (P-450) reductase on drug oxidations

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The current view of the liver microsomal monooxygenase enzyme system is that in the microsomal membranes cytochrome P-450 catalyzes the biotransformation of a variety of drugs, toxic compounds including carcinogens, and endogeneous substrates such as steroids and fatty acids utilizing molecular oxygen and electrons [1–5]. Recent efforts made by several laboratories have realized the almost complete purification of cytochrome P-450 and NADPH-cytochrome *c* (P-450) reductase [6–11]. The determination of molecular weight and specific activity of these purified enzymes has led to the conclusion that cytochrome P-450 is present in 10 to 25 times larger amounts than NADPH-cytochrome *c* (P-450) reductase in microsomal membranes, and that at least six species of cytochrome P-450 exist in microsomes from phenobarbital- and 3-methylcholanthrene-treated rats [12]. As regards the possibility of whether or not NADPH-cytochrome *c* (P-450) reductase is the rate limiting enzyme in drug oxidations in whole microsomes, not many reports have appeared. From results obtained using reconstituted mixed function oxidase system, Kamataki *et al.* [10] proposed that in whole microsomes NADPH-cytochrome *c* (P-450) reductase rather than cytochrome P-450 is the rate limiting enzyme for benzphetamine *N*-demethylation. In support of this hypothesis, quite recent reports by Miwa and Cho [13] and Miwa *et al.* [14] demonstrated that a detergent solubilized NADPH-cytochrome *c* (P-450) reductase was incorporated into microsomes to enhance drug oxidation activities. If cytochrome P-450 and NADPH-cytochrome *c* (P-450) reductase are not rigidly organized in the membranes as mentioned by Yang and Strickhart [15] and Yang [16], then it seems reasonable to hypothesize that multiple species of cytochrome P-450 compete with each other in functional binding to the limited amount of NADPH-cytochrome *c* (P-450) reductase in microsomal membranes.

Thus, in this paper we would like to report the results of the experiments showing the effects of fortification of rat liver microsomes with purified NADPH-cytochrome *c* (P-450) reductase to further support the idea that there is an order in the cytochrome P-450 species for receiving electrons from limited NADPH-cytochrome *c* (P-450) reductase in microsomal membranes.

Materials and methods. Male rats of Cr1:CD(SD) strain weighing 120 to 150 g were used throughout this study. The animals, which were maintained on a commercial rat chow, CE-2 Nippon Clea Co., Japan, were starved for about 20 hr prior to sacrifice, but were given tap water *ad lib.* When necessary, intraperitoneal (i.p.) injection of 3-methylcholanthrene (25 mg/kg) dissolved in olive oil and subcutaneous (s.c.) injection of phenobarbital (80 mg/kg) dissolved in saline were conducted simultaneously once a day for three days.

DEAE-Sephadex (A-50) and 2′,5′ ADP-Sepharose 4B were purchased from Pharmacia Fine Chemicals Co., and hydroxylapatite from Bio-Rad. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Grade I) and cytochrome *c* (horse heart) were purchased from Boehringer Mannheim. Emulgen 913, a non-ionic detergent, was kindly provided by Kao-Atlas Co. Commercial aniline was redistilled under vacuum and the distillate was stored at about −10° under an atmosphere of nitrogen. Other chemicals were of highest purity commercially available. Microsomes were prepared as described previously [17]. Protein was determined by the method of Lowry *et al.* [18] using bovine serum