Short Communications

Specific Binding Sites for 5-Hydroxytryptamine on Rat Blood Platelets

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(Received 21 March 1975)

5-Hydroxytryptamine changes the shape of rat blood platelets by combination with a cinanserin-sensitive receptor which is not associated with the active uptake of 5-hydroxytryptamine. Binding of 5-hydroxy[³H]tryptamine to platelets at 4°C demonstrates the presence of three saturable sites, and the highest-affinity site is apparently this 5-hydroxytryptamine receptor.

The ability of blood platelets to accumulate 5-hydroxytryptamine by active transport is well documented (for a review see Sneddon, 1973). Also, when 5-hydroxytryptamine is added to platelet-rich plasma, the platelets rapidly change in shape, and those of some animal species go on to form aggregates (Mitchell & Sharp, 1964; O'Brien & Heywood, 1966; Mills, 1970). Baumgartner & Born (1968) proposed that these stimulatory effects of 5-hydroxytryptamine on the platelet were the result of 5-hydroxytryptamine uptake through the plasma membrane (Baumgartner & Born, 1968, 1969; Baumgartner, 1969). In later experiments, Born *et al.* (1972) demonstrated that some analogues of 5-hydroxytryptamine, e.g. 5-methoxy- α -methyltryptamine, were more potent platelet stimulants than was 5-hydroxytryptamine, although they are not actively transported by the cell. This strongly suggests that there are at least two separate receptors for 5-hydroxytryptamine on platelets. To investigate this possibility we studied the platelet shape-change induced by both 5-hydroxytryptamine and 5-methoxy- α -methyltryptamine and compared the potency of inhibitors against shape-change and 5-hydroxytryptamine uptake. We have also correlated the binding of radioactively labelled 5-hydroxytryptamine to platelets with its stimulatory effect. Our results indicate that there are three receptors for 5-hydroxytryptamine on blood platelets, one of which is responsible for its stimulatory effect.

Materials and methods

All drugs used in this study were dissolved in 0.9% NaCl. 5-Hydroxytryptamine creatinine sulphate and 5-hydroxyindol-3-ylacetic acid were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. DL-5-Methoxy- α -methyltryptamine hydrochloride was given by I.C.I. Pharmaceuticals, Macclesfield, Cheshire, U.K. Cinanserin hydrochloride was given

by Squibb Research Laboratories, Princeton, N. J., U.S.A. Cyproheptadine hydrochloride was given by Merck, Sharp and Dohme, Hoddesdon, Herts., U.K. Compound Lilly 110140, 3-(*p*-trifluoromethylphenoxy)-*N*-methyl-3-phenylpropylamine, was given by Lilly Research Laboratories, Indianapolis, Ind., U.S.A. Chloroimipramine hydrochloride and imipramine hydrochloride were gifts from Geigy Pharmaceuticals, Macclesfield, Cheshire, U.K.

The platelet shape-change was measured photometrically in rat platelet-rich citrated plasma (Gordon & Drummond, 1974). Samples (0.1 ml) were preincubated at 37°C for 60s with the drugs or an equivalent volume of 0.9% NaCl, then stirred for 30s with 5-hydroxytryptamine or 5-methoxy- α -methyltryptamine, and the shape-change was measured as the maximum rate of decrease in light transmission.

The active uptake of 5-hydroxytryptamine was estimated by using 5-hydroxy[G-³H]tryptamine creatinine sulphate (500mCi/mmol; supplied by The Radiochemical Centre, Amersham, Bucks., U.K.). Samples (0.1 ml) of platelet-rich plasma were preincubated with drugs or 0.9% NaCl for 60s at 37°C before addition of 0.8 μ M-5-hydroxy[³H]tryptamine. After 120s incubation with 5-hydroxytryptamine, 0.4 ml of ice-cold 0.4% EDTA in 0.9% NaCl was added and the samples were centrifuged (120s at 14700g). The platelet pellet was washed with 1 ml of ice-cold 0.4% EDTA in 0.9% NaCl and then digested at 60°C for 10 min with 0.5 ml of 19M-formic acid before transfer to a counting vial containing 10 ml of scintillant [toluene containing 0.33% 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole plus 30% (v/v) ethoxyethanol]. Radioactivity was measured in a Nuclear-Chicago Mark 2 liquid-scintillation counter.

Receptor binding of 5-hydroxy[³H]tryptamine was measured by using the same technique as for the active-uptake studies, except that all steps until formic acid digestion were carried out at 4°C. Preliminary experiments indicated that binding was maximal by

2 min. EDTA was added during the separation step in these experiments in order to prevent the loss of endogenous platelet 5-hydroxytryptamine, which can occur during centrifugation. Extracellular space in the platelet pellet was estimated by performing parallel experiments in the presence of a 100-fold excess of unlabelled 5-hydroxytryptamine. Under these conditions, less than 5% of the radioactivity remained in the pellet, and this value was subtracted from the total 5-hydroxy[^3H]tryptamine bound in order to correct for extracellular space.

Results and discussion

The concentrations of 5-hydroxytryptamine and 5-methoxy- α -methyltryptamine which produced a half-maximal shape-change response (EC_{50}) were $0.8\mu\text{M}$ and $0.2\mu\text{M}$ respectively. Using these agonist concentrations, we compared the effects of cinanserin, a specific 5-hydroxytryptamine antagonist in other tissues (Rubin *et al.*, 1964), and compound Lilly 110140, an inhibitor of 5-hydroxytryptamine uptake (Wong *et al.*, 1974). For both 5-hydroxytryptamine and 5-methoxy- α -methyltryptamine, the concentration of cinanserin which inhibited the shape-change by 50% (IC_{50}) was $0.003\mu\text{M}$. The IC_{50} for compound Lilly 110140 was $4\mu\text{M}$ against 5-hydroxytryptamine and $1.5\mu\text{M}$ against 5-methoxy- α -methyltryptamine. When we investigated the effects of these inhibitors on the uptake of 5-hydroxy[^3H]tryptamine ($0.8\mu\text{M}$) by platelets, the IC_{50} values were $0.45\mu\text{M}$ for compound Lilly 110140 and $100\mu\text{M}$ for cinanserin. These results demonstrate that cinanserin is much more potent at inhibiting shape-change than uptake, whereas compound Lilly 110140 is a more effective inhibitor of uptake than of shape-change.

It has been previously found that condensation of 5-methoxytryptamine with *o*-phthalaldehyde produces an even more intensely emitting fluorophore than that formed between 5-hydroxytryptamine and *o*-phthalaldehyde (Maickel & Miller, 1966). We found that 5-methoxy- α -methyltryptamine formed a fluorophore with similar characteristics (maximum excitation 360 nm, emission 462 nm; uncorrected instrument readings) and made use of this property to investigate the interaction of 5-methoxy- α -methyltryptamine with blood platelets, based on the assay procedure for 5-hydroxytryptamine that we developed (Drummond & Gordon, 1974). Born *et al.* (1972), who measured the native fluorescence of 5-methoxy- α -methyltryptamine at neutral pH, could not detect any uptake by the platelets, and our results confirmed this observation. However, because of the sensitivity of the *o*-phthalaldehyde assay, we were able to use concentrations of 5-methoxy- α -methyltryptamine below $1\mu\text{M}$, and under these conditions we could detect binding of 5-methoxy- α -

methyltryptamine to the platelets. This binding was essentially instantaneous, and was unaffected by temperature within the range 4–37°C. The amount bound at saturation was about 7 pmol/ 10^8 cells. A concentration ($10\mu\text{M}$) of 5-methoxy- α -methyltryptamine that produced a maximal shape-change and gave maximal saturable binding did not significantly inhibit the uptake of 5-hydroxytryptamine.

The relative lack of effect of 5-methoxy- α -methyltryptamine or cinanserin on 5-hydroxytryptamine uptake suggests that a specific receptor for 5-hydroxytryptamine exists on the platelet that is distinct from the uptake site, and that is responsible for initiating the platelet shape-change. Inhibition of the shape-change by high concentrations of compound Lilly 110140 indicates merely that the platelet is similar to other 5-hydroxytryptamine-sensitive

Fig. 1. Scatchard analysis of the binding of 5-hydroxy- ^3H tryptamine to rat blood platelets at 4°C

5-Hydroxy[^3H]tryptamine in the concentration range 0.001 – $10\mu\text{M}$ was incubated with 0.1 ml portions of rat citrated platelet-rich plasma containing 0.1 mM-5-hydroxytryptamine (unlabelled) or an equivalent volume of 0.9% NaCl for 120 s at 4°C. The amount of 5-hydroxy[^3H]tryptamine binding to platelets (7.85×10^7 cells) under both conditions was measured as described in the text and the value obtained in the presence of 0.1 mM-5-hydroxytryptamine (unlabelled) was subtracted to give specific binding. B , Amount of specifically bound 5-hydroxytryptamine (pmol/ 10^8 cells); C , molar concentration of 5-hydroxytryptamine; K , apparent dissociation constant. Calculated values for K are: site A, $2.3 \times 10^{-8}\text{M}$; site B, $1.5 \times 10^{-7}\text{M}$; site C, $2.0 \times 10^{-6}\text{M}$. The calculated capacity of each site for 5-hydroxytryptamine (number of molecules binding at saturation/cell) is: at site A, 6600; at site B, 1800; at site C, 57300.

Table 1. Effects of 5-hydroxytryptamine antagonists on the binding of 5-hydroxy[³H]tryptamine to rat blood platelets and on the platelet shape-change induced by 5-hydroxytryptamine

Samples (0.1 ml) of platelet-rich plasma were preincubated for 60 s at 37°C with drugs or 0.9% NaCl (10 μ l) before being stirred (800 rev./min) with 0.8 μ M 5-hydroxytryptamine. The shape-change velocity was measured as described in the text. The drug concentration that decreased the shape-change velocity by 50% was calculated. For the binding studies, 0.1 ml of platelet-rich plasma was preincubated for 1 min at 4°C with drugs or 0.9% NaCl (10 μ l) before the addition of 0.04 μ M 5-hydroxy[³H]tryptamine in 5 μ l. After 2 min, 0.4 ml of ice-cold 0.4% EDTA in 0.9% NaCl was added, and the sample was immediately centrifuged (120 s at 14700g). ³H radioactivity in the platelet pellet was measured as described in the text. Results were corrected for non-specific binding as indicated in Fig. 1. In each experiment the 5-hydroxy[³H]tryptamine which could be displaced by cinanserin (0.0008–0.8 μ M) was measured, and the amount of this cinanserin-sensitive binding that remained in the presence of several concentrations of the other antagonists was estimated. The concentrations of those compounds that decreased cinanserin-sensitive binding by 50% are shown below.

Compound	IC ₅₀ value (μ M) against	
	5-Hydroxytryptamine-induced shape-change	5-Hydroxy[³ H]tryptamine binding at 4°C
Cinanserin	0.0028	0.0030
Cyproheptadine	0.0030	0.0010
Imipramine	0.25	0.10
Chloroimipramine	0.40	0.50
Compound Lilly 110140	4.0	0.70
5-Hydroxyindol-3-ylacetic acid	No effect at 50 μ M	No effect at 50 μ M

tissues, in which uptake inhibitors at high concentrations can act as 5-hydroxytryptamine antagonists (Domenjoz & Theobald, 1959). In summary, our results so far are consistent with the view that stimulation of the platelet by 5-hydroxytryptamine can proceed independently of any interaction with the uptake site. This is directly opposed to the hypothesis proposed by Baumgartner & Born (1968), but is entirely consistent with the data of Born *et al.* (1972). These latter workers concluded that the uptake site for 5-hydroxytryptamine had stringent structural requirements, whereas the receptor concerned with the shape-change had lower structural specificity; however, they reached no conclusions about the possibility of these sites existing as separate entities, nor about the functional interaction of uptake and stimulation. To clarify these points we measured the binding of labelled 5-hydroxytryptamine to platelets at 4°C.

Under these conditions no active transport occurs, and binding of 5-hydroxy[³H]tryptamine within the concentration range 0.001–10 μ M revealed the existence of three sites (Fig. 1). Although in Fig. 1 the apparent dissociation constant for site A is derived from only three points, the value of 2×10^{-8} M for this constant was substantiated in several other experiments. Since cinanserin is a potent inhibitor of the shape-change, it was important to test whether the binding of 5-hydroxytryptamine to any of these sites could be blocked by cinanserin. The amount of 5-hydroxytryptamine interacting with each of the three sites was calculated for a range of 5-hydroxy-

tryptamine concentrations. Binding experiments were then performed by using these concentrations and the 5-hydroxy[³H]tryptamine that could be displaced by cinanserin was determined in each case. The cinanserin-sensitive binding always corresponded to the amount of 5-hydroxytryptamine calculated to interact with the highest-affinity site ($K = 2 \times 10^{-8}$ M).

IC₅₀ values for cinanserin against this 5-hydroxy[³H]tryptamine binding and the 5-hydroxytryptamine-induced shape-change were almost identical, being 0.0028 and 0.0030 μ M respectively. When several 5-hydroxytryptamine antagonists were tested, each was almost equipotent against 5-hydroxy[³H]tryptamine binding and the 5-hydroxytryptamine-induced shape-change (Table 1). 5-Hydroxyindol-3-ylacetic acid, a metabolite of 5-hydroxytryptamine that neither induces the shape-change itself nor inhibits that induced by 5-hydroxytryptamine, did not affect 5-hydroxy[³H]tryptamine binding, even at 50 μ M.

The close correlation between the inhibition of cinanserin-sensitive 5-hydroxy[³H]tryptamine binding and the inhibition of the platelet shape-change suggests that the binding of 5-hydroxy[³H]tryptamine to this site represents an interaction that initiates the platelet shape-change. To our knowledge, this is the first demonstration of a correlation between binding of 5-hydroxytryptamine and the production of a physiological response in any tissue. Further studies of this type may help to elucidate the basic mechanisms that underly the physiological effects of 5-hydroxytryptamine in other tissues.

A. H. D. is an M.R.C. Scholar. This work was supported in part by a grant from Beecham Research Laboratories.

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