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UPTAKE OF 5-HYDROXYTRYPTAMINE BY RAT BLOOD PLATELETS AND ITS INHIBITION BY ADENOSINE 5′-DIPHOSPHATE

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1 The uptake of 5-hydroxytryptamine (5-HT) by rat blood platelets in citrated plasma was linear for at most 10 s and was substantially complete within 3 minutes.

2 Adenosine 5′-diphosphate (ADP) was a potent inhibitor of 5-HT uptake ($K_i = 0.38$ μM) and kinetic analysis revealed that the inhibition was not competitive.

3 Inhibition of 5-HT uptake by ADP was abolished in the presence of prostaglandin E_1 and 2-*n*-amylthio-AMP, which also inhibit the stimulant actions of ADP on blood platelets.

4 It is concluded that ADP could inhibit 5-HT uptake by changing the Na^+/K^+ distribution across the cell membrane, and the biological significance of this is discussed.

Introduction

Adenosine 5′-diphosphate (ADP) and 5-hydroxytryptamine (5-HT) exert stimulatory effects on blood platelets, that is, they cause the cells to change in shape and, under appropriate conditions, to form aggregates (Gaarder, Jonsen, Laland, Hellem & Owren, 1961; Mitchell & Sharp, 1964). These agents exert their effects by interacting with specific recognition sites on the cell membrane (Born, 1965; Baumgartner & Born, 1968; Nachman & Ferris, 1974; Drummond & Gordon, 1975). Platelets also contain a highly efficient uptake process for 5-HT (for review see Sneddon, 1973), and this would be expected to reduce the stimulatory effect of the amine.

Both ADP and 5-HT are stored in platelet intracellular organelles and are released in parallel when platelets are stimulated by agents such as collagen or thrombin (Holmsen, Day & Stormorken, 1969). Since the growth of a haemostatic plug is promoted by the stimulatory effects of extracellular ADP and 5-HT, the interaction of these agents at the platelet membrane is of some biological significance. We have found that ADP is a potent inhibitor of 5-HT uptake by rat blood platelets and suggest that this interaction could contribute to the efficiency of the haemostatic process.

Methods

Preparation of platelet-rich plasma (PRP)

PRP was prepared from citrated rat blood as described previously (Gordon & Drummond, 1974). Platelet counts in PRP ranged from 0.8 to $1{\cdot}2 \times 10^9$ cells/ml.

Uptake of 5-hydroxy [^{3}H] tryptamine by platelets

Uptake of 5-HT was measured by incubating 0.1 ml samples of PRP at 37°C with 0.2 to 2 μM 5-hydroxy [^{3}H] tryptamine ([^{3}H]-5-HT). PRP samples were pre-incubated for 60 s at 37°C before adding [^{3}H]-5-HT in a volume of 5 μl and briefly vortex-mixing. After 10 s incubation with [^{3}H]-5-HT, 0.5 ml volumes of ice-cold iso-osmotic saline containing 0.4% w/v disodium edetate (EDTA) were added and samples centrifuged for 30 s at 14,700 *g* in a Quickfit microcentrifuge; control experiments showed that the ice-cold EDTA-saline halted 5-HT uptake. The supernatants were decanted and the platelet pellets washed once with 1.0 ml volumes of ice-cold EDTA-saline; the amount of ^{3}H removed by washing was comparable with that in control experiments using samples of cell-free plasma. The pellets were then digested by incubation at 80°C for 10 min with 0.5 ml volumes of 19 M formic acid, and transferred to vials containing 10 ml of scintillant (toluene with 0.33% w/v 5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa,3,4 diazole (butyl PBD) plus 30% (v/v ethoxyethanol). Radioactivity was measured in a Nuclear Chicago Mk. 2 liquid scintillation counter. In some experiments uptake of [^{3}H]-5-HT was measured as the decrease in supernatant radioactivity after separation of platelets and plasma by centrifugation (see legend of Figure 1).

Measurement of endogenous 5-hydroxytryptamine

Endogenous 5-HT in platelets and plasma was measured fluorimetrically after condensation with *o*-phthaldialdehyde as previously described (Drummond & Gordon, 1974).

Materials

5-Hydroxy [G-^{3}H] tryptamine creatinine sulphate (0.5 Ci/mmol), from the Radiochemical Centre, Amersham, Bucks., was dissolved in 0.2 M sodium acetate-acetic acid buffer, pH 5.5, to a concentration of 10^{-4} M and stored in 0.5 ml volumes at −20°C. It was diluted before use to the required concentration with 0.5 M Tris(hydroxymethyl)aminomethane buffer, pH 7.4. ADP and EDTA, from the Sigma Chemical Co., Kingston-upon-Thames, Surrey were dissolved in iso-osmotic saline. Prostaglandin E_1 (Upjohn Co., Kalamazoo, Michigan, U.S.A.) was dissolved and stored as described previously (MacIntyre & Gordon, 1974). Indomethacin (Merck, Sharp & Dohme, Hoddesdon, Herts) was dissolved in 0.5% w/v sodium carbonate (pH 10.3). The pH of the solution was adjusted to 7.5 by addition of 1 N HCl and further dilutions were made with iso-osmotic saline. 2-*n*-Amylthio AMP (Kohjin Co. Ltd., Saiki, Oita, Japan) was dissolved in iso-osmotic saline.

Results

Uptake of 5-hydroxy [^{3}H]-tryptamine

Using substrate concentrations of 0.5 to 2.0 μM, uptake of [^{3}H]-5-HT was approximately linear for 10 s and was largely completed within 3 min (Figure 1). To determine whether any exchange occurred between endogenous platelet 5-HT and added [^{3}H]-5-HT during the uptake process, the total 5-HT in the cell-free supernatant was measured fluorimetrically over a 30 min period in aliquots of the samples taken for radioactivity measurements. Changes in total 5-HT paralleled the changes in radioactivity, indicating that the uptake process was not accompanied by measurable 5-HT exchange (Figure 1). Lineweaver-Burk analysis revealed that the uptake process was saturable; the K_m value for 5-HT uptake by rat blood platelets was 0.83 ± 0.29 μM (mean ± s.e.; $n = 9$) and the V_{max} was 63.2 ± 23.9 pmol/10^8 cells per 10 seconds. These values were derived from 9 separate experiments, and the results of one experiment using a small range of 5-HT concentrations are shown in Figure 2.

Inhibition of uptake by adenosine 5'-diphosphate

When PRP samples were pre-incubated for 60 s at 37°C with ADP (0.4 to 3.3 μM) before the addition of [^{3}H]-5-HT, uptake was inhibited. The pattern of inhibition was not competitive (Figure 2). Analysis of the data in Figure 2 by the method of Plowman (1972) revealed that the K_i for ADP was 0.38 μM. Inhibition of [^{3}H]-5-HT uptake by ADP was short-lived. When PRP samples were pre-incubated with ADP, inhibition

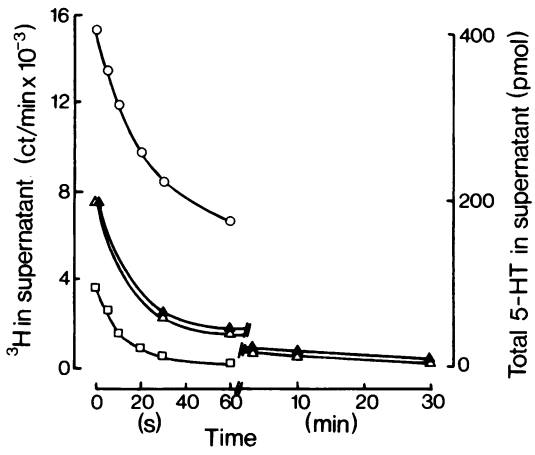

Figure 1 Rate of uptake of 5-hydroxytryptamine (5-HT) by rat blood platelets in citrated platelet-rich plasma (PRP). PRP (0.2 ml) was pre-incubated at 37°C for 1 min before the addition of [^{3}H]-5-HT. At each time-point 0.5 ml of ice-cold 0.4% w/v EDTA in saline was added and samples were spun at 14,700 *g* for 30 seconds. Samples (0.25 ml) of the supernatant were taken for total 5-HT assay (closed symbols) and 0.1 ml samples were taken for the measurement of radioactivity (open symbols) as described in the methods section. Initial [^{3}H]-5-HT concentrations added were: (○) 2 μM, (△) 1 μM, (□) 0.5 μM. Each point is the mean of duplicate determinations.

of 5-HT uptake decreased with increasing time of pre-incubation. In the presence of 1.66 μM ADP uptake had returned to control levels after 10 min; using 6.6 μM, 5-HT uptake was inhibited by 85% after 1 min pre-incubation with ADP, 53.6% inhibited after 10 min and 34.4% inhibited after 20 min (Figure 3).

Modification of the inhibitory effect of adenosine 5'-diphosphate by drugs

The observed inhibition of [^{3}H]-5-HT uptake by ADP could conceivably have resulted from ADP releasing endogenous platelet 5-HT, but when rat platelets were exposed to ADP in the concentration range used for these experiments we could detect no release of platelet 5-HT.

In support of this conclusion, incubation of PRP samples with drugs which inhibit the release of platelet constituents (e.g. 0.1 mM indomethacin) did not affect the inhibition of [^{3}H]-5-HT uptake by ADP. However, agents which inhibit the direct platelet-stimulating action of ADP (0.6 μM prostaglandin E_1; 20 μM 2-*n*-amylthio AMP) abolished the inhibition of [^{3}H]-5-HT uptake by ADP (Table 1). 2-*n*Amylthio AMP (0.1 mM) and prostaglandin E_1 (0.6 μM) had no direct effect on the uptake of [^{3}H]-5-HT.

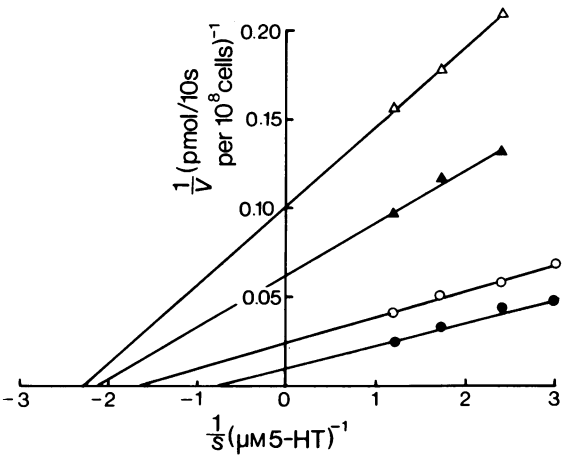

Figure 2 Double reciprocal analysis of the effect of adenosine 5′-diphosphate (ADP) on 5-hydroxytryptamine (5-HT) uptake by rat platelets. Initial velocity (V) is expressed as [^{3}H]-5-HT uptake in units of pmol/10 s per 10^8 cells. [^{3}H]-5-HT concentrations used in the experiment shown were in the range 0.3 to 0.8 μM, but in other experiments concentrations up to 2 μM were used. Additions were: (●) saline; (○) 0.415 μM ADP; (▲) 1.66 μM ADP; (△) 3.32 μM ADP. Lines were constructed by linear regression analysis, and each point is the mean of duplicate determinations.

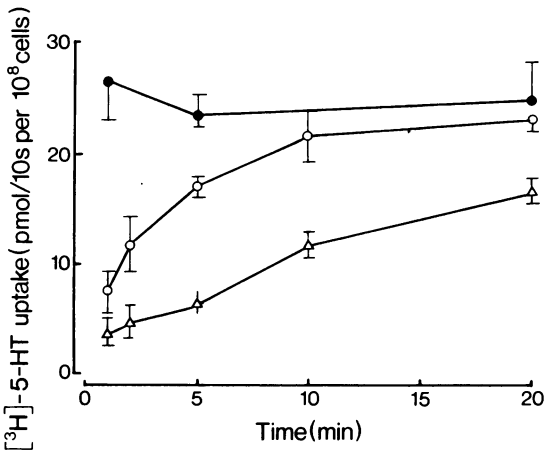

Figure 3 Adenosine 5′-diphosphate (ADP) inhibition of 5-hydroxytryptamine (5-HT) uptake by rat blood platelets: effect of pre-incubating ADP in platelet-rich plasma (PRP). PRP was pre-incubated at 37°C with: saline (●); 1.6 μM ADP (○); or 6.6 μM ADP (△); for 1, 2, 5, 10 or 20 min as indicated before addition of 0.415 μM [^{3}H]-5-HT. Uptake was measured as described in the text. Each point is the mean of triplicate determinations, and the vertical bars show the range of values obtained (in three cases, only one half of the range is indicated).

Table 1 Adenosine 5′-diphosphate (ADP) inhibition of 5-hydroxytryptamine (5-HT) uptake by rat blood platelets: effect of drugs

Compound (μM)	*% inhibition of 5-HT uptake by ADP*
Saline	96
2-*n*-Amylthio AMP (1)	95
2-*n*-Amylthio AMP (10)	46
2-*n*-Amylthio AMP (20)	8
Prostaglandin (0.03)	93
Prostaglandin (0.15)	59
Prostaglandin (0.60)	16
Indomethacin (10)	96
Indomethacin (30)	94
Indomethacin (100)	97

PRP (0.1 ml) was pre-incubated at 37°C for 1 min with drugs or an equivalent volume of isotonic saline prior to the addition of 10 μM ADP or saline. One min later, 0.6 μM [^{3}H]-5-HT was added and the uptake was terminated after 10 s by the addition of EDTA-saline. Uptake was measured as described in the text. Each value is the mean of triplicate determinations on PRP samples from at least two rats.

Discussion

The uptake of submicromolar concentrations of [^{3}H]-5-HT by rat blood platelets in citrated plasma was largely complete within 1 min, with a linear uptake component which lasted, at most, 10 seconds. These findings are in close agreement with the results of Buczko, de Gaetano & Garattini (1975), who studied uptake of [^{14}C]-5-HT by rat platelets in citrated plasma. Sneddon (1969), using rat platelets washed free of plasma and suspended in a protein-free salt solution, reported that uptake of [^{3}H]-5-HT was linear for at least 12 min, but analysis of his data reveals that the rate of 5-HT uptake was much lower in his experiments than in ours. The reason for this may be that the platelets were damaged during the washing procedure or that the medium in which they were finally suspended lacked some plasma component essential for the efficient function of the 5-HT uptake process.

Although the results of the present study reveal a hitherto unrecognized action of ADP on blood platelets, which could be of some biological significance, the mechanism of action remains unclear.

5-HT uptake by blood platelets is an active process obeying Michaelis-Menten kinetics which apparently utilizes the energy inherent in the spontaneous movement of Na^+ and K^+ across the platelet membrane. Platelets have low sodium and high potassium levels relative to plasma and if this asymmetric ion distribution is not maintained the 5-HT uptake process does not function efficiently (Sneddon, 1969; 1971). The uptake of 5-HT by platelets can be inhibited by agents which combine directly with the uptake site, such as chlorimipramine and other tricyclic anti-depressants. Kinetic studies of the effect of these compounds have invariably shown the inhibition to be competitive in nature (Stacey, 1961; Yates, Todrick & Tait, 1964; Tuomisto, 1974) and we can therefore exclude the possibility that ADP is acting in this manner. Uptake can be non-competitively inhibited by agents which block glycolysis (Hughes & Brodie, 1959; Weissbach & Redfield, 1960; Sneddon, 1971; Campbell & Todrick, 1973) or by inhibitors of $(Na^+ + K^+)$-ATP-ases, such as ouabain (Weissbach, Redfield & Titus, 1960; Sneddon, 1971; Campbell & Todrick, 1973). Both these types of inhibitor appear to act by interfering with the asymmetric ion distribution across the membrane which is necessary for efficient 5-HT uptake (Tissari, Schönhöfer, Bogdanski & Brodie, 1969; Sneddon, 1971).

At physiological pH values ADP is an impermeant anion, and available evidence indicates that its stimulant effects on platelets are mediated by specific interaction with sites on the exterior of the cell membrane (Nachman & Ferris, 1974). The rapid onset of ADP's inhibitory effect on 5-HT uptake is consistent with a similar mode of action, and the reduced potency of ADP when preincubated in PRP could be the result of its degradation by plasma enzymes. Moake, Ahmed, Bachur & Gutfreund (1970) reported that ADP was a potent non-competitive inhibitor of platelet membrane ATP-ase activity, and suggested that this might mediate its stimulant action. Inhibition of the ATP-ase should alter the distribution of Na^+ and K^+ across the platelet membrane, and indeed Feinberg, Scorer, Lebreton, Grossman & Born (1975) found that ^{22}Na uptake by platelets was rapidly and transiently stimulated by 10 μM ADP. It therefore seems possible that the stimulant effects of ADP and its inhibition of 5-HT uptake may both be linked to a change in trans-membrane ionic distribution.

In support of the concept that these actions of ADP share a common mechanism are our findings that inhibition of 5-HT uptake is blocked by prostaglandin E_1 and 2-*n*-amylthio AMP, at concentrations which also inhibit the platelet shape change induced by ADP. Furthermore, the concentration of ADP (0.1 to 0.2 μM) which induces a half-maximal platelet shape change in rat citrated PRP is close to its K_i value (0.38 μM) for inhibition of 5-HT uptake (Drummond & Gordon, unpublished observations). It is also conceivable that ADP might inhibit 5-HT uptake simply by inducing the platelets to change in shape, if this resulted in masking of uptake sites on the cell membrane. This seems most unlikely, however, because other agents (e.g. 5-methoxy-α-methyl-tryptamine) can induce a shape change in rat platelets without affecting 5-HT uptake (Drummond & Gordon, 1975). We can therefore conclude that inhibition of 5-HT uptake is a specific property of ADP, and not a general effect associated with the shape change.

Although further work is necessary to establish the mechanisms by which ADP inhibits 5-HT uptake, the immediate biological significance of this effect is evident: maintaining a higher extracellular 5-HT level helps promote the growth of platelet aggregates and can thus increase haemostatic efficiency. Of greater potential significance, however, is the possibility that this effect may not be restricted to platelets: the transmembrane Na^+/K^+ distribution regulates amine uptake in the central nervous system (CNS) as well as in platelets (Bogdanski, Tissari & Brodie, 1968; Tissari *et al.*, 1969) and stimulating Na^+ influx by batrachotoxin or veratridine inhibits dopamine uptake by rat striatal synaptosomes (Holz & Coyle, 1974). Since adenine nucleotides are released in conjunction with amine neurotransmitters, it would seem important to establish whether ADP can stimulate Na^+ influx in the CNS as well as in platelets (Feinberg *et al.*, 1975), and, if so, whether this provides a basis for control of transmitter re-uptake.

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