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IRREVERSIBLE AGGREGATION OF PIG PLATELETS AND RELEASE OF INTRACELLULAR CONSTITUENTS INDUCED BY 5-HYDROXYTRYPTAMINE

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Abstract—Platelet aggregation and the release of intracellular constituents induced by 5-hydroxytryptamine (5-HT) and by ADP were measured at 37° in stirred pig platelet-rich plasma (PRP) anticoagulated with citrate or heparin. In citrated PRP (with calcium partly chelated) aggregation responses to 0·1–10 μM ADP were reversible, but 50 μM ADP or more caused apparently irreversible aggregation. Aggregation induced by 5-HT was always small and reversible. In heparinised PRP, ADP was around 10 times more potent than in citrated PRP, but the pattern of responses was similar. 5-HT-induced aggregation in heparinised PRP was reversible below 1 μM, but higher concentrations caused irreversible aggregation and released adenine nucleotides from the platelets. 5-HT was more effective than was ADP at releasing platelet constituents. Responses in heparinised PRP were not due to the presence of heparin, but to the absence of a chelating anticoagulant: adding calcium to citrated PRP potentiated responses to 5-HT more than those to ADP.

Platelet aggregation induced by 5-hydroxytryptamine (5-HT) was first observed by Mitchell and Sharp [1] in citrated platelet-rich plasma (PRP) from man and rabbit. The aggregation response was transient and reversible. When reviewing the aggregating action of 5-HT in platelets from different species Mills [2] concluded that the cat was unique inasmuch as a biphasic aggregation response could be evoked by 5-HT, while in all other species studied the response to 5-HT was either small and reversible or absent. However, biphasic responses to 5-HT have also been found in a small percentage of human volunteers [3, 4]. It has been assumed that the second phase of this aggregation is associated with the selective release of platelet constituents such as adenine nucleotides and 5-HT since a similar biphasic response to ADP and adrenaline is accompanied by the platelet release reaction [5, 6].

Ionised calcium is a cofactor for platelet aggregation and the release reaction [7], and the ionised calcium concentration is more critical in the aggregation of pig platelets than in other species [8]. Most studies on platelet aggregation have used PRP anticoagulated with citrate, which reduces the ionised calcium concentration in plasma. The addition of small amounts of ionised calcium to citrated rabbit PRP enhances 5-HT-induced aggregation, and to a lesser extent that induced by ADP [1]. We have found that 5-HT induces little aggregation and no release in citrated pig PRP, but in heparinised pig PRP or citrated PRP to which small amounts of calcium have been added, 5-HT induces irreversible aggregation and release. Less release is induced by ADP than by 5-HT under these conditions. A preliminary report of part of this work has been presented [9].

MATERIALS AND METHODS

Preparation of PRP. Pig blood was collected from a local abattoir and anticoagulated with either 6% (v/v) acid–citrate–dextrose [10] or 5 units/ml heparin. PRP was prepared within 30 min of blood collection by differential centrifugation (1500 *g*; 80 sec).

Platelet aggregation. Aggregation was measured photometrically [11] in 0·5 ml volumes of PRP using a Bryston aggregometer (Polystan, Hitchin). After preincubation for 2 min at 37°, samples were stirred for 4 min at 800 rev/min with an aggregating agent or with an equal volume of isotonic saline. Aggregation responses were measured as the maximum change in light transmission units (LTU). In each experiment, light transmission was adjusted to read 10 LTU with unaggregated PRP and to a full-scale reading (120 LTU) with platelet-free plasma.

Measurement of released platelet constituents. After stirring with an aggregating agent or with isotonic saline, 0·5 ml PRP was diluted with 0·6 ml ice-cold 0·2% (w/v) EDTA in saline. Samples were centrifuged (3500 *g*; 15 min), the supernatant plasma was decanted, and the platelet pellet was resuspended in 1 ml glass-distilled water. After precipitation of proteins in platelets and plasma samples with ice-cold trichloroacetic acid (0·1 ml; 6 M), 0·25 ml of the protein-free supernatant was taken for measurement of 5-HT [12] or adenine nucleotides [13].

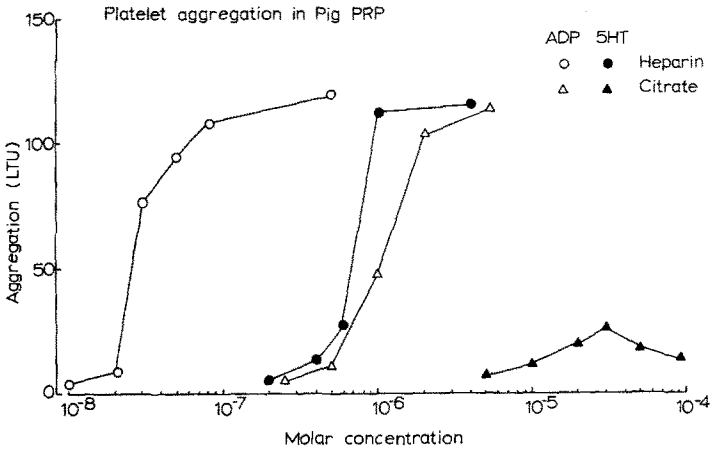

Fig. 1. Dose-response curves for platelet aggregation induced by ADP and 5-HT in pig PRP anticoagulated with heparin (○——○ ADP; ●——● 5-HT) or citrate (△——△ ADP; ▲——▲ 5-HT).

Materials. Recrystallized *o*-phthaldialdehyde [14] was dissolved (0·5% w/v) in ethanol and 10 volumes of 8 M HCl were added. This solution was stable for at least 2 months when stored at 4° in an amber bottle.

5-Hydroxytryptamine creatinine sulphate, ADP, and ATP were obtained from Sigma Chemical Co. (Kingston on Thames). Chloroacetaldehyde was obtained as the dimethyl acetal (R.N. Emanuel Ltd, London), and *o*-phthaldialdehyde was from British Drug Houses (Atherstone, Warwicks.).

RESULTS

In citrated PRP platelet aggregation was detectable with 0·1–0·2 μM ADP. Responses were wholly or partly reversible at concentrations below 10 μM, but with 50 μM or above, little or no disaggregation was observed during the 4-min period of observation. Aggregation induced by 5-HT in citrated PRP was always small and reversible: responses were detectable around 1 μM, increased slightly up to 30–40 μM, and decreased with higher concentrations.

In heparinised PRP, aggregation responses to ADP were qualitatively similar to those in citrated PRP but were produced by ADP concentrations 10–12 times lower. 5-HT caused small, reversible aggregation at 0·2–0·3 μM, but responses became biphasic around 1 μM and monophasic, apparently irreversible aggregation was obtained with all higher concentrations tested. Figure 1 shows dose–response curves for aggregation induced by ADP and 5-HT in citrated and heparinised PRP.

To establish that the different responses in citrated and heparinised PRP were mainly due to the difference in ionised calcium concentration, increasing amounts of calcium chloride (1·0–5 mM) were added to citrated PRP samples and the effects on aggregation responses to 5-HT (20 μM) and ADP (0·5 μM) were recorded. The control aggregation responses to the two agents were similar in size. 5-HT-induced aggregation was markedly enhanced by 1 mM calcium chloride: responses became biphasic in the presence of about 1·2 mM added calcium, and the addition of higher concentrations produced monophasic, apparently irreversible aggregation. ADP-induced aggregation was slightly increased by 1–2 mM calcium chloride but even after the addition of 5 mM calcium, responses were only moderately enhanced and did not become biphasic. Although the addition of more than 2·5 mM calcium chloride necessitated the addition of low concentrations (0·3 units/ml) of heparin to the plasma to prevent clotting, irreversible 5-HT-induced aggregation was observed at lower calcium concentrations without any added heparin; this indicates that the response to 5-HT was not a consequence of the presence of heparin.

Total adenine nucleotides in platelets and in plasma were measured in heparinised PRP before and after aggregation by 5-HT at concentrations of 0·5 μM (which produced reversible aggregation). 5 μM (biphasic aggregation), and 50 μM (monophasic, irreversible aggregation). The two higher concentrations of 5-HT released 20·7 and 23·7 per cent respectively of platelet adenine nucleotides, all of which were recovered from the supernatant plasma (Table 1).

A similar experiment was performed in which ADP (0·5, 5 and 50 μM) was the aggregating agent and the content of 5-HT in platelets and plasma was measured. All three concentrations of ADP caused aggregation which was apparently irreversible during the 4 min period of observation. The content of platelet 5-HT was reduced by 11–14 per cent in the samples treated with the two higher ADP concentrations, and a corresponding increase was found in the supernatant plasmas. Although the reduction in platelet 5-HT was not statistically significant in either group, the increase in supernatant 5-HT was significant at the 1 per cent level in both cases (Table 2).

Table 1. Adenine nucleotides in platelets and plasma, before and after aggregation induced by 5-HT

Sample	n	Nucleotide content (nmoles/10^8 cells) Platelets	Plasma	Release (%) from platelets
Control	39	10·22 ± 0·32	1·30 ± 0·10	—
+ 0·5 μM 5-HT	20	10·00 ± 0·46	1·94 ± 0·22*	2·1
+ 5·0 μM 5-HT	19	8·1 ± 0·47†	3·52 ± 0·20†	20·7
+ 50·0 μM 5-HT	19	7·8 ± 0·45†	3·74 ± 0·23†	23·7

Amounts are shown as means ± S. E.
Significance (unpaired *t*-test) vs control values: *P < 0·01; †P < 0·001; other values were not significantly (P > 0·05) different from controls.

Aggregation induced by 5 μM 5-HT in heparinised PRP was abolished when cyproheptadine (0·5 μM) or methysergide (0·2 μM) was added simultaneously with the 5-HT. When acetylsalicylic acid (0·4 mM) was preincubated in the PRP for 2 min at 37° before adding the 5-HT, only the second phase of aggregation was inhibited.

DISCUSSION

In the presence of physiological concentrations of ionised calcium, 5-HT at micromolar concentrations causes irreversible platelet aggregation and releases adenine nucleotides from pig platelets. Under similar conditions ADP also causes irreversible aggregation and releases 5-HT from the platelets. Although we used different markers to measure the release reaction induced by 5-HT and ADP, it is important to note that the percentage release induced by 50 μM 5-HT was twice that induced by the same concentration of ADP. We should have liked to use the same release marker for both aggregating agents but it is difficult to measure the release from platelets of any agent which had itself been used as the release inducer. It has previously been shown that during the platelet release reaction a higher percentage of platelet 5-HT than of platelet nucleotides is released [6]. Our results therefore indicate that, in pig PRP, 5-HT is a more potent inducer of the platelet release reaction than is ADP, provided that physiological levels of ionised calcium are present in the plasma.

In citrated pig PRP, ADP can induce irreversible platelet aggregation although higher concentrations are required than in heparinised PRP. Aggregation responses to 5HT, however, were small and reversible at all concentrations tested. The addition of 1–2 mM calcium chloride to pig citrated PRP potentiated aggregation responses to 5-HT more than those to ADP; Mitchell and Sharp [1] reported similar observations in rabbit PRP. No evidence for release induced by 5-HT has been previously reported, probably because citrated PRP is routinely used for platelet aggregation studies. Pig platelets are particularly well suited for studies on calcium-sensitive reactions because they show a greater functional dependence on ionised calcium than do platelets from other animal species [8]. As a consequence of this, however, restoring the physiological concentration of ionised calcium in PRP from other species may not cause such a dramatic change in aggregation responses to 5-HT and our findings may therefore not be directly applicable to human platelets. Besterman and Gillett [4] reported that 8 per cent of volunteers showed biphasic aggregation responses to 5-HT in citrated PRP; it might therefore be important to determine the percentage of 'biphasic responders' when the ionised calcium is not chelated.

Our results indicate that previous studies using citrated PRP may have underestimated the ability of 5-HT to induce platelet aggregation and the release reaction. In view of the importance of the platelet release reaction in haemostasis and thrombosis [15], we suggest that the role of 5-HT in platelet function merits critical re-examination.

Table 2. 5-HT in platelets and in plasma, before and after aggregation induced by ADP

Sample	n	5-HT content (μg/10^9 cells) Platelets	Plasma	Release (%) from platelets
Control	42	3·64 ± 0·25	0·23 ± 0·02	—
+ 0·5 μM ADP	25	3·40 ± 0·24	0·32 ± 0·02	6·6
+ 5·0 μM ADP	24	3·13 ± 0·23	0·55 ± 0·07*	14·0
+ 50·0 μM ADP	19	3·21 ± 0·24	0·55 ± 0·08*	11·8

Amounts are shown as means ± S.E.
* Significantly (P < 0·001; unpaired *t*-test) different from control; all other values were not significantly (P > 0·05) different from controls.

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