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Enhancement of ADP-induced aggregation by 5-HT in rabbit platelets[1]

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KEY WORDS serotonin; adenosine diphosphate; platelet aggregation; calcium; inositol 1,4,5-triphosphate

AIM: To study the enhanced effects of 5-hydroxytryptamine (5-HT) on ADP-induced aggregation. **METHODS**: Platelet aggregation was quantified by the light transmission, the cytosolic-free calcium ($[Ca^{2+}]_i$) was measured by digital fluorescent microscopy, and inositol 1,4,5-triphosphate (IP_3) was determined by receptor binding assay. **RESULTS**: In rabbit platelet-rich plasma (PRP), 5-HT 0.03 - 3 $\mu mol \cdot L^{-1}$ induced a decrease in light transmission (DLT) in a concentration-dependent manner with centralization of granules, as revealed by electron microscopy. The DLT was accompanied with neither platelet aggregation nor a release reaction. In single washed platelets loaded with Fura-2, 5-HT caused a concentration-dependent elevation of $[Ca^{2+}]_i$, and IP_3 level was also transiently increased in washed platelets at 15 s after stimulation by 5-HT. Adenosine diphosphate (ADP) also caused DLT transiently in PRP before its own aggregation without a release reaction. Pretreatment of PRP or washed platelets with 5-HT, the DLT by ADP was reduced concentration-dependently and ADP-induced aggregation and $[Ca^{2+}]_i$ mobilization were enhanced. **CONCLUSION**: The enhancement of ADP-induced aggregation was attributed to the superimposition of the calcium release from the storage sites and calcium influx induced by ADP over the calcium release from the storage sites by 5-HT.

5-Hydroxytryptamine (5-HT) is stored in the platelet with adenine nucleotides and calcium, and released upon suitable stimulation of platelets[1]. Aggregation has been induced by 5-HT in cats and pigs, but it is usually weak and reversible in human platelets, and rabbit platelets are not aggregated by 5-HT[2]. 5-HT enhanced the aggregation by other agents, such as ADP[3], epinephrine[4], and thrombin[5]. When ADP was added immediately after or together with 5-HT, the second aggregation by ADP was enhanced[6]. This enhanced effect was reduced in proportion to the logarithm of the time interval between the addition of two aggregating agents. On the measurement of the level of signal transduction, simultaneous addition of submaximal amounts of epinephrine together with 5-HT amplified $[Ca^{2+}]_i$, inositol 1,4,5-triphosphate (IP_3) metabolism, activation of protein kinase C and myosin light chain kinase, as compared with the alternation induced by 5-HT alone[7].

Rabbit platelets do not respond to 5-HT with aggregation[2], so, it is easier to analyze the subcellular mechanisms underlying the decrease in light transmission (DLT) and the enhancement of aggregation by 5-HT at the level of the signal transduction. The purpose of present experiments was to observe the effects of 5-HT on ADP-induced DLT and aggregation in rabbit platelets.

MATERIALS AND METHODS

Reagents Luciferase-luciferin (Chrono-Log Corp, Havertown PA) for measuring the secretion of ATP was dissolved in saline ($40\ g \cdot L^{-1}$) and stored in a cold and dark place. ATP (Sigma) was dissolved in distilled water ($1\ mmol \cdot L^{-1}$) in plastic microtubes and stored at -20 ℃. The stored solution was diluted with saline to $1\ \mu mol \cdot L^{-1}$ before use. 5-HT (Wako Pure Chemicals, Osaka), ADP (Sigma), and methysergide (Sandoz) were stored in Tris-buffer saline at 4 ℃. Egtazic acid (Sigma) was dissolved in water with NaOH 1 $mol \cdot L^{-1}$ and, after the pH was adjusted to 7.4 with HCl 1 $mol \cdot L^{-1}$, stored at room temperature. Indomethacin (Merck, Sharp & Dohme Research Lab Rahway, NJ) was dissolved in ethanol at 14 $mmol \cdot L^{-1}$ and kept at 4 ℃. It was diluted with Tris-buffer saline before use.

PGE_1 and a stable analogue of thromboxane A_2 (STA_2), kindly provided by Ono Pharmaceutical Co, were dissolved in ethanol and stored at -20 ℃. They were diluted with Tris-

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buffer saline prior to use. Fura-2 and Fura 2-AM (Wako Pure Chemicals, Osaka) were used for Ca^{2+} calibration and platelet loading.

Preparation of platelet-rich plasma (*PRP*)[8]

Platelet aggregation Aggregation was quantified by the change of light transmission[9]. After 2 min of preincubation at 37 ℃, PRP (250 μL) in a cuvette was further incubated for 2 min with Tris-buffer saline (20 μL, Tris-buffer 150 mmol·L^{-1}; 0.9 % saline = 1:4, pH 7.4) or inhibitors (20 μL) before the addition of aggregating agents (10 μL) during stirring with a siliconized magnetic bar.

The tracing of aggregation showed 3 patterns of light transmission changes after the addition of aggregation agents: an initial small increase due to the dilution of the addition of aggregating agents; a DLT attributable to the so-called shape change; and a marked increase in the light transmission attributable to aggregation. We gave particular attention to the *DLT*.

Preparation of washed platelet, Fura-2 loading, and measurement of $[Ca^{2+}]_i$ in single platelets[8]

Measurement of IP_3 Washed platelets (4×10^{12}·L^{-1}) were incubated with 5-HT 3 μmol·L^{-1} for 25 s. IP_3 was determined according to receptor binding assay method[10, 11].

Preparations for electron microscopy The PRP in the cuvette (250 μL) was incubated for 2 min at 37 ℃ with Tris-buffer (20 μL) in a 4-channel aggregometer with stirring, and then for another 2 min with 5-HT or ADP in a final concentration of 3 μmol·L^{-1}. The PRP was spun at 800 × *g*, 37 ℃ for 5 min, the platelet pellet was fixed by 2.5% of glutaraldehyde with 0.1% cacodylate buffer, pH 7.2.

Statistical analysis Data were expressed as $x \pm s$ and compared with *t*-test.

RESULTS

DLT by 5-HT DLT was induced by 5-HT 0.1, 0.3, 1, and 3 μmol·L^{-1} by −5.3 %, −6.4 %, −6.9 %, and −7.2 %, respectively. The duration of DLT was also prolonged (Fig 1).

The DLT induced by 5-HT 3 μmol·L^{-1} was not inhibited by indometacin 3 μmol·L^{-1} and egtazic acid 0.1 − 3 mmol·L^{-1}, but was dose-dependently and completely inhibited by preincubation with methysergide 1 − 30 μmol·L^{-1}, PGE_1 0.1 − 3 μmol·L^{-1} (Tab 1).

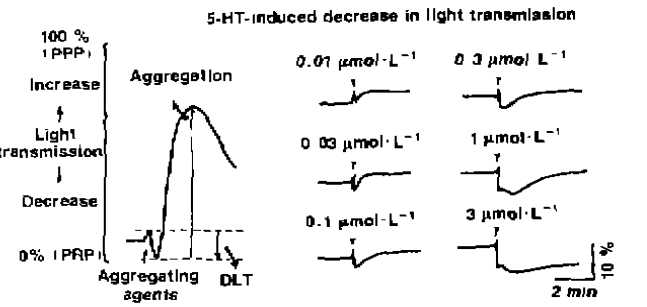

Fig 1. Quantification of platelet aggregation by light transmission (LT) and effect of 5-HT on LT of rabbit PRP.

5-HT did not induce aggregation in rabbit platelets, but it elevated $[Ca^{2+}]_i$ (Fig 2A). The resting level of $[Ca^{2+}]_i$ was 78.5 ± 3.4 nmol·L^{-1}. 5-HT 0.3, 1, and 3 μmol·L^{-1} induced a dose-dependent increase in $[Ca^{2+}]_i$ and the peak value, obtained with 5-HT 3 μmol·L^{-1}, was 150 ± 10 nmol·L^{-1} at 100 s after stimulation.

The IP_3 level in platelets with 5-HT 3 μmol·L^{-1} was increased and reached peak level at 15 s and returned to the resting level within 25 s (Fig 2B).

Enhancement of ADP-induced aggregation by 5-HT ADP 3 μmol·L^{-1} induced a transient DLT in rabbit platelets immediately before its own aggregation (Fig 3, upper panel). The DLT induced by ADP was not inhibited by methysergide or indometacin, but was partially inhibited (36 %) by PGE_1 0.01 − 3 μmol·L^{-1}. The aggregation was not accompanied with detectable amounts of ATP or with the release of the granular contents (Fig 3, lower

Tab 1. 5-HT-induced DLT and the effects of methysergide, indometacin, egtazic acid, and PGE_1 on the DLT by 5-HT. $\bar{x} \pm s$. $^bP < 0.01$, $^cP < 0.01$ *vs* control.

5-HT 3 μmol·L^{-1}	*n*	Concentration/μmol·L^{-1} 0	0.01	0.03	0.1	0.3	1	3	10	30
−	8 − 13		−1.4±0.5	−3.2±0.5	−5.3±0.4	−6.2±0.3	−6.9±0.5	−7.2±0.4		
Indometacin	7 − 16		−1.3±0.3	−2.8±0.3	−5.1±0.4	−6.0±0.4	−7.4±0.4	−7.6±0.5		
Methysergide	9 − 14	−7.3±0.6		−7.4±0.8	−7.3±0.8	−6.9±0.9	−4.9±0.8^b	−2.6±0.5^c	−1.1±0.3^c	−0.3±0.4^c
Egtazic acid	6 − 11	−7.6±0.7			−7.3±0.3	−7.9±0.6	−8.6±0.7	−8.5±0.5		
PGE_1	9 − 11	−7.2±0.3	−6.4±0.4	−6.2±0.4	−4.2±0.5^b	−2.4±0.3^c	−1.2±0.3^c	−0.49±0.26^c		

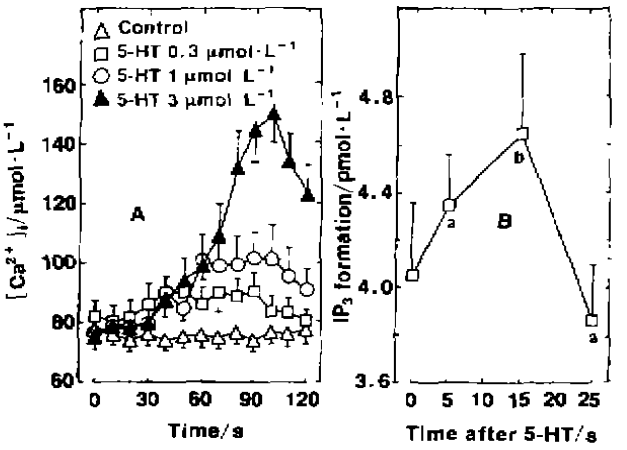

Fig 2. A: Intracellular calcium mobilization by 5-HT in single washed platelets loaded with Fura-2. n = 14 – 36 cells from >6 rabbits. B: IP_3 formation by 5-HT (3 μmol·L⁻¹). n = 8 preparations from 6 rabbits. $x \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* 0 s.

panel). The pretreatment with 5-HT 0.3 μmol·L⁻¹ reduced the magnitude of the DLT induced by ADP and enhanced its aggregation (Fig 3, upper panel). 5-HT 3 μmol·L⁻¹ abolished the DLT by ADP and the aggregation was further enhanced.

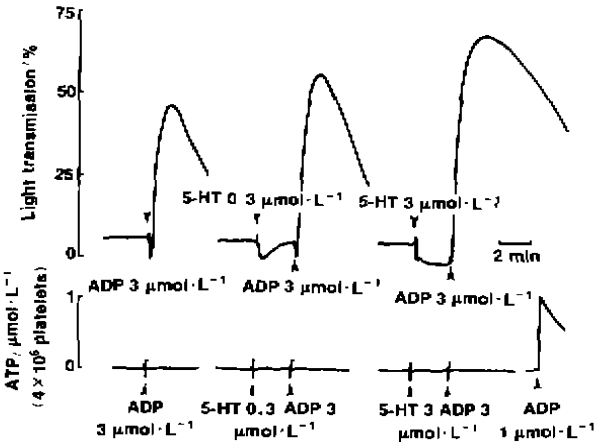

Fig 3. Effects of 5-HT on ADP-induced DLT and aggregation; and ADP-induced release reaction without or with preincubation of 5-HT.

The ratios of the enhancement of ADP-induced aggregation by the preceding addition of 5-HT were dependent upon the time intervals between the addition of 5-HT 3 μmol·L⁻¹ and ADP 0.3 μmol·L⁻¹ (Fig 4A). The maximal enhancement (37.8 % ± 2.1 % of light transmission) was obtained at 10 s after the addition of 5-HT.

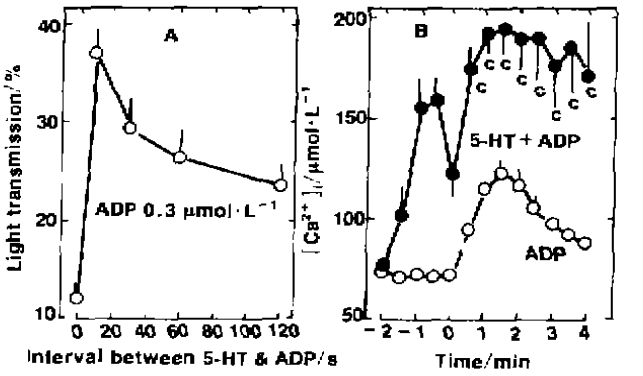

Fig 4. A: Influences of the time intervals between the addition of 5-HT and that of ADP, and the effects of 5-HT on ADP-induced aggregation. ADP was added at 10, 30, 60, and 120 s after the stimulation by 5-HT. n = 8 – 13 preparations, $x \pm s$. B: effects of 5-HT on $[Ca^{2+}]_i$ mobilization by ADP. n = 19 – 21 cells from 6 rabbits, $x \pm s$. $^cP < 0.01$ *vs* the value from ADP only.

ADP-induced $[Ca^{2+}]_i$ mobilization The enhancement of ADP-induced aggregation by 5-HT was accompanied by an increase in the level of $[Ca^{2+}]_i$ (Fig 4B). ADP 3 μmol·L⁻¹ alone increased $[Ca^{2+}]_i$ from 74 ± 3 nmol·L⁻¹ to 131 ± 5 nmol·L⁻¹ at 90 s. 5-HT itself raised the $[Ca^{2+}]_i$ to 166 ± 19 nmol·L⁻¹ and further addition ADP on the platelets resulted in a further increase in the $[Ca^{2+}]_i$ to 186 ± 15 nmol·L⁻¹ ($P < 0.01$).

Morphological changes The resting rabbit platelets (Fig 5 upper left) contained dense granules, but the stimulation of the platelets with 5-HT induced centralization of the granules with the formation of small numbers of pseudopods, indicating that the granules remained (lower left). The addition of ADP caused aggregation with increased numbers of pseudopods, but the dense granules were not released (upper right). The stimulation of the platelets with a stable analogue of thromboxane A_2 (STA_2) released the contents of the granules and the platelets were aggregated together (lower right).

DISCUSSION

Rabbit platelets responded to 5-HT with DLT and the responses did not proceed further to aggregation. Thus, the rabbit platelets are a good model for analyzing the mechanism of the signal transduction of the responses, since the DLT is the response of the

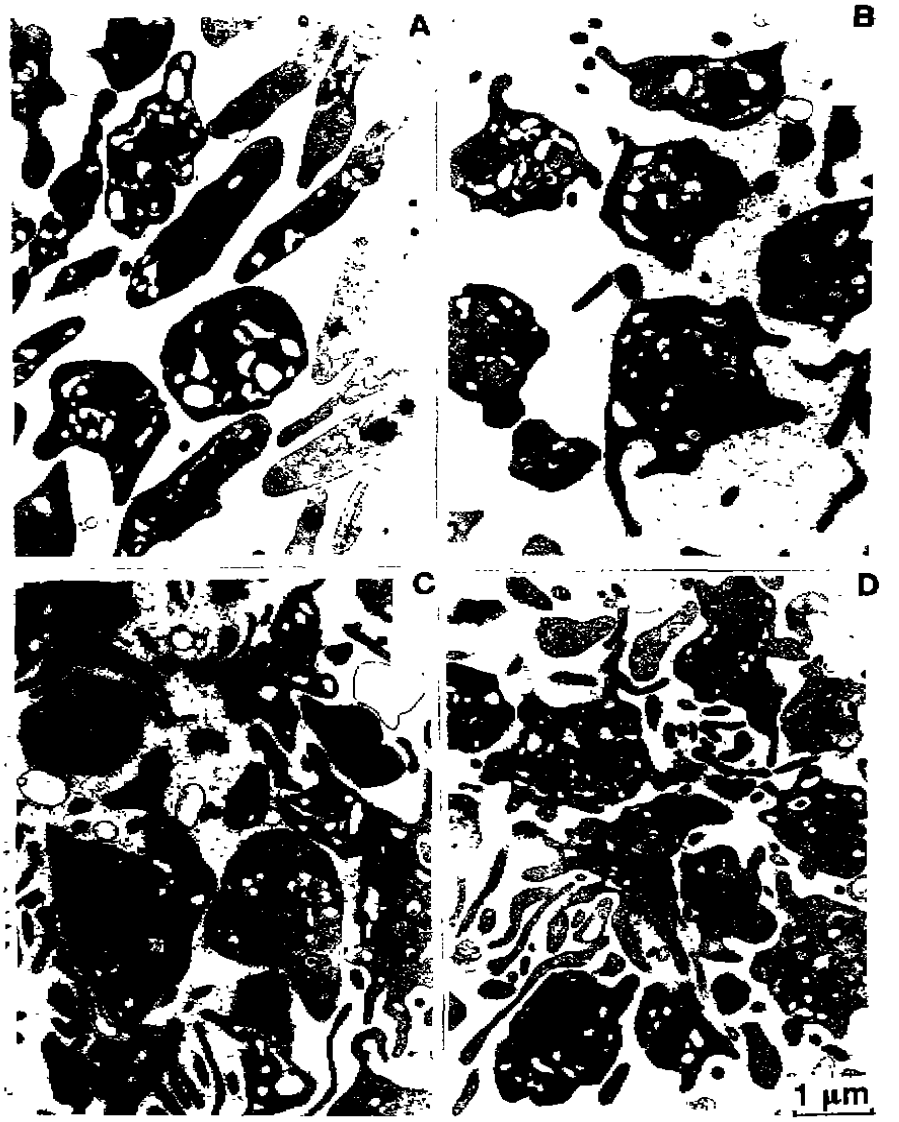

Fig 5. Electron microscopy (× 10 000). A: resting stage of platelets; B: centralization of granules and pseudopods formation by 5-HT without aggregation; C: ADP-induced aggregation without release reaction; and D: aggregation with release reaction by STA_2.

platelets not only to 5-HT, but also to all other stimulants, such as, ADP in the present experiment, arachidonic acid, STA_2, collagen and thrombin in the following studies. The DLT is attributed to the so-called shape change and the centralization of dense granules[12] revealed by an electron micrograph in the present study (Fig 5). The DLT due to 5-HT was receptor mediated, and accompanied with small increase in $[Ca^{2+}]_i$, but did not require calcium influx or TXA_2 because ADP-induced DLT was inhibited by methysergide, but not by egtazic acid and indomethacin. The DLT required the IP_3 generation, as shown in the present experiment. The inhibition of DLT by PGE_1 indicated that it required a contraction of the cytoskeleton, since PGE_1 activates adenylate cyclase and accumulates cyclic AMP[13]. With these data taken together, it can be concluded that the DLT due to 5-HT was induced by centralization of the dense granules or other organelles as results of the contraction of platelet microtubules, which was induced by increasing $[Ca^{2+}]_i$ mobilized from the storage sites by IP_3 through 5-HT receptors.

IP_3 formation was reported to be accompanied with the aggregation by thrombin[12]. The total level of inositol triphosphate consists of at least two isomers[13], 1,4,5-IP_3 measured in this experiment and 1,3,4-IP_3, so, the increase in [^{3}H]-inositol triphosphate does not mean an increase in the IP_3 level confirmed by HPLC. The receptor binding assay method using a specific receptor for 1, 4, 5-IP_3[11], employed in this experiment, guaranteed the reliability of the measurement of IP_3. The results indicated that IP_3 was very rapid generation and short in duration after the addition of 5-HT.

The enhancement of ADP-induced aggregation by pretreatment of 5-HT was accompanied with a further increase in the level of $[Ca^{2+}]_i$. This enhancement was related to the concentration of 5-HT and time-dependent, which reached the maximum at 10 s after the addition of 5-HT and then decreased with time, the similar observation was reported[12]. It is concluded that $[Ca^{2+}]_i$ released through IP_3 formation by 5-HT may be supplemented by both calcium influx and the release from storage sites after the stimulation of platelets with ADP. The decline with time of $[Ca^{2+}]_i$ released from storage sites by 5-HT resulting in changes in the enhancement ratios.

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58-62 (15)

5-HT 增强家兔 ADP 介导的血小板聚集反应[1]

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关键词 血清素; 腺苷二磷酸; 血小板聚集; 钙; 肌醇 1,4,5-三磷酸

目的: 研究 5-HT 对 ADP 介导的血小板聚集反应的增强作用. **方法**: 以透光法、图像法和受体结合法评价聚集反应、单细胞内钙和三磷酸肌醇的含量. **结果**: 5-HT (0.03 – 3 $\mu mol \cdot L^{-1}$)浓度依赖性地引起 PRP 的透光度降低(DLT), 电镜结果显示血小板变形的同时伴有颗粒中心化, 无聚集和释放反应. Fura-2 负载后, 5-HT 升高$[Ca^{2+}]_i$、90 秒达峰值, IP_3 一过性升高. ADP 同样引起 DLT, 但可被 5-HT 消除、呈浓度依赖性. ADP 的聚集反应和$[Ca^{2+}]_i$ 动员则由于 5-HT 预处理而升高. **结论**: 5-HT 增强 ADP 的聚集反应与 5-HT 的细胞内钙动员及 ADP 的外钙内流两者的叠加作用有关.