Short Communication: Possible Association of Newly Absorbed Serotonin With Nonmetabolic, Granule-located Adenine Nucleotides in Human Blood Platelets

By Holm Holmsen, Carol A. Setkowsky, and H. James Day

[3H]-adenine-labeled human platelets in plasma were incubated with or without nonradioactive serotonin. Release reaction was then induced by ADP, epinephrine, collagen, or thrombin. Platelets that had been incubated with serotonin released four times as much serotonin as platelets incubated without serotonin. The specific radioactivities of the ATP and ADP released to plasma during release reaction induced with all four inducers were the same in both systems. This shows that when serotonin is taken up by human platelets, it enters the compartment containing nonmetabolic, granula-stored ATP, and not the compartment with metabolic extragrannular ATP. These results suggest that the mechanism of serotonin storage in human platelets is similar to that in other species investigated, i.e., rabbit, guinea pig, and pig.

Serotonin storage has been studied in rabbit, guinea pig, pig, and human platelets and has been found to occur exclusively within dense granules.

Isolated dense granules from rabbit platelets contained, in addition to serotonin, large amounts of nucleoside tri- and diphosphates as well as Ca2+ and Mg2+. These findings led to in vitro experiments that indicated that divalent cations and ATP in concentrations similar to those found in dense granules formed micellar complexes that were able to incorporate serotonin. It was suggested by Berneis et al. and Pletscher et al. that such metal-ATP micellar complexes existed in dense granules, and the affinity of this complex for serotonin was responsible for uptake and storage of the amine in the granules. This hypothesis would explain how the platelet cytosol serotonin level can be maintained at a concentration low enough to establish a gradient across the platelet membrane favorable for the accumulation of the amine from the extracellular medium, despite the high serotonin content of the platelet.

Although it has not yet been possible to isolate large amounts of human platelet-dense granules with a comparable degree of purity as species mentioned above, there is indirect evidence that human platelet-dense granules are more or less similar to those of other species with regard to serotonin and adenine nucleotide storage. Radioautographic techniques have established that serotonin is stored in the dense granules. The low levels of ATP, ADP, and serotonin in platelets that contain a markedly reduced number of dense granules.

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(characteristic of platelets from patients with "storage pool deficiency") indicate that nucleotides are associated with serotonin in human platelets.\textsuperscript{14,15} In release reaction experiments, the metabolically active adenine nucleotide pool was labeled with \textsuperscript{3}H-adine for 30–60 min to distinguish it from storage pool ATP and ADP which was not labeled in that time period; the stoichiometry of the released ATP plus ADP (with low specific radioactivity) and serotonin was the same as that found in crude dense granules that were isolated in low yield.\textsuperscript{16–18}

This same technique was used in the work described here to determine whether exogenous serotonin that is taken up by the intact platelet combines with granular or extragranular ATP (or ADP) as distinguished by radio-labeling.\textsuperscript{17} According to the storage hypothesis of Pletscher et al.,\textsuperscript{13} exogenous serotonin should combine with the stored ATP and be released with it, since newly absorbed serotonin behaves exactly the same as the endogenous amine.\textsuperscript{19}

MATERIALS AND METHODS

\textsuperscript{3}H]-adenine-labeled human platelet-rich plasma was prepared as described previously.\textsuperscript{20} Portions of 0.9 ml of this platelet-rich plasma were incubated with 0.1 ml of either 0.15 M NaCl or 25.0 \( \mu M \) serotonin (in 0.15 M NaCl) at 37°C for 10 min and then stirred in an aggregometer (EEL titrator, see reference 20) at 37°C with 0.1 ml of aggregating (releasing) agent (ADP, adrenaline, collagen, or thrombin; these substances were obtained and prepared as described previously)\textsuperscript{20} and with 0.1 ml of 0.15 M NaCl or 0.1% acetic acid (control). Final concentrations were: 3 \( \mu M \) ADP, 5 \( \mu M \) adrenaline, 100 \( \mu g/ml \) of collagen, or 0.4 U/ml of thrombin. The mixtures were stirred for 4 min, which produced maximal aggregation, with biphasic aggregation responses for ADP and adrenaline. The thrombin samples often clotted during this incubation period. After the 4-min aggregation period, 0.1 ml of 77 mM EDTA, pH 7.4, was added and the mixtures cooled in ice and centrifuged at 11,000 \( g \) for 10 min at 4°C. The sediments were resuspended in 1.1 ml of 0.1 M NaCl/5 mM EDTA/30 mM Tris HCl, 96 mM glucose (pH 7.4), 0.2-ml portions of these suspensions and of the supernatants were each mixed with 0.2 ml EDTA-ethanol (1 volume 0.1 M EDTA, pH 7.4 + 9 volumes 96% ethanol) for determination of the amounts\textsuperscript{21} and radioactivity\textsuperscript{22} of ADP and ATP. To the remainder of supernatant and sediment-suspension was added 5 \( \mu l \) of 20% Triton X-100, and these mixtures were analyzed for serotonin (total 5-hydroxyindoles) as described elsewhere.\textsuperscript{17}

RESULTS AND DISCUSSION

Table 1 shows the results from a typical experiment with ADP. Incubation of the platelets with serotonin caused a fourfold increase in the level of cell-bound serotonin. This produced no changes in the amounts or specific radioactivity of ATP and ADP (Table 1A). Stirring with ADP gave secondary platelet aggregation, after which the isolated cells contained distinctly lower levels of ATP and ADP with specific radioactivities that were higher (especially for ADP) than the control (NaCl-treated) cells. Both the levels and the specific radioactivity of cellular ADP and ATP in the ADP-treated platelets were the same in platelets that had been loaded previously with serotonin or not. The level of serotonin had dropped in the ADP-treated platelets as compared to the control cells, and the per cent drop was the same in serotonin-loaded as in nonloaded platelets (Table 1B). The serotonin-loaded platelets released about four times more serotonin to plasma than the nonloaded platelets, and, hence, the same per cent of the cell-bound amine was released from the two types of cells. The amounts and specific radioactivity of ATP and ADP in plasma were the same whether the nucleotides were released from nonloaded or loaded
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Table 1. ADP-induced Release of Serotonin, ATP, and ADP From Platelet Incubated With and Without (Nonloaded) Added Serotonin

<table>
<thead>
<tr>
<th></th>
<th>Nonloaded*</th>
<th>Loaded*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>cpm</td>
</tr>
<tr>
<td></td>
<td>10¹¹ cells</td>
<td>μmole</td>
</tr>
<tr>
<td>A. NaCl- treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>5.5</td>
<td>11,388</td>
</tr>
<tr>
<td>ADP</td>
<td>2.8</td>
<td>6,066</td>
</tr>
<tr>
<td>Serot.</td>
<td>0.096</td>
<td>—</td>
</tr>
<tr>
<td>B. ADP- treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>3.8</td>
<td>12,399</td>
</tr>
<tr>
<td>ADP</td>
<td>0.9</td>
<td>11,737</td>
</tr>
<tr>
<td>Serot.</td>
<td>0.060</td>
<td>—</td>
</tr>
<tr>
<td>C. Released† to plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>by ADP</td>
<td>0.9</td>
<td>1,560</td>
</tr>
<tr>
<td>ADP</td>
<td>2.0</td>
<td>137</td>
</tr>
<tr>
<td>Serot.</td>
<td>0.032</td>
<td>—</td>
</tr>
</tbody>
</table>

The experimental procedure is described in the text. Platelet count was 3.8 x 10⁸ cells/ml.
*Nonloaded, not exposed to added serotonin; loaded, exposed to added serotonin.
†The values show the difference in plasma levels between the ADP-treated and NaCl-treated sample. The ADP values are corrected for added ADP.

platelets (Table 1C). The increase extracellularly in ADP corresponded well to the decrease intracellularly during release. For ATP, the intracellular decrease was always greater than the extracellular increase, a phenomenon due to intercellular conversion of metabolic ATP to hypoxanthine and rapid breakdown of ATP in plasma after release.¹⁷

Experiments with adrenaline, collagen, and thrombin as release inducers gave the same results as those recorded in Table 1.

These results indicate that serotonin does not combine with extragranular ATP and support the view that the amine is stored in the granules of human platelets by the same mechanism as suggested for platelets from other species by Pletscher et al.⁹ However, the possibility that some serotonin combined with extragranular ATP and was not released cannot be excluded.

This finding further explains the lack of serotonin in platelets from patients with "storage pool deficiency" that lack inexchangeable or storage ADP and ATP. Since 5HT does not combine with extra granular ATP, and this constitutes more than three-fifths of total ATP, comparison between 5HT and total ATP is erroneous. Such comparisons are often done in order to see if ATP is a limiting factor in 5HT uptake.²³²⁴

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