Active Transport of 5-Hydroxytryptamine by Plasma Membrane Vesicles Isolated from Human Blood Platelets

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Plasma membrane vesicles isolated from human platelets accumulate 5-hydroxytryptamine when an electrical potential (interior negative) or an Na⁺ gradient (out > in) is imposed across the vesicle membrane. Kinetic studies reveal a Kᵢ of 0.5 μM for the transport process. Uptake is inhibited strongly by tricyclic antidepressants and by ionophores such as gramicidin which catalyze transmembrane exchange of Na⁺ for K⁺. Transport is absolutely dependent upon external Na⁺ and Cl⁻ and is only mildly, if at all, inhibited by reserpine, cinanserin, ouabain, or arsenate. Experiments are presented which suggest that a single positive charge crosses the vesicle membrane with each molecule of 5-hydroxytryptamine. The results provide direct evidence for Na⁺-coupled active 5-hydroxytryptamine transport by the platelet plasma membrane.

Blood platelets, because of their many similarities to nerve cells, have been proposed as a model for aminergic neurons (1-3). Foremost among these similarities is the ability of platelets to accumulate 5-hydroxytryptamine to high internal concentrations (4-7). In platelets, intracellular 5-HT³ is associated with electron-dense storage granules which may be analogous to the synaptic vesicles of 5-HT containing neurons (8-10). Moreover, inhibitors such as imipramine and reserpine, which block uptake and storage respectively in neurons, have identical effects on uptake and storage of 5-HT in platelets.

Uptake of 5-HT by platelets is an energy-requiring, carrier-mediated process with an absolute requirement for Na⁺ in the external medium (4, 5, 11, 12). The Kᵢ for 5-HT uptake into intact platelets decreases with increasing Na⁺ (11). In these respects it is similar to mammalian active transport systems for sugars (13) and amino acids (14) where it is believed that substrate accumulation is accomplished by co-transport with Na⁺ which moves down its electrochemical gradient, into the cell (15). Sneddon has shown that intact platelets poisoned with 2,4-dinitrophenol and sodium fluoride accumulate 5-HT when intracellular Na⁺ is low with respect to medium Na⁺ (16). However, in this and other previous studies, 5-HT accumulation by platelets has been complicated by the presence of intracellular 5-HT storage granules, which may contribute to uptake of the amine.

Plasma membrane vesicles isolated from a number of bacte-
3H over 14C in the pellet fraction as compared with the supernatant solution, intravesicular volumes were calculated. Isolated platelet plasma membrane vesicles enclosed an intravesicular space of 12.2 ± 1.1 μg/ml of membrane protein. In the presence of 0.1 M sucrose the volume collapses to 6.1 ± 1.4 μg/ml of membrane protein.

**Materials**

[1,2-3H]5-HT, [3H]OH, and [carboxyl-3H]Cinulin were obtained from New England Nuclear. Imipramine was a gift of Mr. Charles Brownley, Geigy Pharmaceuticals. Amtriptyline and protriptyline were gifts of Mr. Walter B. Gall, Merck, Sharp and Dohme Research Laboratories. Doxepin was a gift of Pfizer, Inc. Nortriptyline and monensin were gifts of Dr. Robert Holley, Lilly Research Laboratories. Cinanserin was a gift of Dr. William Brown, Squibb Institute of Medical Research. Nigericin was a gift of Dr. Julius Berger, Hoffman-La Roche, Inc.

All other materials were reagent grade obtained through commercial sources.

**RESULTS**

**Uptake of 5-HT into Membrane Vesicles**

Data presented in Fig. 1 demonstrate uptake of [1,2-3H]5-HT by isolated platelet plasma membrane vesicles. At zero time, membrane vesicles equilibrated with potassium phosphate buffer are diluted 10-fold into NaCl solution containing [1,2-3H]5-HT. The imbalance of Na⁺ and K⁺ across the vesicle membrane provides a driving force for the uptake of approximately 85 pmol of 5-HT/mg of membrane protein in 5 min. This corresponds to an internal 5-HT concentration of 7 μM, or a concentration gradient of 80-fold, a value significantly higher than has been reported for other mammalian membrane vesicle transport systems (19, 20). The uptake of 5-HT reaches a maximum by 5 min at 25° (2 min at 37°) and then slowly declines to a low limiting value of 5 to 10 pmol/mg of membrane protein (approximately 1 h after dilution) as the initial Na⁺ and K⁺ gradients decay. Maximal uptake varies from 50 to 120 pmol/mg of membrane protein in various preparations. In the absence of transmembrane ion gradients, uptake of 5-HT into membrane vesicles is very slow and never reaches the high levels attained in the presence of high internal K⁺ and high external Na⁺ (Fig. 1), demonstrating that these vesicles lack endogenous energy sources for transport. If the Na⁺-K⁺ gradient is dissipated by addition of the ionophore gramicidin (24) after uptake of 5-HT, accumulated 5-HT rapidly effluxes from the vesicles, demonstrating that maintaining the internal 5-HT concentration requires continued Na⁺-K⁺ imbalance across the membrane. The radioactivity released by gramicidin co-chromatographs with authentic 5-HT on Eastman thin layer silica gel sheets developed with chloroform/methanol:acetic acid (90:5:5), a system which separates 5-HT from the metabolites 5-hydroxyindole-3-acetic acid and 5-hydroxyindole-3-ethanol.

Platelet plasma membrane vesicles are osmotically intact as demonstrated by the fact that they collapse under osmotic pressure. Moreover, if the osmolarity of the uptake medium is increased during transport, uptake of 5-HT decreases (Fig. 2), a finding consistent with the suggestion that 5-HT accumulates in the intravesicular space and is not predominantly bound to sites on the membrane surface.

In addition to gramicidin, two other ionophores, monensin and nigericin, inhibit 5-HT uptake (Table I). Inhibition of transport may be related to the ability of all these ionophores to dissipate Na⁺ and K⁺ gradients across the vesicle membrane. Carbonyl cyanide m-chlorophenylhydrazone, a proton-conducting ionophore, inhibits partially at high concentrations, while valinomycin, an ionophore which is relatively specific for K⁺ (24) has little effect on maximal uptake of 5-HT. Uptake of 5-HT is absolutely dependent on the presence of external Na⁺ and Cl⁻ and is only slightly stimulated by Mg²⁺ (Table I). Na⁺ and Cl⁻ are both required for transport of 5-HT into intact platelets (11, 12). Little or no inhibition of transport is observed on replacing internal phosphate with arsenate, or adding ouabain to the external medium. Thus the possibility that high internal K⁺ and high external Na⁺ gradients stimulate 5-HT uptake by synthesis of ATP via reversal of the Na⁺, K⁺-dependent adenosine triphosphatase appears unlikely. Moreover, addition of ATP in the equilibration or dilution medium has no effect on transport (data not shown). Finally, Table I shows that reserpine, a potent inhibitor of 5-HT storage in platelet dense granules, and cinanserin, an inhibitor of 5-HT induced aggregation have little effect on uptake into isolated plasma membrane vesicles.

The effect of various inhibitors on the initial rate of 5-HT uptake is presented in Table II. The tricyclic antidepressants...
Inhibition of maximal 5-HT uptake by various inhibitors

Transport was assayed at 37°C as described under "Experimental Procedures." Inhibitors were added to membrane vesicles approximately 1 min before dilution into NaCl medium except in the case of reserpine which was added 5 min prior to assay. Na+ and Cl− were replaced by Li+ and PO4−3, respectively, in the Na+ = 0 and Cl− = 0 experiments. In the AsO4−3 experiment, vesicles were equilibrated with 0.04 M potassium arsenate, pH 6.7, containing 1 mM MgSO4 and 0.12 M mellibiose and uptake is expressed relative to vesicles equilibrated with 0.4 M potassium phosphate, pH 6.7, containing 1 mM MgSO4 and 0.12 M mellibiose. Results are averages of at least four assays in each case.

Table I

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration of inhibitor μM</th>
<th>5-HT uptake % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gramicidin</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>Nigericin</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Monensin</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Carbonyl cyanide m-chloro-phenyl hydrazone</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Na+ = 0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Cl− = 0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>MgSO4</td>
<td>1000</td>
<td>130</td>
</tr>
<tr>
<td>Reserpine</td>
<td>3.3</td>
<td>80</td>
</tr>
<tr>
<td>AsO4−3</td>
<td>4 × 10−6</td>
<td>89</td>
</tr>
<tr>
<td>Ouabain</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>25</td>
<td>90-150</td>
</tr>
<tr>
<td>Cinanserin</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

Effect of inhibitors on initial rate of 5-HT uptake

Transport assays were carried out at 37°C at a 5-HT concentration of 0.17 μM as described under "Experimental Procedures." Reactions were stopped at 5 and 10 s after dilution of membrane vesicles into NaCl medium. Inhibitors were added to vesicles approximately 1 min before dilution except in the case of reserpine, which was preincubated with vesicles for 5 min at 25°C prior to assay.

Table II

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration of inhibitor μM</th>
<th>5-HT uptake rate % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Protriptyline</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Doxepin</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>Ouabain</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>Reserpine</td>
<td>3.3</td>
<td>75</td>
</tr>
</tbody>
</table>

Imipramine, nortriptyline, amitriptyline, protriptyline, and doxepin inhibit norepinephrine and 5-HT uptake into neurons and also into intact platelets (23-29). These compounds are also potent inhibitors of 5-HT transport into isolated platelet plasma membrane vesicles. Imipramine inhibition is competitive, with a Ki of 8 to 10 nM (data not shown). Ouabain and reserpine have little effect on initial rates of uptake, consistent with their lack of effect on maximal uptake (Tables I and II).

Kinetics of 5-HT Uptake— During the first 10 to 15 s after dilution of K+-loaded vesicles into Na+ medium, carrier-mediated uptake of 5-HT proceeds at a constant rate which is directly proportional to external 5-HT at low concentrations but saturates at high external 5-HT (Fig. 3). Passive diffusion accounts for approximately 25% of total 5-HT uptake under these conditions. Analysis of the kinetic data by the method of Hofstee (30) (Fig. 3, inset) yields kinetic constants of 0.6 μM and 750 pmol/mg of membrane protein/min for K+, and Vmax, respectively, at 37°C. Reported values of K+ for uptake of 5-HT into intact platelets range from 0.13 to 45 μM with most values between 0.1 and 1.0 μM (7, 11, 31-33).

Driving Force for Active Transport— In the experiment shown in Fig. 1, two possible sources of energy exist for 5-HT uptake: (a) an outward directed K+ concentration gradient and an inward directed Cl− gradient, which will generate a transmembrane electrical potential (interior negative) if the membrane is sufficiently permeable to these ions, and (b) the asymmetric distribution of Na+ (out > in). A recent report indicates that a membrane potential (interior negative) exists in intact platelets (34). Fig. 4 demonstrates the contribution of each of these energy sources to 5-HT accumulation. In Curve A, a membrane potential is generated in the absence of a Na+ gradient by diluting membrane vesicles equilibrated with sodium phosphate into a medium containing NaSCN and NaCl. Uptake of the highly permeant thiocyanate anion generates a membrane potential (interior negative) and approximately 17 pmol of 5-HT/mg of membrane protein are accumulated. Curve B shows the results of an experiment in which a Na+ gradient is created in the absence of a membrane potential. In this case, K+ and Cl− are present at the same concentration on each side of the vesicle membrane and external sodium phosphate is replaced internally by isotonic sucrose or mellibiose. Under these conditions vesicles accumulate approximately 11 pmol of 5-HT/mg of membrane protein. Essentially identical results are obtained in the presence of valinomycin. In the experiment shown in Curve C, the Na+ and SCN− gradients are combined by diluting membrane vesicles equilibrated with lithium phosphate into NaSCN medium. Addition of a SCN− potential to the Na+ gradient increases 5-HT uptake 3.4-fold to 38 pmol/mg of membrane protein. In this system, Li+ cannot substitute for Na+ or K+. Curve D is a control where Na+ and K+ are present.
gradients. Thus, a net charge of +1 enters the vesicle with an electrical potential (interior negative) across the vesicle membrane shows the linear relationship between the K' and 5HT uptake, no uptake of 5-HT would occur at an infinite K' gradient (out > in). If active transport of 5-HT is driven by a membrane potential, the concentration gradient of 5-HT across the vesicle membrane should be given by the Nernst equation:

$$E_m = \frac{RT}{nF} \ln \frac{[K^+]_{in}}{[K^+]_{out}}$$

Combining the two equations,

$$n \ln \frac{[K^+]_{in}}{[K^+]_{out}} = \ln \frac{[5-HT]_{in}}{[5-HT]_{out}}$$

If n = 1, a linear relationship will exist between the K' gradient and the 5-HT gradient. If n = 2, the 5-HT gradient will increase as the square of the K' gradient, etc. Fig. 5 demonstrates the linear relationship between the K' and 5-HT gradients. Thus, a net charge of +1 enters the vesicle with each molecule of 5-HT. Extrapolation of the line suggests that no uptake of 5-HT would occur at an infinite K' gradient (out > in). It should be noted that in the experiment shown in Fig. 5 the absolute magnitude of the membrane potential is not known due to the contribution of Cl' influx. The 5-HT gradient formed at equimolar K' internally and externally is due to the NaCl gradient (high externally) across the vesicle membrane.

**DISCUSSION**

Active transport in membrane vesicles is similar in many aspects to 5-HT transport in intact platelets; Na' and Cl' are required for activity, the K' for 5-HT is about 0.5 \(\mu\)M, and tricyclic antidepressants inhibit transport. One advantage of plasma membrane vesicles compared to intact cells is that accumulation of 5-HT remains free in the intravesicular space and is not metabolized or sequestered in subcellular compartments. Accumulation of 5-HT by platelet plasma membrane vesicles under physiological conditions (high internal K', low internal Na') strongly suggests that 5-HT is actively transported into intact platelets prior to accumulation within storage organelles.

Two lines of evidence suggest that uptake into vesicles represents transport as opposed to binding. First, uptake of 5-HT decreases when the intravesicular space shrinks upon addition of external sucrose (Fig. 2). Second, the initial rate of 5-HT uptake saturates at high external 5-HT (Fig. 3). If 5-HT uptake represented binding to sites on the membrane, a bimolecular process, the rate would be expected to increase linearly with free 5-HT. Another indication that binding is not involved is that cinanserin, a compound which inhibits the 5-HT-induced shape change in platelets, does not inhibit 5-HT uptake in membrane vesicles at concentrations as high as 1 \(\mu\)M. Drummond and Gordon have shown that at 5-HT concentrations below 0.2 \(\mu\)M, most of the 5-HT bound to platelets is displaced by cinanserin with an IC\textsubscript{50} of 30 nM (35).
Transport of 5-HT into intact platelets is sensitive to a number of metabolic inhibitors, including ouabain (7, 40-42). Sneddon has proposed that this is because intact platelets use ATP to generate transmembrane Na⁺ and K⁺ gradients via the membrane-bound (Na⁺, K⁺)-dependent adenosine triphosphatase, and these ion gradients directly drive active transport of 5-HT (11, 16). This hypothesis is supported by the fact that ouabain and arsenate do not abolish transport in vesicles (11, 16). This hypothesis is supported by the fact that ouabain and arsenate do not abolish transport in vesicles (11, 16).

Sneddon has suggested that Na⁺ and 5-HT are co-transported into intact platelets (11) while Lingjaerde has proposed a model in which Na⁺, Cl⁻, and 5-HT all cross the membrane directly involved in transport. If it is assumed that 5-HT crosses the membrane in its cationic form (the predominant form at neutral pH), then Sneddon’s mechanism predicts a net influx of two positive charges per molecule of 5-HT while Lingjaerde’s model predicts one. The results presented in Fig. 5 of this paper indicate that one positive charge enters the vesicle with each molecule of 5-HT. This result does not prove or disprove either of the above models, however, since it is not known which form of 5-HT. This result does not prove or disprove either of the above models, however, since it is not known which form of 5-HT is cationic.

**Acknowledgments** — We would like to thank Ms. Pamela Nelson for excellent technical assistance, and Drs. P. Hinkle and H. R. Kaback for helpful discussions during this investigation.

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