EFFECT OF CONCANAVALIN A ON $^3$H-5-HYDROXYTRYPTAMINE UPTAKE IN RABBIT BLOOD PLATELETS: INTERACTION WITH ADENYLATED CYCLASE ACTIVITY

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Abstract—Concanavalin A (Con A) was shown to have inhibitory effect on platelet adenylate cyclase as well as 5-hydroxytryptamine (5HT) uptake. These effects of Con A were antagonized by $\alpha$-methyl-D-mannoside, a specific inhibitor of Con A binding to glycoprotein. The effect of Con A on adenylate cyclase was partial inhibition, and Con A had no effect on prostaglandin E$_1$ stimulated activity, indicating the adenylate cyclase which is thought to be involved in 5HT uptake might be a minor component. The same result as Con A was demonstrated in the inhibitory effect of N-ethylmaleimide (NEM) on this activity. Impermeable sulfhydryl blocking reagents and iodoacetamide had no effect on 5HT uptake. It might be necessary for the sulfhydryl blocking reagent to pass through the cell membrane in order to exert its inhibitory effect on 5HT uptake. Furthermore, the inhibitory effect of Con A on 5HT uptake was antagonized by sulfhydryl reducing reagents and adenosine. It is postulated that a NEM sensitive, sub-membrane contractile protein system might mediate the effect of Con A, and Con A might inhibit platelet 5HT uptake by affecting the adenylate cyclase system through some possible transmembrane regulatory mechanism.

Concanavalin A (Con A), a lectin from *Canavalia ensiformis*, binds specifically to sugars with D-arabinose configuration like D-mannose or D-glucose and membrane glycoproteins containing such a sugar residue. Con A binding on the plasma membrane of various cell types induced changes in their biological or biochemical properties.

In our previous studies (1), Con A was shown to have a potent inhibitory effect on the uptake of 5-hydroxytryptamine (5HT) by blood platelets, and this effect of Con A was antagonized by $\alpha$-methyl-D-mannoside ($\alpha$-MM), colchicine, theophylline and dibutyryl cyclic adenosine-3',5'-monophosphate. It was postulated that the microtubules, contractile protein and membrane adenylate cyclase system of blood platelets might be involved in the mechanism of the inhibitory action of Con A. Majerus and Brodie (2) showed that E-phytohemagglutinin, a lectin from *Phaseolus vulgaris*, inhibited the adenylate cyclase activity of human blood platelet. Furthermore, Bonnafous et al. (3) discussed the possible involvement of adenylate cyclase in the stimulation of lymphocytes by Con A.

In this paper, therefore, the effect of Con A on adenylate cyclase has been investigated in rabbit blood platelets, and the possible involvement of adenylate cyclase in 5HT uptake by blood platelets is discussed.

Materials and Methods
Reagents: 5-Hydroxy(G-$^3$H)-tryptamine creatinine sulphate ($^3$H-5HT) and (2-$^3$H)-
ATP ammonium salt were obtained from the Radiochemical Center, Amersham. Concanavalin A (Con A) was obtained from the Sigma Chemical Co., Saint Louis, Mo., U.S.A. Prostaglandin E, (PG-E,) was a gift from the Ono Pharmaceutical Co., Ltd., Osaka, Japan. Iodoacetamide (IAA), N-ethylmaleimide (NEM), azodicarboxylic acid bisdimethylamide (Diamide), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), ρ-chloromercuribenzoic acid (PCMB), and all other reagents were purchased from Nakarai Chemicals Co., Ltd., Kyoto, Japan.

Animals: Rabbits of either sex weighing 2–2.5 kg were used. Whole blood was collected from the carotid artery, mixed with 1/10 volume of 3.8% sodium citrate and centrifuged at 150xg for 20 min at room temperature. The supernatant (platelets rich plasma: PRP) was collected.

Measurement of adenylate cyclase activity: Platelet adenylate cyclase activity was assayed by a modification of the method of Zieve and Greenough (4). PRP (30–50 ml) was washed twice with 40 ml buffered salt solution (BSS) (composition in mM: NaCl, 134; MgCl₂, 3; D-glucose, 5; Tris-HCl buffer, pH 7.4, 15) and re-suspended in 4 ml BSS. The suspensions were mixed with 36 ml glass distilled water and rapidly frozen in a dry ice-ethanol bath and thawed at 37°C. After homogenization with a Polytron (Kinematica, Switzerland), the suspensions were centrifuged at 10,000xg for 10 min. The sediments were washed with TM-buffer (40 mM Tris-HCl buffer, pH 7.4, 15) and re-suspended in 4 ml BSS. The suspensions were mixed with 36 ml glass distilled water and rapidly frozen in a dry ice-ethanol bath and thawed at 37°C. After homogenization with a Polytron (Kinematica, Switzerland), the suspensions were centrifuged at 10,000xg for 10 min. The sediments were washed with TM-buffer (40 mM Tris-HCl buffer, pH 7.4, 15) and re-suspended in 4 ml BSS. This suspension was used as an enzyme solution. The final incubation mixture consisted of 100 μl of enzyme solution, 100 μl of 4 mM cyclic AMP, 100 μl of a solution containing 4 mM ³H-ATP (0.63 μCi/ml), and 100 μl of test substance in TM-buffer. The mixture was incubated at 37°C in a water bath incubator with gentle shaking for 10 min. The reaction were stopped by placing the tubes in boiling water for 3 min. In all experiments, a control tube was exposed to boiling water for 3 min prior to incubation. The mixture was then centrifuged at 10,000xg for 10 min. The supernatant from each tube was placed on 2.3 cm columns of Dowex 50W×4, 200–400 mesh, hydrogen form, packed in tuberculin syringes. The columns were washed with 1 ml glass distilled water, and the effluent containing residual ATP was discarded. Remaining nucleotides were eluted with an additional 1.5 ml of water. Nucleotides other than cyclic AMP were precipitated by the addition of 0.2 ml of balanced solutions of zinc sulfate and saturated barium hydroxide. One ml of the supernatant solution was added to 10 ml of a scintillation medium and counted in a Packard Tri-Carb Scintillation Counter. The results were expressed as the percent activity of the control experiment. The mean activity in control experiments was 0.521±0.058 nmole cyclic AMP formed/mg protein/10 min (in 8 experiments). The amount of cyclic AMP in this assay system was linear over the first 20 min. The activity of platelet cyclase, when assayed with a 10 min incubation time, increased linearly with the amount of enzyme protein up to at least 0.625 mg (data not shown).

Measurements of ³H-5HT uptake: Experiments of ³H-5HT uptake by rabbit blood platelets were performed by the method previously described (1, 5). PRP (15 ml) was diluted with 35 ml BSS, to which heparin solution was added to yield a final concentration of 10 units/ml. The final dilution of PRP, containing 2.37±0.15×10⁸ platelets/ml (n=11), was separated into 1 ml aliquots, each of which was transferred into a polypropylene test tube containing 0.4 ml BSS or the drug solution to be tested. After aerobic pre-incubation for 30 min at 37°C with gentle shaking (80 strokes/min), ³H-5HT (1.27×10⁻⁷ M) was added to the
sample, and the mixtures were further incubated for 3 min. The incubation was terminated by adding 3 ml of ice cold BSS, and the mixtures were centrifuged at 1,500 x g for 30 min at 4°C. Platelets thus sedimented were washed twice with ice cold BSS. After draining, platelets were dissolved in 0.1 ml of 1 N NaOH. After neutralization with 1 N HCl, the solution was placed in a scintillation vial containing 10 ml Bray’s scintillant. Radioactivity was determined in the scintillation counter. Blank values were obtained from samples to which the radioactive material was added after the test tube had been placed in ice water.

Results

Effect of Con A on adenylate cyclase activity of rabbit blood platelets: Figure 1 shows the effect of Con A on the activity of platelet adenylate cyclase. Con A (2x10^-4 and 5x10^-4 g/ml) significantly inhibited the activity by 20% and 23%, respectively. This inhibitory effect was antagonized by α-MM, a specific inhibitor of Con A binding to glycoprotein. However, Con A had no effect on PG-E1 stimulated activity, which was about 600% of the basal activity (Fig. 2).

Effect of sulfhydryl reagents on 3H-5HT uptake activity of rabbit blood platelets: Effect of sulfhydryl (SH) reagents with different types of action and cell permeability were tested to see possible involvement of SH groups in the 5HT uptake system of platelets (Fig. 3). Only NEM at 10^-3 M, which alkylates SH groups and rapidly penetrates the cell membrane (6), had a strong inhibitory effect. IAA, which also alkylates SH groups (although by a different mechanism from NEM) and penetrates the membrane.

Fig. 1. Effect of concanavalin A (10^-4-5x10^-4 g/ml) on adenylate cyclase activity of rabbit blood platelets, and its antagonism by α-methyl-D-mannoside. The mean activity in control experiments was 0.521 ± 0.058 nmole cyclic AMP formed/mg protein/10 min (n=8). Mean values ± S.E.M. for at least 4 experiments. *Significant at P<0.02 (vs. None).

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<th>Drugs</th>
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<tr>
<td>Con A 5x10^-4 g/ml</td>
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Fig. 2. Effect of concanavalin A (2x10^-4 g/ml) on prostaglandin E1 (2.5x10^-6 M) stimulated adenylate cyclase activity of rabbit blood platelets. For legend see Fig. 1. *Significant at P<0.05, **P<0.02 (vs. None).

Fig. 3. Effect of sulfhydryl reagents (10^-5-10^-3 M) on 3H-5-hydroxytryptamine uptake activity in rabbit blood platelets. IAA: Iodoacetamide, NEM: N-Ethylmaleimide, Diamide: Azodicarboxylic acid biddimethylamide, DTNB: 5,5'-Dithio-bis(2-nitrobenzoic acid), PCMB: p-Chloromercuribenzoic acid. Mean values ± S.E.M. for at least 4 experiments. *Significant at P<0.001 (vs. control).
rapidly, had no effect on 5HT uptake. Cell impermeable SH reagents such as DTNB and PCMB had no effect at concentrations which did not cause endogenous 5HT release from blood platelets. Diamide (10^-3 M), a SH oxidizing reagent, which inhibits the polymerization of tubulin (7), interfered with 3H-5HT uptake by 40%.

Effect of IAA and NEM on adenylate cyclase activity: Figure 4 describes the effect of IAA (10^-4 and 10^-3 M) and NEM (10^-4 and 10^-3 M) on the adenylate cyclase activity. IAA had no effect. In contrast, NEM (10^-3 M), which caused the inhibition of 3H-5HT uptake (Fig. 3), significantly inhibited the activity.

Effect of sulfhydryl reducing reagents on Con A induced 3H-5HT uptake inhibition: Dithiothreitol (DTT) and 2-mercaptoethanol (2ME), SH reducing reagents, had no effect on 3H-5HT uptake, but antagonized the effect of Con A on 3H-5HT uptake (Fig. 5).

Effect of adenosine on Con A induced 3H-5HT uptake inhibition: Adenosine, which was reported to stimulate platelet adenylate cyclase (8), antagonized the inhibitory effect of Con A on 3H-5HT uptake (Fig. 6).

**Discussion**

It was already demonstrated in our previous study (5) that theophylline significantly augmented the uptake of 5HT by rabbit blood platelets, and this effect of theophylline was potentiated by PG-E_1_, but not by the same concentration of PG-E_2_. Con A, at the concentration which
inhibited $^3$H-5HT uptake (1), significantly attenuated adenylate cyclase activity (Fig. 1). These inhibitory effects of Con A were antagonized by α-MM (a specific inhibitor of Con A binding to glycoprotein) as well as its effect on platelet morphology (1); therefore, it is suggested that these effects were caused by Con A binding to the receptor glycoprotein on the surface of the cell membrane. The inhibitory effect of Con A on adenylate cyclase activity (20–23%) was much less than that on 5HT uptake activity (92%). Con A had no effect on PG-E$_2$ stimulated activity of adenylate cyclase (Fig. 2). Judging from these results, adenylate cyclase which is thought to be involved in 5HT uptake (1, 5), might be a minor component in the blood platelet. The same result was demonstrated in the inhibitory effect of NEM on these activities. NEM ($10^{-3}$ M) inhibited $^3$H-5HT uptake completely (95%), whereas it inhibited adenylate cyclase only partially (50%) (Figs. 3 and 4).

Impermeable SH blocking reagents such as DTNB and PCMB, could not inhibit 5HT uptake (Fig. 4). Our data are consistent with the concept that ecto-SH groups of human blood platelets were apparently not involved in regulating platelet active transport processes or responses to stimuli (9). Also in the experiments of the inhibitory effect of NEM on human erythrocyte hexose transport, it was suggested that the hexose transport mechanism requires a SH group which is not accessible at the cell surface (10). Hence, it might be reasonable to assume that it is necessary for the SH blocking reagent to pass through the membrane in order for it to exert the inhibitory effect on 5HT uptake by blood platelets.

IAA, though it was reported to pass the cell membrane rapidly (11), failed to inhibit both adenylate cyclase and 5HT uptake. In the experiment with the erythrocyte membrane, Carraway and Shin (12) reported different sites of binding for IAA and NEM and suggested that the high molecular weight protein spectrin might be most heavily labeled with radioactive NEM. Existence of a spectrin-like, NEM sensitive, sub-membrane contractile protein system might be postulated in blood platelets. In our previous paper (1), we demonstrated the involvement of a colchicine sensitive contractile system in the inhibitory effect of Con A on 5HT uptake. Thus, the similarity between the inhibitory action of Con A and NEM, though the former acts indirectly and the latter directly, is suggestive. This speculation is further supported by the fact that the inhibitory effect of Con A on 5HT uptake was antagonized by thiol reagents such as DTT and 2ME (Fig. 5).

It is concluded that Con A might inhibit platelet 5HT uptake by affecting the adenylate cyclase system through some possible trans-membrane regulatory mechanism. As to this trans-membrane mechanism in blood platelets, there is no direct evidence at present. Dornand et al. (13, 14) recently suggested the involvement of 5'-nucleotidase in the inhibition of lymphocyte adenylate cyclase by Con A. They showed that lymphocyte 5'-nucleotidase, described as an ecto-enzyme, was directly inhibited by Con A binding to its receptor and as a consequence resulting reduction of the adenosine level might interfere with the activity of lymphocyte adenylate cyclase. They concluded that Con A might be a target of Con A, and adenosine might be a second messenger for its action. In fact, as shown in Fig. 6, the inhibitory effect of Con A on $^3$H-5HT uptake was antagonized by adenosine. Further studies should be performed to clarify the involvement of the 5'-nucleotidase—adenosine system in the action of Con A on blood platelets.

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References


