Effect of Concanavalin A on Serotonin Transport into Blood Platelets: Possible Involvement of Protein Kinase C

Yuki JIKOH, Hiroaki NISHIO*, Kimiko OKUGAWA* and Tomio SEGAWA

Department of Pharmacology, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734, Japan

Accepted April 3, 1990

Abstract—Possible involvement of protein kinases in the serotonin (5-HT) transport system in platelets and the inhibitory effect of concanavalin A (Con A) on platelet 5-HT uptake were investigated. Staurosporine and K-252a, highly active inhibitors of protein kinases, did not inhibit 5-HT transport, but they antagonized the inhibitory effect of Con A on 5-HT uptake. KT5720, a protein kinase A inhibitor that has no effect on protein kinase C, neither affected 5-HT transport nor antagonized the inhibitory effect of Con A on 5-HT uptake. The Con A effect on 5-HT uptake was also antagonized by LaCl₃, a Ca++ entry blocker. When the activity of Ca++ transport into platelets was estimated, Con A was shown to have a stimulative effect, which was antagonized by α-methyl-D-mannoside, a specific antagonist of Con A binding to cell membrane glycoproteins. Furthermore, Con A was shown to stimulate the protein kinase C activity of platelets, which phosphorylates a 40-kDa platelet protein; the Con A effects were antagonized by α-methyl-D-mannoside, staurosporine and K-252a, but not by KT5720. We suggest that the activation of protein kinase C and phosphorylation of 40-kDa protein might be involved in the inhibitory effect of Con A on platelet 5-HT transport.

Blood platelets, which originate from megakaryocytes in the bone marrow, have been shown to have unique signal transduction system in response to external stimuli. Because platelets have a very rapid and active transport system for serotonin (5-hydroxytryptamine; 5-HT), which has shown to have the same pharmacological characteristics as serotonergic nerve endings (1), they have been proposed as a potential model for 5-HT neurons (2). Platelets contain, as do neurons, receptor sites for the antidepressant drug imipramine, which are associated with, but not identical to, 5-HT uptake sites (3). Platelets have proved to be an integral model for studying 5-HT₂ receptor sites (4). It is suggested that the signal transducing system coupled to 5-HT₂ receptors on platelets involves metabolism of inositol containing phospholipids, elevation of intracellular free Ca++, and activation of protein kinase C (5).

In order to elucidate the mechanism by which transport of 5-HT in blood platelets is regulated, we have been utilizing concanavalin A (Con A) as an experimental tool. 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 sugar residues. Con A binding on the plasma membrane of various cell types induced changes in their biological or biochemical properties (6). In our previous studies, Con A was shown to have a potent inhibitory effect on the uptake of 5-HT without a 5-HT release reaction (7). This effect of Con A on 5-HT transport was antagonized by α-methyl-D-
mannoside, a specific antagonist of Con A binding to glycoproteins of the cell membrane. It has been suggested that the effect of Con A in platelets might be expressed through stimulation of 5'-nucleotidase (8), which may be connected to the inhibition of adenylyl cyclase activity and inhibition of 5-HT uptake (9). The signal transducing system, by which 5-HT uptake is directly regulated, should be elucidated in more details. There have been no reports concerning the relationship between 5-HT uptake and protein kinase in platelets.

We investigated the possible involvement of protein kinases in the 5-HT transport system and the inhibitory effect of Con A on 5-HT uptake in platelets. The effect of various protein kinase inhibitors such as staurosporine (10), K-252a (11) and KT5720 (11), all derived from micro-organelles, on 5-HT transport and on the inhibitory effect of Con A were tested. Also, the effects of Con A on Ca++ transport into platelets and protein phosphorylation in platelets were examined. Finally, the possibility of the involvement of both protein kinase A and protein kinase C activity of platelets in the inhibitory effect of Con A on the uptake of 5-HT was discussed.

Materials and Methods

Chemicals: 5-[1,2-3H(N)-Hydroxytryptamine creatinine sulfate (3H-5-HT, 1036 GBq/mmol), and 32P-orthophosphate (315–337 TBq/mmol) were purchased from NEN Research Products (Boston, U.S.A.). 45CaCl2 (0.37–1.5 GBq/mg calcium) was purchased from Amersham International plc (England). Concanavalin A (Con A) was obtained from Boehringer Mannheim (West Germany). Staurosporine, K-252a and KT5720 were generously donated by Dr. H. Kase (Kyowa Hakko Kogyo Co., Ltd., Japan). Other chemicals used were of analytical grade.

Preparation of rabbit platelets: Whole blood collected from rabbits was mixed with 1/10 volume of 3.8% sodium citrate and centrifuged at 150 × g for 20 min at room temperature. The supernatant (platelet-rich plasma, PRP) was collected and diluted with buffered salt solution (BSS: 134 mM NaCl, 3 mM MgCl2, 5 mM D-glucose, 15 mM Tris-HCl buffer, pH 7.4) to make diluted PRP (ca. 1.2 × 10^8 platelets/ml).

Assay of serotonin transport into rabbit platelets: Determination of 5-HT transport into platelets was performed by the previously described method (9), but with some modifications. Briefly, aliquots (1 ml) of diluted PRP were pre-incubated with or without drug in the presence of heparin (10 units/ml) at 37°C. Then 3H-5-HT (0.1 nM, 9.25 KBq) was added to the sample, and the mixtures were further incubated for 3 min at 37°C. The incubation was terminated by adding ice-cold BSS. Platelets in the sample medium were then separated by filtration with Whatman GF/C filters, and the radioactivity in the platelets was counted with a liquid scintillation counter. Blank values were obtained from the samples to which 3H-5-HT was added after the test tube had been placed in an ice water bath. The transport activity was expressed as % of the control experiment. The mean activity of 3H-5-HT uptake in the control experiment was 0.383 ± 0.045 nmol/10^9 platelets/min (n = 9).

Assay of 45Ca++ transport into rabbit platelets: Aliquots (1 ml) of diluted PRP were pre-incubated with or without drug in the presence of heparin (10 units/ml) at 37°C. Then 0.5 mM 45CaCl2 (ca. 37 KBq/ml) was added to the sample, and the mixtures were further incubated for 10 or 30 min at 37°C. The incubation was terminated by adding ice-cold BSS, the platelets in the sample medium were separated by filtration with Whatman GF/C filters, and then the radioactivity in the platelets was counted with a liquid scintillation counter. Blank values were obtained from the samples to which 45CaCl2 was added after the test tube had been placed in an ice water bath. The transport activity was expressed as radioactivity (dpm) per 10^8 platelets.

Protein phosphorylation of rabbit platelets: Protein phosphorylation was assayed according to the method described by Santoro (12), but with some modifications. PRP (ca. 10^9 platelets/ml) was incubated for 30 min at room temperature with 18.5 MBq/ml carrier-free 32P-orthophosphate. After the addition of 1/10 volume buffer A (0.8% citric acid, 2.2% trisodium citrate, 2.45% glucose, pH 4.5), the platelets were washed by centrifugation at 1,000 × g for 10 min at room tempera-
ture. The pellet was resuspended with diluted platelet-free plasma to make a platelet suspension of ca. 2.5×10^8 platelets/ml. The samples (110 µl aliquots) were then incubated with drug and/or Con A at 37°C for 20 min, and an equal volume of buffer B (10% glycerol, 10% 2-mercaptoethanol, 0.5 M Tris-HCl buffer, pH 6.8) was added to stop the reaction, followed by heating at 100°C for 4 min. Aliquots (100 µl) were subjected to electrophoretic analysis, performed according to Laemmli (13), employing a 4% acrylamide stacking gel and 10% acrylamide resolving gel. The gels were dried and subjected to autoradiography.

Statistical analysis: Results are expressed as a mean±S.E. Student’s t-test and Duncan’s test were used to compare mean values.

Results

Effects of protein kinase inhibitors and LaCl₃ on ³H-serotonin transport into rabbit platelets: Figure 1 shows the effect of protein kinase inhibitors, staurosporine, K-252a and KT5720, on ³H-5-HT transport. Staurosporine, at the concentration of 10⁻⁶ M, caused a small acceleration of 5-HT transport, whereas K-252a and KT5720 had no effect on 5-HT transport. LaCl₃, a Ca²⁺ entry blocker, also had no effect (Fig. 2).

Effects of protein kinase inhibitors and LaCl₃ on the inhibitory effect of concanavalin A on ³H-serotonin transport into rabbit platelets: We have already shown that Con A, which binds specifically to cell surface membrane glycoproteins with mannoside residues, inhibits 5-HT transport into platelets (7). A transmembrane signaling system, induced by Con A binding, had been suggested by Nishio and Segawa (9). The inhibitory effect of Con A was antagonized by staurosporine and K-252a (Figs. 3 and 4). However, KT-5720, protein kinase A specific inhibitor without protein kinase C inhibitory activity (11), had no effect on Con A induced inhibition (Fig. 5). The inhibitory effect of Con A was also antagonized by LaCl₃ as shown in Fig. 6.

Effect of concanavalin A on ⁴⁵Ca⁺⁺ transport into rabbit platelets: We investigated the effect of Con A on the Ca²⁺ transport. As seen in Fig. 7, net influx of ⁴⁵Ca⁺⁺ into platelets was significantly enhanced by Con A treatment, and the effect of Con A was antagonized by α-methyl-D-mannoside (α-MM), a specific inhibitor of Con A binding to glycoproteins of the cell membrane.

![Graph](image-url)

**Fig. 1.** Effect of microbial alkaloids with protein kinase inhibiting activity on ³H-5-HT uptake into platelets. The platelet suspension (dil-PRP) was preincubated with staurosporine, K-252a or KT5720 at 37°C for 5 min, and ³H-5-HT uptake activity was measured as described in the Methods. Each point shows the mean±S.E. of three independent experiments. *Significant at P<0.05 (vs. control).
Effect of concanavalin A and protein kinase inhibitors on protein phosphorylation of rabbit platelets: We checked if Con A actually activates protein kinases of platelets.

Fig. 2. Effect of LaCl₃ on ³H-5-HT uptake into platelets. The platelet suspension (dil-PRP) was preincubated with LaCl₃ at 37°C for 5 min, and ³H-5-HT uptake activity was measured as described in the Methods. Each point shows the mean±S.E. of three independent experiments.

Fig. 3. Effect of staurosporine (Stauro.) on the ³H-5-HT uptake inhibition by Con A (0.135 mg/ml). Each value shows the mean±S.E. of three independent experiments. **Significant at P<0.02 (vs. control or Con A alone).

Fig. 4. Effect of K-252a on the ³H-5-HT uptake inhibition by Con A (0.135 mg/ml). Each value shows the mean±S.E. of three independent experiments. *Significant at P<0.05 (vs. control). **Significant at P<0.02 (vs. control or Con A alone).
Fig. 5. Effect of KT5720 on the $^3$H-5-HT uptake inhibition by Con A (0.135 mg/ml). Each value shows the mean±S.E. of three independent experiments. **Significant at P<0.02 (vs. control).

Fig. 6. Effect of LaCl$_3$ on the $^3$H-5-HT uptake inhibition by Con A (0.135 mg/ml). Each value shows the mean±S.E. of three independent experiments. *Significant at P<0.05 (vs. control or Con A alone). **Significant at P<0.02 (vs. control).

Fig. 7. Effect of Con A (0.135 mg/ml) on the Ca$^{++}$ transport into platelets and its antagonism by α-methyl-D-mannoside (α-MM, 20 mM). The platelet suspension (dil-PRP) was preincubated with drugs, and the influx of $^{45}$Ca$^{++}$ into platelets was estimated as described in the Methods. Each value shows the mean±S.E. of three independent experiments. **Significant at P<0.02 (vs. control). ***Significant at P<0.01 (vs. control or Con A alone).
Figure 8 illustrates the autoradiogram of the SDS-gel. The upper and lower panel reveals the autoradiograms with and without Ca++ in the medium, respectively. By Con A treatment, 40-kDa protein, reported to be one of the substrates for protein kinase C in platelets (14), was phosphorylated. Phosphorylation was antagonized by α-MM, staurosporine and K-252a, but not by KT5720. In the presence of Ca++ in the medium, however, much more phosphorylation was observed in the control platelets, so the effect of Con A could not be clearly identified.

Discussion

Staurosporine at the concentration of $10^{-6}$ M, caused a small acceleration of 5-HT transport, and K-252a and KT5720 had no effect on 5-HT transport (Fig. 1). Staurosporine and K-252a, protein kinase inhibitors derived from micro-organells, were shown to have the strongest inhibitory effect on protein kinase C among the inhibitors which are now available (10).

We have already shown that Con A selectively inhibits 5-HT transport into platelets
without the release reaction (7), and some transmembrane signaling system, including ecto-5'-nucleotidase (8) and/or adenylate cyclase (9) system, that operates via Con A binding has been suggested. In our present results, the inhibitory effect of Con A was antagonized by staurosporine and K-252a, highly active inhibitors of protein kinase C (10, 11). However, KT5720, a protein kinase A selective inhibitor with no protein kinase C inhibitory activity (11), did not antagonize Con A-induced inhibition. From these results, it may be suggested that the effect of Con A on 5-HT transport into platelets might be exerted through the activation of protein kinase C. This speculation is supported by the results that the net influx of \(^{45}\text{Ca}\) to platelets was significantly enhanced by Con A treatment, and this effect of Con A was antagonized by \(\alpha\)-MM (Fig. 7). These results coincide with the antagonistic influence of \(\text{LaCl}_3\) on the inhibitory effect of Con A on \(^3\text{H}-5\text{-HT}\) uptake (Fig. 6). In many cell types, including lymphocytes (15), monocytes (16) and spleen cells (17), it has been reported that Con A enhanced the influx of \(^{45}\text{Ca}\)^{2+} and stimulated protein kinase C activity by changing intracellular localization of Ca^{2+}.

We checked if Con A actually activated protein kinase of platelets, using \(^{32}\text{P}\) and the SDS electrophoresis technique. By Con A treatment, 40-kDa protein (Fig. 8), which has been reported to be one of the substrates for protein kinase C in platelets (14), was phosphorylated. Antagonism by \(\alpha\)-MM indicates that the effect of Con A is exerted by its binding to cell surface glycoproteins. This phosphorylation is also antagonized by either \(\alpha\)-MM, staurosporine or K-252a, but not by KT5720. These results strongly support the hypothesis that protein kinase C of platelets is involved in the inhibitory effect of Con A on 5-HT transport.

In our previous studies (8, 9), we reported that c-AMP might regulate 5-HT transport and the inhibitory effect of Con A through the activation of protein kinase A activity. In mouse T lymphocytes, 'gated channels' for Ca^{2+}, which could be opened directly by Con A binding to membrane glycoproteins, have been postulated, the operation of which may be regulated by cyclic nucleotides (18). From these results, combined with the present data, co-interaction between protein kinase A and protein kinase C in platelets is suggested to be involved in the inhibitory effect of Con A on 5-HT transport. It has suggested that c-AMP may interrupt IP\(_3\) formation/Ca^{2+} release/protein kinase C pathway through a mechanism in which protein kinase A activation is involved (19). This possibility is especially interesting in view of a recent report (20) suggesting c-AMP mediated phosphorylation of a putative IP\(_3\) receptor. The mechanism of interaction between the 5-HT transport system and the protein kinase system in the platelet should be clarified in more detail. Our results, however, can not rule out the possibility that the myosin light chain kinase of platelets, which is also inhibited by staurosporine (21) and K-252a (11) with high affinity, might be involved in the regulation of the 5-HT transport system in platelets.

Acknowledgment: The authors are grateful to Kyowa Hakko Kogyo Co., Ltd., Japan for the generous supply of staurosporine, K-252a and KT5720.

References
8 Nishio, H., Takeshita, K., Okugawa, K. and Segawa, T.: Effect of concanavalin A on 5'-


