Effect of Concanavalin A on Intracellular Calcium Concentration in Single Blood Platelets

Yuri Ikegami, Hiroaki Nishio*, Toshio Fukuda, Yoshihiro Nakata and Tomio Segawa

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

Received June 17, 1991 Accepted July 8, 1991

ABSTRACT—Mobilization of Ca\(^{++}\) was estimated in single rabbit blood platelets with digital imaging microscopy. Concanavalin A (Con A) caused a rapid initial increase in intracellular concentration of Ca\(^{++}\) ([Ca\(^{++}\)]\(_i\)) with a latent time of about 20 sec, followed by a sustained increase in [Ca\(^{++}\)]\(_i\). This effect of Con A was antagonized by \(\alpha\)-methyl-D-mannose, which already was shown to antagonize the inhibitory effect of Con A on 5-HT transport, indicating that this effect of Con A was also derived from its binding to cell surface glycoproteins. The presence of EGTA in the medium did not affect the initial rise, but inhibited the latter phase of sustained rise. Thus, Con A induced elevation of [Ca\(^{++}\)]\(_i\) was suggested to consist of two different processes: mobilization of Ca\(^{++}\) from the intracellular storage sites and successive Ca\(^{++}\) influx through Ca\(^{++}\) channels. The effect of Con A on the 5-HT transport was tested in the presence of EGTA, a condition where no Ca\(^{++}\) influx occurs. The results indicate that Con A induced inhibition of 5-HT transport was not influenced by EGTA in the medium. It is suggested that the effect of Con A on 5-HT transport might be exerted through the Ca\(^{++}\) mobilization from its intracellular storage sites.

Since platelets have a very rapid and active transport system for 5-hydroxytryptamine (5-HT), which has been shown to have the same pharmacological characteristics as serotonergic nerve endings (1), platelets are proposed to be a potential model for 5-HT neurons (2). To elucidate the mechanism by which the transport of 5-HT into blood platelets is regulated, we have been utilizing concanavalin A (Con A) as a experimental tool. Con A, a lectin from Canavalia ensiformis, binds specifically to sugars with a 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 (3). In our previous studies, Con A was shown to have a potent inhibitory effect on 5-HT uptake without the 5-HT release reaction (4), and suggested that some transmembrane signaling system, including ecto-5'-nucleotidase (5) and/or the adenylate cyclase (6) system, might be involved in the effect of Con A. Adenosine, a product of ecto-5'-nucleotidase and endogenous inhibitor of adenylate cyclase activity, has been suggested to act as the modulator in these transmembrane signaling systems (5, 6).

It has become increasingly clear that adeno-
sine has a potent modulatory effect on synaptic transmission in the mammalian nervous system. Fredholm and Dunwiddie (7) has suggested that adenosine may depress transmitter release in several ways, including inhibition of adenylate cyclase, opening K⁺ channels and reduction of Ca²⁺ influx. It has also been speculated that c-AMP may interrupt IP₃ formation/Ca²⁺ release/protein kinase C pathway through a mechanism in which protein kinase A activation is involved (8). The idea that the activation of protein kinase C exert a regulatory role on adenylate cyclase was also suggested in many cell systems (9-11) including human platelets (12). These interactions between protein kinase A and C have received a great deal of attention as the so-called “cross talk” of the second messengers (13, 14). It is therefore very important to get accurate information about cytoplasmic Ca²⁺ mobilization to elucidate the biochemical mechanism of cell function.

Early studies of cytoplasmic Ca²⁺ changes used Quin-2 as the indicator. Fura-2 has recently been introduced and has a number of technical advantages over Quin-2, including a lower buffer capacity for Ca²⁺, a 30-fold higher fluorescence yield, better selectivity against Mg²⁺ and other heavy metals, and a wavelength shift upon binding Ca²⁺. This wavelength shift endows Fura-2 with the additional advantage that cytoplasmic Ca²⁺ can be quantitated by calculation of the ratio of fluorescence emissions at two different wavelengths, which is independent of the cell size and concentration of the probe within the cell (15). These technical advantages of Fura-2 enable us to continuously monitor [Ca²⁺] in single platelets with high accuracy, sensitivity and resolution by fluorescence microscopy and digital image processing (16).

In this report we analyzed the effect of Con A on the mobilization of Ca²⁺ using fluorescence indicative dye; namely, the effect of Con A on [Ca²⁺] was estimated continuously in single rabbit blood platelets. We found that Con A induced increase in [Ca²⁺] in a single platelet with oscillation. Furthermore, a possible involvement of this action of Con A in the inhibitory effect on 5-HT uptake into platelets was suggested.

MATERIALS AND METHODS

Materials
The following reagents were obtained from the sources indicated: Fura-2/AM from Dojin Laboratories (Kumamoto, Japan); Concanavalin A (Con A) from Boehringer Mannheim (Germany); α-methyl-D-mannose (α-MM) from Tokyo Kasei (Japan); and 5-[1,2-³H(N)-hydroxytryptamine creatinine sulfate (³H-5-HT, 1036 GBq/mmol) from NEN Research Products (Boston, U.S.A.). All other chemicals used here were analytical grades from commercial sources.

Platelet suspension preparation and Fura-2 loading of platelets.
Whole blood collected from rabbits was mixed with 1/10 volume of 3.8% sodium citrate and centrifuged at 150 x 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 X 10⁸ platelets/ml). The diluted PRP was incubated with Fura-2/AM at the final concentration of 5 X 10⁻⁶ M at 37°C for 60 min, washed with Ca²⁺-free buffer, layered on a thin cover-glass coated with 0.1% polyethyleneimine, in a Flexiperm-disc (W.C. Heraeus GmbH, Germany), and then submitted to digital imaging microscopy.

Digital imaging microscopy
Digital imaging microscopy was carried out as described previously (17), but with some modifications (16). Briefly, Fura-2 loaded platelets, which were layered on the thin cover-glass, were put on the microscope stage previously warmed to 37°C by a thermostatted heater controller, and incubated in BSS with or without 1 mM CaCl₂. The microscopic system consisted of a Nikon TMD-EFQ
(Nikon, Japan) with a CF Fluor 100 × objective. Cells were excited by ultraviolet light at 340 nm and 380 nm through narrow bandpass filters (band width 10 nm), 25% transmission neutral density filters, and a 400 nm dichroic mirror. The sequential image was collected through a single broad band pass filter (500 nm, band width 20 nm). A DC-stabilized Xenon lamp was fitted with a computer-associated excitation filter changer. Video images were acquired by a silicon-intensified target camera (VIM-1, Hamamatsu, Japan). The output was digitized by a color image analyzer ARGUS 100 (Hamamatsu). Images were integrated to improve the S/N ratio and calculated to the 340/380 nm fluorescence ratio image on the image analyzer, and then were reconverted to the analogue signal on the image display. The images were constructed as a three dimensional plot of the 45° angular polygons by a graphic plotter. The 340/380 nm fluorescence ratio was converted to [Ca$$^{++}$$]. The calibration curve was used to calculate [Ca$$^{++}$$] at each point.

Assay of serotonin transport into blood platelets

Determination of 5-HT transport into platelets was performed by the previously described method (8). 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 $^3$H-5-HT (10$$^{-7}$$ M, 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 $^3$H-5-HT was added after the test tube had been placed in an ice water bath. The transport activity was expressed as a % of the control experiment. Student's t-test was used to determine the statistical significance of the data.

RESULTS

Distribution of intracellular Ca$$^{++}$$ in single blood platelets

Figure 1 shows three dimensional mapping of [Ca$$^{++}$$] in the Fura-2-loaded single platelets intensified by a digital image processing microscope. Vertical heights at each pixel in these images are proportional to the [Ca$$^{++}$$], of the cells which was calculated from the 340/380 nm fluorescence ratio. In resting platelets, the distribution of [Ca$$^{++}$$], is not

![Graph](image-url)
uniform, with the tendency of a higher concentration at the marginal region than the center part of the platelets. The mean value of the single platelet $[\text{Ca}^{++}]_i$ was calculated as 71.5 ± 4.5 nM ($n = 53$) in the presence of 1 mM CaCl$_2$ and 46.6 ± 4.8 nM ($n = 12$) in the presence of 1 mM EGTA.

**Effect of Con A on the intracellular concentration of Ca$^{++}$ in single blood platelets**

Figure 2 shows the effect of Con A on the $[\text{Ca}^{++}]_i$ in the presence of 1 mM CaCl$_2$. The mean value of $\text{Ca}^{++}$ in single blood platelets is plotted on the ordinate against the time course expressed in seconds on the abscissa, and the arrow indicates the time when the drug was applied. Con A at 0.1 mg/ml caused a rapid increase in $[\text{Ca}^{++}]_i$ with a latent time of about 20 sec. Oscillation of the $[\text{Ca}^{++}]_i$ was observed for several times during its peak value and then it gradually declined. By the three dimensional presentation of $[\text{Ca}^{++}]_i$ (Fig. 3), the elevation of $[\text{Ca}^{++}]_i$ induced by Con A was shown to be random and the existence of so called "hot spots" were suggested in the single platelets. This effect of Con A was shown to be antagonized by α-MM, a specific antagonist of Con A binding to the sugar residues (Fig. 4).

**Effect of EGTA on Con A induced elevation of the intracellular concentration of Ca$^{++}$ in single blood platelets**

To see if extracellular Ca$^{++}$ influx is involved in the influence of Con A, the effect of EGTA was tested. In the presence of 1 mM EGTA, only the initial transient rise was observed, and then $[\text{Ca}^{++}]_i$ decreased to the base line values 90 sec after the addition of Con A (Fig. 5).

**Effect of Con A on serotonin transport into blood platelets in the presence of EGTA**

As already reported in our previous papers (4, 8), Con A was shown to have a potent inhibitory effect on the transport of 5-HT into blood platelets without a 5-HT release reaction. This inhibitory effect of Con A, however, was not affected by EGTA. Thus, Con A revealed the same inhibitory effect on 5-HT transport as shown in the absence of EGTA (Fig. 6).

![Fig. 2. Effect of concanavalin A (Con A) on $[\text{Ca}^{++}]_i$ in a single blood platelet as monitored by Fura-2. Con A, 0.1 mg/ml, was added at the time indicated by the arrow in the presence of 1 mM CaCl$_2$.](image-url)
Fig. 3. Spatial and temporal changes in $[Ca^{2+}]$, in a blood platelet demonstrated by three dimensional computer graphics during Con A stimulation in the presence of 1 mM CaCl$_2$. Plate-(1) is the pre-stimulation state, and plate-(2) is 18 sec after the application of 0.1 mg/ml Con A; then the images from plate-(3) to plate-(12) were taken every 3 sec.

Fig. 4. Effect of $\alpha$-methyl-D-mannose ($\alpha$-MM) on the Con A induced elevation of $[Ca^{2+}]$, in a single blood platelet as monitored by Fura-2. Con A, 0.1 mg/ml, was added at the time indicated by the arrow in the presence of 1 mM CaCl$_2$. $\alpha$-MM, 20 mM, was added at 2 min before the Con A application.
Fig. 5. Effect of EGTA on the Con A-induced elevation of [Ca$^{2+}$], in a single blood platelet as monitored by Fura-2. Con A, 0.1 mg/ml, was added at the time indicated by the arrow in the absence of 1 mM CaCl$_2$. EGTA, 1 mM, was added at 2 min before the Con A application.

Fig. 6. Effect of EGTA on the $^3$H-5-HT uptake inhibition induced by Con A, 0.1 mg/ml. Each value shows the mean ± S.E. of three independent experiments. *Significant at $P < 0.05$ [vs. control (without Con A)].
DISCUSSION

Mobilization of Ca++ was estimated in single rabbit blood platelets by digital imaging microscopy with the use of the specific Ca++-indicator dye Fura-2. Uneven distribution and low level of [Ca++]i was found in the resting platelet even in the presence of extracellular Ca++. The mean value of [Ca++]i was calculated to be 71.5 ± 4.5 nM, which agrees with the value obtained in the cell suspension system (18); and it was decreased by ca. 35% in the presence of 1 mM EGTA. The effect of EGTA was thought to be caused by the leakage of [Ca++]i.

Con A caused a rapid increase in [Ca++]i with a latent time of about 20 sec. This effect of Con A was antagonized by α-MM, which already was shown to antagonize the inhibitory effect of Con A on 5-HT transport, indicating that this effect of Con A was also derived from its binding to cell surface glycoproteins. Oscillation of the [Ca++]i, which has exclusively been shown in the single cell preparation, was observed for several times during its peak value. The same results have already shown when 5-HT was used to stimulate platelets through the activation of 5-HT2 receptors, in which the involvement of protein kinase C has been suggested (16). It is interesting to clarify whether protein kinase C is also involved in the transient oscillation induced by Con A.

To determine if Ca++ influx from the extracellular space may participate in the elevation of [Ca++]i induced by Con A, we tested the effect of EGTA in the medium. The presence of EGTA in the medium did not affect the initial rise in [Ca++]i, but inhibited the latter phase of sustained rise in [Ca++]i. From this result, it is suggested that the initial rise in [Ca++]i with oscillation might not be caused by Ca++ influx, but rather might be induced by the mobilization of Ca++ from intracellular storage sites such as the platelet dense tubuler system (19). Thus, Con A induced elevation of [Ca++]i was suggested to consist of two different kinds of processes, namely mobilization of Ca++ from the intracellular storage sites and successive Ca++ influx through Ca++ channels. The latter effect of Con A was already described in the experiment using 45Ca++ as a tracer (8). The same effect of Con A on Ca++ channels was also reported with lymphocytes (20), spleen cells (21) and mast cells (22). A mitogenic concentration of Con A was shown to cause the production of inositol triphosphate, which may be involved in generating the rise in Ca++ in thymocytes in response to Con A (23, 24).

This is the first report directly indicating that Con A causes the rise in [Ca++]i by inducing its mobilization from intracellular storage sites as well as its influx through Ca++ channels. Because one of our main interests is to elucidate the mechanism by which 5-HT transport is regulated, we analyzed the relationship between the Ca++ mobilization and 5-HT transport inhibition induced by Con A. The effect of Con A on the 5-HT transport was tested in the presence of EGTA, a condition where no Ca++ influx occurs. The results indicate that Con A induced inhibition of 5-HT transport was not influenced by EGTA in the medium. It is suggested that the effect of Con A on 5-HT transport might be exerted through Ca++ mobilization from its intracellular storage sites. Then, as suggested in our previous paper, protein kinase C might be subsequently activated to inhibit 5-HT transport (8). The phosphorylation of a 40-kDa platelet protein was also demonstrated by Con A treatment through the activation of protein kinase C (8). Protein kinase C is an enzyme that plays a pivotal role in the stimulus-response coupling in many cell systems (25). The inhibition of adenylate cyclase might also be involved in these signal transduction systems in the effect of Con A (6).

In conclusion, Con A was shown to cause the elevation of [Ca++]i, through the mobilization of Ca++ from its intracellular storage sites and Ca++ influx by opening Ca++ channels. These results indicate that Ca++ mobilization and protein kinase C might play a cru-
cial role in the signal transduction system for the inhibitory effect of Con A on 5-HT transport. Furthermore, these experimental system may provide useful information about the cross talk between protein kinase A and protein kinase C as the transmembrane signaling system within the cell (13).

REFERENCES

1 Pletscher, A.: Metabolism, transfer and storage of 5-hydroxytryptamine in blood platelets. Br. J. Pharmacol. 32, 1–16 (1968)

2 Pletscher, A.: Platelets as models: Use and limitations. Experientia 44, 152–155 (1988)

3 Nicolson, G.L.: The interaction of lectins with animal cell surfaces. Int. Rev. Cytol. 39, 89–190 (1974)

4 Nishio, H., Segawa, T. and Takagi, H.: Effects of concanavalin A on 5-hydroxytryptamine uptake by rabbit blood platelets and on their ultrastructure. Br. J. Pharmacol. 65, 557–563 (1979)

5 Nishio, H., Takeshita, K., Okugawa, K. and Segawa, T.: Effect of concanavalin A on S'-nucleotidase activity of rabbit blood platelets. Japan. J. Pharmacol. 43, 230–233 (1987)

6 Nishio, H. and Segawa, T.: Effect of concanavalin A on 3H-5-hydroxytryptamine uptake in rabbit blood platelets: Interaction with adenylate cyclase activity. Japan. J. Pharmacol. 33, 79–84 (1983)

7 Fredholm, B.B. and Dunwiddie, T.V.: How does adenosine inhibit transmitter release? Trends Pharmacol. Sci. 9, 130–134 (1988)

8 Jikoh, Y., Nishio, H., Okugawa, K. and Segawa, T.: Effect of concanavalin A on serotonin transport into blood platelets: Possible involvement of protein kinase C. Japan. J. Pharmacol. 53, 403–410 (1990)

9 Kelleher, D.J., Pessin J.E., Ruoho, A.E. and Johnson, G.L.: Phorbol ester induces desensitization of adenylate cyclase and phosphorylation of the β-adrenergic receptor in turkey erythrocytes. Proc. Natl. Acad. Sci. U.S.A. 81, 4316–4320 (1984)

10 Sugden, D., Vanecck, J., Klein, D.C., Thomas, T.P. and Anderson, W.B.: Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pincalocytes. Nature 314, 359–361 (1985)

11 Rozengurt, E., Murray, M., Zachary I. and Collins, M.: Protein kinase C activation enhances cAMP accumulation in Swiss 3T3 cells: Inhibition by pertussis toxin. Proc. Natl. Acad. Sci. U.S.A. 84, 2282–2286 (1987)

12 Watanabe, Y., Horn, F., Bauer, S. and Jakobs, K.H.: Protein kinase C interferes with Ni-mediated inhibition of human platelet adenylate cyclase. FEBS Lett. 192, 23–27 (1985)

13 Yoshimasa, T., Sibley, D.R., Bouvier, M., Leffkowitz, R.J. and Caron M.G.: Cross-talk between cellular signaling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. Nature 327, 67–70 (1987)

14 Laufer, R. and Changeux, J.-P.: Calictonin gene-related peptide and cyclic AMP stimulate phosphoinositide turnover in skeletal muscle cells. J. Biol. Chem. 264, 2683–2689 (1989)

15 Grynkiewicz, G., Poenie, M. and Tsien, R.Y.: A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450 (1985)

16 Nishio, H., Ikegami, Y. and Segawa, T.: Fluorescence digital image analysis of serotonin-induced calcium oscillations in single blood platelets. Cell Calcium 12, 177–184 (1991)

17 Williams, D.A., Fogarty, K.E., Tsien, R.Y. and Fay, F.S.: Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. Nature 318, 558–561 (1985)

18 Tsien, R.Y., Rink, T.J. and Poenie, M.: Measurement of cytosolic free Ca2+ in individual small cells using fluorescence microscopy with dual excitation wavelengths. Cell Calcium 6, 145–157 (1985)

19 Sage, S.O. and Rink, T.J.: Kinetic differences between thrombin-induced and ADP-induced calcium influx and release from internal stores in fura-2-loaded human platelets. Biochem. Biophys. Res. Commun. 136, 1124–1129 (1986)

20 Averdunk, R. and Gunther, T.: Effect of concanavalin A on Ca2+ binding, Ca2+ uptake and the Ca2+ ATPase of lymphocyte plasma membranes. Biochem. Biophys. Res. Commun. 97, 1146–1153 (1980)

21 Wolff, C.H.J. and Akerman, K.E.O.: Concanavalin A binding and Ca2+ fluxes in rat spleen cells. Biochim. Biophys. Acta 639, 315–319 (1982)

22 Suzuki, T., Morii, K. and Uchida, M.: Inhibition by calcium antagonists of histamine release and calcium influx of rat mast cells: Difference between induction of histamine release by concanavalin A and compound 48/80. Eur. J. Pharmacol. 85, 155–161 (1982)

23 Tsien, R.Y., Pozzan, T. and Rink, T.J.: T-cell mitogens cause early changes in cytoplasmic free Ca++ and membrane potential in lymphocytes. Nature 295, 68–71 (1982)
24 Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V. and Metcalfe, J.C.: Free cytoplasmic calcium concentration and the mitogenic stimulation of lymphocytes. J. Biol. Chem. 258, 4876–4882 (1983)

25 Nishizuka, Y.: The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308, 693–698 (1984)