Inhibitory Effect of EGTA on Serotonin Transport into Rabbit Blood Platelets: Possible Involvement of the Glycoprotein IIb/IIIa Complex

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ABSTRACT—We reported previously that serotonin (5-HT) transport was attenuated by treatment of platelets with EGTA, and that this inhibitory effect of EGTA was restored by CaCl2. In the present study, the inhibitory effect of EGTA was found to be uncompetitive, and no inhibitory effect was observed when EGTA was added at 20°C. Genistein and thyrphostin A47, both protein tyrosine kinase inhibitors, inhibited Ca2+-induced restoration of 5-HT transport. In contrast, the protein tyrosine phosphatase inhibitor phenylarsine oxide significantly augmented Ca2+-induced restoration of 5-HT transport. These results might support the hypothesis that the glycoprotein (GP) IIb/IIIa complex, a platelet membrane integrin protein, might regulate 5-HT transport into blood platelets. It is conceivable that Ca2+ chelation by EGTA might cause temperature-dependent dissociation of the GP IIb/IIIa complex, which results in the reduction of 5-HT transport. Rearrangement of the GP IIb/IIIa complex by replenishment of the Ca2+ binding sites might restore the EGTA-induced reduction of 5-HT transport.

Keywords: Serotonin (5-HT) transport, EGTA, Glycoprotein IIb/IIIa complex

Blood platelets have a very rapid and active transport system for serotonin (5-HT), which has been shown to have the same pharmacological characteristics as serotonergic nerve endings (1). They have been proposed as a potential model for 5-HT nerve terminals. However, physiological regulation of this neurotransmitter transport into blood platelets as well as into nerve terminals has not been clearly demonstrated (2).

In our previous study, we reported that 5-HT transport activity into blood platelets was attenuated by the treatment of platelet-rich plasma with 1 mM EGTA, an extracellular Ca2+ chelator, and that this inhibitory effect of EGTA was ameliorated by the application of 2 mM CaCl2 (3). It was suggested that depletion of Ca2+ storage sites by EGTA would result in the inhibition of 5-HT transport into blood platelets. We postulated that Ca2+ storage sites in the glycoprotein IIb/IIIa (GP IIb/IIIa) complex, an integrin glycoprotein, might be depleted by EGTA treatment (3). Furthermore, it was demonstrated that concanavalin A (Con A), a lectin from Canavalia ensiformis, inhibits 5-HT transport activity into rabbit blood platelets by affecting intracellular Ca2+ mobilization (4). This effect of Con A was suggested to be initiated by its binding to the membrane surface glycoproteins of blood platelets. The GP IIb/IIIa complex is regarded to be the major platelet surface glycoprotein recognized by the lectin Con A, and it represents more than 50% of the retained material when platelet lysate is loaded onto a Con A-Sepharose affinity column (5). These experimental results strongly suggest that GP IIb/IIIa might act as a regulator of 5-HT transport into blood platelets. In the present study, we investigated the possibility that extracellular EGTA and Ca2+ might regulate 5-HT transport activity into platelets through the function of the GP IIb/IIIa complex.

Whole blood collected from rabbits (Shimizu Biomaterials, Kyoto) 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) was collected and diluted with buffered salt solution (134 mM NaCl, 3 mM MgCl2, 5 mM d-glucose, 15 mM Tris-HCl buffer; pH 7.4) to make diluted platelet-rich plasma (1–2 × 108 platelets/ml). Transport of 3H-5-HT (1098.9 GBq/mmol; NEN Research Products, Boston, MA, USA) into the platelets was determined by the previously described method (6). The transport activity was expressed as % activity relative to that in the control experiments. The mean activity of 3H-5-HT uptake in the
control experiment was 244±13 pmol/10^9 platelets/3 min (n=13).

Effects of various reagents on the EGTA-induced inhibition of 5-HT transport were estimated by incubating diluted platelet-rich plasma with 1 mM EGTA for 60 min at 37°C in the absence or presence of the various tested reagents. Kinetic analysis of the EGTA-induced inhibition of 5-HT transport was performed by using different concentrations of ^3H-5-HT (0.1 to 1 μM) as a substrate; the activity was analyzed by double reciprocal plots (Lineweaver-Burk plots). Effects of various reagents on the Ca^{2+}-induced restoration of 5-HT transport were estimated by incubating EGTA-treated blood platelets with 2 mM CaCl_2 for 45 min at 37°C in the absence or presence of the various tested reagents. The activity of Ca^{2+}-induced restoration was expressed as % of the control activity increased by 2 mM CaCl_2. Genistein and tyrophostine 47 were purchased from Research Biochem. Int. (Natick, MA, USA). Phenylarsine oxide (PAO) was obtained from Sigma Chemical (St. Louis, MO, USA).

In the present study, we confirmed that ^3H-5-HT transport activity was reduced to ca. 50% of the control value after incubation of platelets with 1 mM EGTA for 60 min at 37°C. Figure 1 shows double reciprocal plots (Lineweaver-Burk plots) of the 5-HT transport activity into platelets in the absence or presence of 1 mM EGTA. In the absence of 1 mM EGTA, the apparent K_m value estimated from the X-axis and the V_max value estimated from the Y-axis were calculated as 1.84 μM and 4.09 nmol/10^9 platelets/3 min, respectively. When the platelets were treated with 1 mM EGTA for 60 min at 37°C, both K_m and V_max values were decreased (0.34 μM and 0.74 nmol/10^9 platelets/3 min, respectively). This indicates that the EGTA-induced inhibition of 5-HT transport activity is not derived from competition with substrate recognition sites within the 5-HT transporter, but from its action on some EGTA-sensitive component(s) in the platelets.

We suggested previously (3) that depletion of Ca^{2+} pools induced by EGTA might trigger the inhibitory effect of 5-HT transport activity into blood platelets and postulated that Ca^{2+} binding sites within the GP IIb/IIIa complex as well as intracellular Ca^{2+} storage sites might serve as the EGTA-sensitive Ca^{2+} pools responsible for 5-HT transport inhibition. The heterodimeric GP IIb/IIIa complex serves as the receptor for fibrinogen and other adhesive proteins after platelet activation. Integrity of Ca^{2+} storage sites in the extracellular domain of the GP IIb/IIIa complex is essential for its fibrinogen recognition site function, and chelation of Ca^{2+} in the store causes functional inhibition of GP IIb/IIIa (7). In the present study, we examined the possibility that regulation of 5-HT transport by EGTA and/or Ca^{2+} might be achieved...
Fig. 3. Effects of genistein, tyrphostine A47 and phenylarsine oxide (PAO) on \(^{3}H\)-5-HT transport into blood platelets in the absence (a) and in the presence of EGTA (b) and on the Ca\(^{2+}\)-induced increase in \(^{3}H\)-5-HT transport into EGTA-treated blood platelets (c). a: Diluted platelet-rich plasma was incubated with each drug for 45 min at 37°C and \(^{3}H\)-5-HT uptake activity was estimated. \(^{3}H\)-5-HT uptake activity is expressed as % of the control. b: Diluted platelet-rich plasma was incubated with each drug for 45 min at 37°C in the presence of 1 mM EGTA and \(^{3}H\)-5-HT uptake activity was estimated. EGTA-induced inhibition of \(^{3}H\)-5-HT uptake is expressed as % of the control (1 mM EGTA alone). c: After treatment with 1 mM EGTA at 37°C for 60 min, each drug was applied with 2 mM CaCl\(_2\). The mixtures were further incubated for 45 min and \(^{3}H\)-5-HT uptake activity was estimated. \(^{3}H\)-5-HT uptake activity increased by Ca\(^{2+}\) is expressed as % of the control (without drug). Each point represents the mean ± S.E.M. of four to six independent experiments. Significantly different from the control (*P < 0.05, **P < 0.01).
through their effect on GP IIb/IIIa function. It is generally accepted that the inhibitory regulation of the GP IIb/IIIa complex by EGTA is temperature-dependent (8). Thus, dissociation of the GP IIb/IIIa complex occurs exclusively at 37°C, but not at 20°C (9). Therefore, we determined if the effect of EGTA on 5-HT transport activity was also temperature-dependent. As shown in Fig. 2, treatment with EGTA at 20°C did not inhibit the 5-HT transport activity. This strongly suggested that EGTA-induced 5-HT transport inhibition might be elicited through its effect on the GP IIb/IIIa complex.

Intimate relationships between the cellular function of the GP IIb/IIIa complex and protein tyrosine kinase activity have been suggested in blood platelets (10, 11). Interplay between protein tyrosine kinase and protein tyrosine phosphatase activities is assumed to control the phosphorylation status of proteins involved in signal transduction events. So, we examined whether protein tyrosine phosphorylation or dephosphorylation might be involved in the EGTA-induced 5-HT transport inhibition and/or its reversal by CaCl₂. Thus, we analyzed the effects of protein tyrosine phosphatase inhibitors as well as those of protein tyrosine phosphatase inhibitors on 5-HT transport activity. Neither genistein, a competitive inhibitor of ATP binding to tyrosine kinase, nor tyrphostin A47, a competitive inhibitor of substrate binding to tyrosine kinase, affected 5-HT transport activity. Neither PAO, a protein tyrosine phosphatase inhibitor, significantly augmented Ca²⁺-induced restoration of 5-HT transport activity (Fig. 3c). In contrast, PAO, a protein tyrosine phosphatase inhibitor, significantly augmented Ca²⁺-induced restoration of 5-HT transport activity without affecting EGTA-induced 5-HT transport inhibition (Fig. 3: a, b and c). It was reported that several tyrosine phosphorylated protein bands (52–62 kDa) were observed in unstimulated platelets (12). Vostal et al. (13) suggested that homeostatic levels of Ca²⁺ in storage compartments favor tyrosine dephosphorylation of specific proteins and that the level of intracellular Ca²⁺ and stored Ca²⁺ appear to control tyrosine dephosphorylation antagonistically. Our present results might suggest that the Ca²⁺-induced restoration of 5-HT transport activity is mediated by tyrosine kinase activation. Thus, application of Ca²⁺ might cause functional restoration of the GP IIb/IIIa complex that had been inhibited by EGTA treatment, followed by activation of protein tyrosine phosphorylation and the restoration of 5-HT transport activity. At present, which tyrosine phosphorylated protein(s) might directly regulate 5-HT transport activity remains unclear. It has been reported that low molecular weight (21 kDa) GTP binding proteins, which serve as a substrate for tyrosine kinase, may mediated the function of GP IIb/IIIa in platelets (14).

Recently, Helmeste and Tang (15) reported that tyrosine kinase regulated 5-HT transport into human blood platelets. They suggested that the effects of genistein, a tyrosine kinase inhibitor, might be mediated by an as yet ill-defined non-Ca²⁺-related mechanism. However, it should be kept in mind that 5-HT uptake inhibition of genistein was observed in the literature (15), while we found no inhibitory effect of genistein on 5-HT uptake into rabbit blood platelets (Fig. 3a). It remains to be established whether the discrepancy is due to experimental conditions or whether it is related to the involvement of species variations.

It is conceivable that the GP IIb/IIIa complex, a platelet membrane integrin protein with Ca²⁺ binding activity, might regulate 5-HT transport activity into blood platelets. An observation that pentamidine, a non-peptide antagonist of GP IIb/IIIa, significantly inhibited 5-HT transport activity into blood platelets (H. Nishio et al., unpublished observation) further supports the involvement of the GP IIb/IIIa complex in the regulation of 5-HT transport. Ca²⁺ chelation by EGTA might cause temperature-dependent dissociation of the GP IIb/IIIa complex, resulting in reduction of 5-HT transport activity. Rearrangement of the GP IIb/IIIa complex by replenishment of the Ca²⁺ binding sites might ameliorate the EGTA-induced reduction of 5-HT uptake activity. This effect of Ca²⁺ replenishment seems to be dependent, at least in part, on tyrosine kinase activity. The proposed mechanism for EGTA-induced inhibition and its restoration by Ca²⁺ will require further investigation, where identification of the substrate(s) for tyrosine phosphorylation involved in the 5-HT transport regulation would appear to be a most promising approach.

It was concluded that EGTA induced depletion of Ca²⁺ from its storage sites on the GP IIb/IIIa complex, indicating its dissociation and therefore its dysfunction, which might result in the inhibition of 5-HT transport activity. Replenishment of the Ca²⁺ storage sites evoked recovery of the function of GP IIb/IIIa and restored the 5-HT transport activity in a protein tyrosine kinase-dependent manner.

REFERENCES

4 Ikegami Y, Nishio H, Fukuda T, Nakata Y and Segawa T: Effect of concanavalin A on intracellular calcium concentration


10 Findik D, Reuter C and Presek P: Platelet membrane glycoproteins IIb and IIIa are substrates of purified pp60src protein tyrosine kinase. FEBS Lett 262, 1–4 (1990)


