Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 306-311

INHIBITORY EFFECTS OF 4,4'-DIISOTHIOCYANOSTILBENE-2,2'-DISULFONATE(DIDS) ON THE ADP-STIMULATED AGGREGATION OF GEL-FILTERED BOVINE BLOOD PLATELETS

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Received January 19, 1983

The effect of DIDS, a specific inhibitor of anion transport in the erythrocyte membrane, on the ADP-stimulated aggregation of gel-filtered bovine blood platelets was examined. Marked inhibition of aggregation was observed at concentrations of more than 5×10^{-5}M DIDS. On preincubation with platelets for 30 min, DIDS was more potent and significant inhibition was observed at concentrations of over 2×10^{-7}M. Since ADP-stimulated aggregation of bovine gel-filtered platelets precedes the release reaction, these results suggest that an anion transport system in the plasma membrane is involved in platelet aggregation.

Platelet aggregation is affected by the ionic milieu, and has been shown to depend on the concentration of cations such as Ca^{2+} and K^{+}(1,2,3). Moreover, platelet activation is associated with marked change of cation transport(4,5). These results suggest that cation transport is involved in platelet reactivity(4,5). Our previous paper reported significant anion dependency of the ADP-stimulated aggregation of bovine blood platelets(6), and also suggested the involvement of anion transport in platelet reactivity. To test this suggestion, we examined the effect of DIDS, which is known to be specific anion transport inhibitor in the erythrocyte membrane(7,8), on ADP-stimulated aggregation of bovine blood platelets.

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Abbreviation: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate.

0006-291X/83/040306-06$01.50/0

MATERIALS AND METHODS

DIDS, bovine serum albumin(essentially fatty acid free) and bovine fibrinogen were purchased from Sigma Chemicals Co. (St.Louis, MO.). ADP was purchased from Oriental Yeast Co. (Tokyo, Japan), highly purified luciferin-luciferase reagent from LUMAC B.V.(Schaesberg, Netherlands) and all other chemicals were purchased from Wako Pure Chemical Industries(Osaka, Japan).

Platelet rich plasma(PRP) of bovine(holstein) blood was obtained as described in the previous paper(6). PRP was applied to a Sepharose 2B column equilibrated with Ca^{2+}-free tyrode solution to obtain plasma-free suspensions of platelets(9). Platelet suspensions were mixed with a half volume of various concentrations of DIDS in Ca^{2+}-free tyrode solution. The final platelet concentration was about $1.8 \times 10^5 (\mu l^{-1})$. $CaCl_2$ was added to give a final concentration of 0.4 mM and the suspensions were incubated at 37°C for certain times. Then solution of 1 $mg \cdot ml^{-1}$ albumin and fibrinogen containing equi-volume of Ca^{2+}-free tyrode solution was added, and the aggregation induced by 1×10^{-5} M ADP was measured in an aggregometer(Bio-Data Co., Horsham, PA).

The rate of aggregation was measured as the steepest tangential slope of the downward deflection of the records. Aggregation inhibition was expressed as the percentage decrease of the aggregation rate in the presence of DIDS relative to that without DIDS.

Release of ATP was measured simultaneously with aggregation by the luminescence method using luciferin-luciferase(10,11). Aliquots of the platelet suspensions were withdrawn at intervals and mixed with equi-volume of the solution containing luciferin -luciferase reagent. Luminescence was measured with Biocounter 2010(LUMAC B.V.).

RESULTS AND DISCUSSION

Gel-filtered platelets are good material to use in fundamental studies on the effects of drugs on platelet activities, because binding of drugs to plasma proteins can be prevented. As seen in Figure 1, addition of 1×10^{-5} M ADP induced aggre-

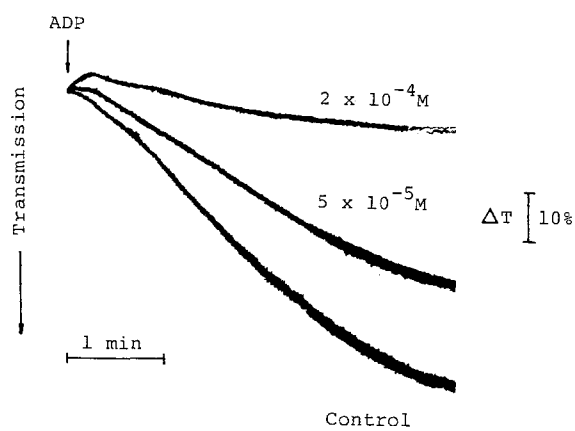

Figure 1. Aggregation of gel-filtered bovine blood platelets and its inhibition by DIDS. The concentrations of DIDS are given in the figure. ΔT = percent change in optical density.

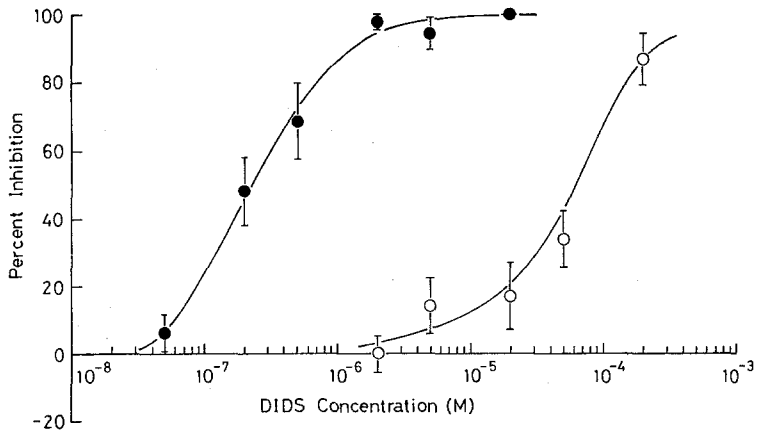

Figure 2. Inhibition of aggregation of platelets by preincubation with DIDS for 0 min (○) and 30 min (●). Data are mean values ± S.D. for three to six experiments.

gation of bovine gel-filtered platelets, and the aggregation was inhibited by DIDS. When DIDS was added without preincubation, it caused marked inhibition at concentrations of more than 5×10^{-5} M and aggregation was almost completely inhibited at a concentration of 2×10^{-4} M, as shown in Figure 2. However, on preincubation for 30 min with platelets, DIDS was more effective, significant inhibition being observed even at a concentration of 2×10^{-7} M, and almost complete inhibition at 2×10^{-6} M.

We examined the effects of the preincubation time with 2×10^{-6} M DIDS on platelet aggregation in detail. The results in Figure 3 show that during the first 5 min of preincubation the inhibitory effect increased markedly and preincubation for 15 min or more caused maximum inhibition. This potent inhibitory effect of DIDS and its dependency on the preincubation time were quite similar with those of the inhibitory effect of DIDS on anion transport through the erythrocyte membrane(7,12).

In experiments on human platelets, Pollard's group found that 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate(SITS) inhibited thrombin-induced serotonin release reaction(13,14).

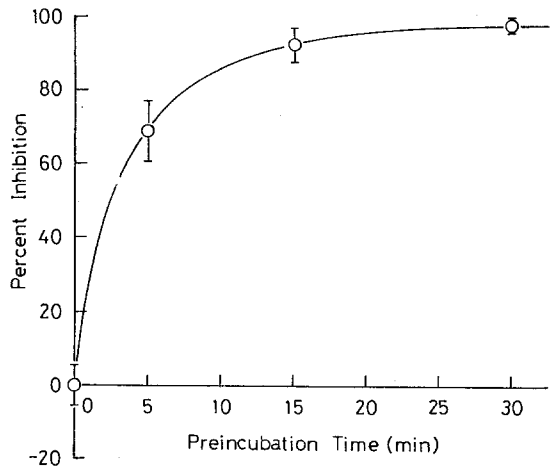

Figure 3. Effect of preincubation time with DIDS on inhibition of platelet aggregation. Data are mean values ± S.D. for three to six experiments with 2 x 10^{-6} M DIDS.

SITS is also a potent inhibitor of anion transport through the erythrocyte membrane, but its specificity to anion channel protein is much lower than that of DIDS(12). Considering the impermeability of disulfonic stilbene derivatives through the cell membranes(8), they proposed that the reaction site of SITS was the vesicular membrane fused with the plasma membrane because of the similarity of the reaction of SITS with that chromaffin granules(15) and the pH- and osmolarity-dependent release of serotonin from platelets(13,14). However, unlike thrombin-induced aggregation of human platelets(16), ADP-induced aggregation proceeds before the release reaction as shown in Figure 4 for bovine platelets, which is consistent with the results for human platelets(16). Therefore, the target membrane of stilbene derivatives must be the plasma membrane itself. Moreover, high specificity of DIDS for the anion channel protein in the erythrocyte membrane suggests that the anion transport system in the platelet plasma membrane, like that in the erythrocyte membrane, may be involved in platelet aggregation. Anion transport in

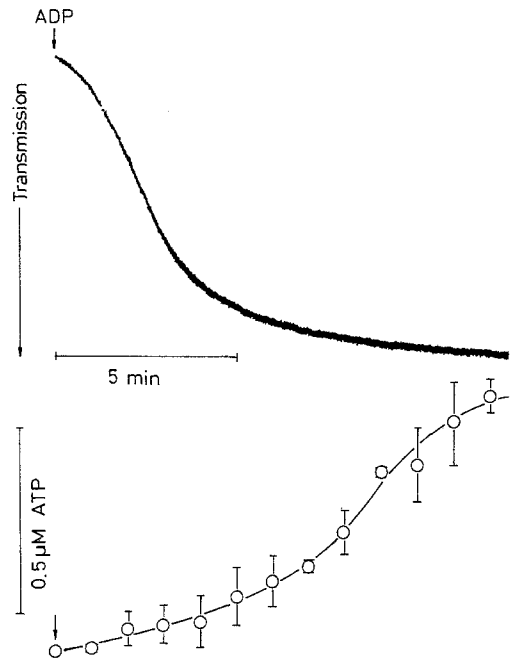

Figure 4. Simultaneous measurements of aggregation and secretion induced by 1 x 10^{-5} M ADP. Upper and lower curves show aggregation and release of ATP, respectively. Measurements of ATP release were done at intervals by the luminescence assay, immediately after withdrawing aliquots of the platelet suspensions containing 0.1 mg·ml^{-1} albumin and fibrinogen. Data of ATP release expressed as concentration in the medium are mean values ± S.D. for three experiments.

the platelet plasma membrane may be related to control of the transmembrane potential or maintenance of cytoplasmic pH.

However, the action of DIDS on other protein cannot be excluded completely, although DIDS has been revealed to interact with the anion channel protein in highly specific manner. In fact DIDS was recently found to inhibit ATPase activity in the erythrocyte membrane(17), although it may also be related with an anion transport(18). Further studies on the inhibitory mechanism of DIDS on the platelet aggregation is now in progress.

ACKNOWLEDGEMENTS

We are grateful to Dr. J. Isobe, University Hospital of Tokushima, for help in measurement of aggregation. This work was supported in part by a grant from the Japanese Ministry of Education, Science and Culture.

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