

# The platelet activation induced by wheat germ agglutinin

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In human platelets, wheat germ agglutinin (WGA) induced serotonin release without cell agglutination. WGA induced the phosphorylation of both 40-kDa and 20-kDa proteins in a parallel manner, and at least, the phosphorylation of 40-kDa protein was preceded by transient formation of endogenous diacylglycerol (DG) accompanied by a decrease in phosphatidylinositol (PI). Both phosphorylation of these two proteins and serotonin release were inhibited by prior treatment of platelets with dibutyl cyclic AMP, W-7, or TMB-8. These results suggest that both phosphatidylinositol turnover and  $\text{Ca}^{2+}$  mobilization play an essential role in WGA-induced platelet activation.

*Wheat germ agglutinin      Platelet      Calcium      Diacylglycerol      Fluorescent indicator*

## 1. INTRODUCTION

Wheat germ agglutinin (WGA), which shows specificity for sialic acid, *N*-acetylglucosamine, and *N*-acetylgalactosamine, strongly activates platelets [1–3]. This activation is accompanied by serotonin release, which may be independent of cell agglutination [4]. Although sialic acid may be important in the activation by WGA [5,6], the precise mechanism of WGA-induced platelet activation remains obscure.

In a preliminary study, we observed that WGA-induced platelet activation was accompanied by the incorporation of  $^{32}\text{P}$  into peptides of  $M_r$  40 000 and 20 000 [7]. Here, we show that WGA induced diacylglycerol (DG) formation followed by 40-kDa protein phosphorylation and [ $^{14}\text{C}$ ]serotonin release. These results suggest that phosphatidylinositol (PI) turnover plays an important role in the platelet ac-

tivation induced by WGA as in the case of thrombin [8–10]. The mobilization of both cytoplasmic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) and membrane-bound  $\text{Ca}^{2+}$  in response to WGA will also be briefly described by using a new fluorescent calcium indicator, quin2, and chlortetracycline (CTC), respectively.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

WGA, apyrase (grade II) and imipramine were purchased from Sigma, St. Louis, MO. [ $^{14}\text{C}$ ]Serotonin (58 mCi/mmol) was purchased from Amersham, Arlington Heights, IL. [ $^3\text{H}$ ]Arachidonate (62.2 Ci/mmol) and carrier-free  $^{32}\text{P}_i$  (350 mCi/ml) were obtained from New England Nuclear, Boston, MA. All other chemicals were of reagent grade or were the best commercially available.

### 2.2. Platelet preparation

Blood was collected from volunteers in plastic syringes utilizing 3.8% (w/v) sodium citrate as anticoagulant (9:1). Platelet-rich plasma (PRP) was

*Abbreviations:* W-7, *N*-(6-aminoethyl)-5-chloro-1-naphthalensulfonamide hydrochloride; TMB-8, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride

obtained by centrifugation at  $120 \times g$  for 10 min. Washed platelets were prepared by the method of Mustard et al. [11] except that the final platelet pellet was resuspended in  $\text{Ca}^{2+}$ -free Tyrode's buffer with glucose (1 mg/ml). Formaline-fixed washed platelets were prepared as described by Allain et al. [12].

### 2.3. Aggregation study and assay for serotonin release

Platelet aggregation was measured on a Chlono-Log lumiaggregometer with or without stirring (1100 rpm) as described [7]. The release of serotonin was measured with platelets preloaded with [ $^{14}\text{C}$ ]serotonin according to Haslam et al. [13]. Imipramine (1  $\mu\text{M}$ ) was used to prevent the re-uptake of released serotonin.

### 2.4. Assay for platelet protein phosphorylation

Washed platelets were labelled with  $^{32}\text{P}_i$  by the method of Haslam and Lynham [14]. The radioactive platelets were finally resuspended in  $\text{Ca}^{2+}$ -free Tyrode's buffer with glucose (1 mg/ml). A suspension of platelets (190  $\mu\text{l}$ ) was stimulated with 10  $\mu\text{l}$  WGA at  $37^\circ\text{C}$  without stirring. The incubation was terminated by addition of 100  $\mu\text{l}$  SDS-stop solution [188 mM Tris-HCl, 6.9% SDS, 30% glycerol (w/v), 15% 2-mercaptoethanol, pH 6.8]. The radioactive platelets were then directly subjected to SDS-polyacrylamide gel electrophoresis [15], stained, dried on a filter paper and then exposed to a Kodak X-Omat film. The relative intensity of each band was quantitated by measuring the absorbance at 430 nm using a Shimazu dual-wavelength chromatogram scanner, model CS-910.

### 2.5. Analysis of DG and PI

Labelling of platelets with [ $^3\text{H}$ ]arachidonate was carried out as described by Rittenhouse-Simmons [8]. The incubation was terminated by addition of chloroform/methanol (1:2) and the lipids were directly extracted from the platelet suspension as described by Bligh and Dyer [16]. Radioactive DG and PI were separated and quantified by thin-layer chromatography on silical gel 60 F254 (Merck) plates as described by Rittenhouse-Simmons [8] and Vitiello and Zancella [17], respectively.

### 2.6. Quin2 loading and fluorescence measurements

The platelets were loaded with the fluorescent calcium-indicator, quin2 by incubating the PRP with 15  $\mu\text{M}$  quin2 acetoxymethyl ester (Lancaster Synthesis, Morecambe, England) added from 50 mM stock solution in dimethyl sulfoxide (DMSO) for 30 min at  $37^\circ\text{C}$ . The platelets were washed twice by the method of Mustard et al. [11], and resuspended in a medium containing 145 mM NaCl, 5 mM KCl, 1 mM  $\text{MgSO}_4$ , 10 mM Hepes, 5 mM glucose, pH 7.4, at  $37^\circ\text{C}$ . Fluorescence was recorded at  $37^\circ\text{C}$  in a Hitachi fluorescence spectrometer 650-60. Excitation was at 339 nm and emission at 492 nm.  $[\text{Ca}^{2+}]_i$  was calculated from the fluorescence signal as described by Rink et al. [18].

### 2.7. CTC loading and fluorescence measurements

PRP were incubated with 50  $\mu\text{M}$  CTC for 30 min at room temperature, washed twice [11], and final pellet was resuspended in  $\text{Ca}^{2+}$ -free Tyrode's buffer with glucose (1 mg/ml). CTC fluorescence in platelets was measured in a Hitachi fluorescence spectrometer 650-60 (400 nm excitation, 530 nm emission).

### 2.8. Other procedures

Lactic dehydrogenase (LDH) activity was determined according to the method of Wroblewski and La Due [19].

## 3. RESULTS

WGA-induced platelet aggregation in washed platelets is illustrated in fig.1A. Maximal aggregation induced by WGA (100  $\mu\text{g}/\text{ml}$ ) after 5 min was  $52 \pm 6\%$  (mean  $\pm$  SD,  $n = 5$ ). On the other hand, the extent of WGA-induced agglutination in formalin-fixed washed platelets was small ( $4 \pm 2\%$ ,  $n = 8$ ). In the absence of stirring, at least in a lumiaggregometer, WGA-induced agglutination did not occur (fig.1A). Fig.1B shows the time course of [ $^{14}\text{C}$ ]serotonin release induced by WGA without stirring. WGA induced serotonin release rapidly and about 50% of [ $^{14}\text{C}$ ]serotonin was released within 1 min. The release reaction was not due to cytolysis of the platelets because LDH release by WGA (100  $\mu\text{g}/\text{ml}$ ) was very slight ( $2.3 \pm 1.1\%$ , mean  $\pm$  SD,  $n = 5$ ). Serotonin release

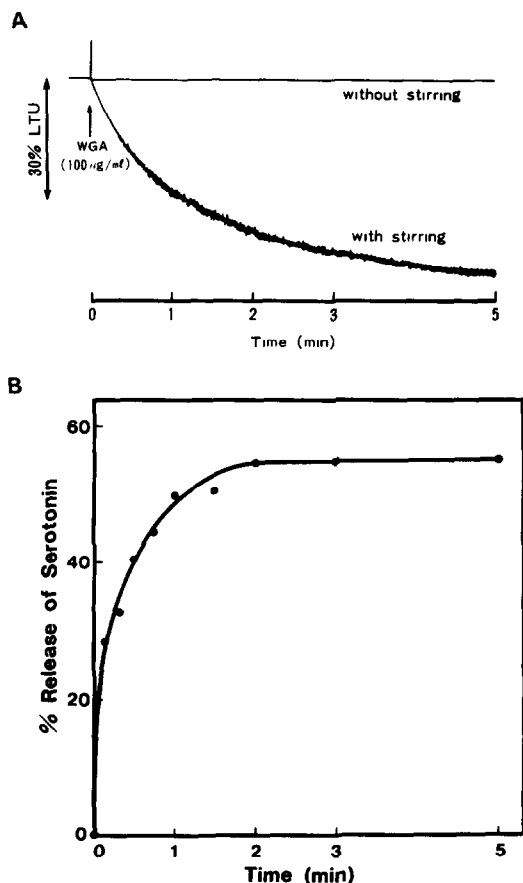


Fig.1. (A) Platelet aggregation induced by WGA (100  $\mu\text{g/ml}$ ). WGA-induced aggregation proceeded in a dose-dependent manner (not shown). No agglutination was seen without stirring in a lumiaggregometer. On the other hand, with stirring (1100 rpm), maximal aggregation was  $52 \pm 6\%$  (mean  $\pm$  SD,  $n=5$ ). (B) Serotonin release induced by WGA (100  $\mu\text{g/ml}$ ) without stirring. Values are means from 3 experiments.

induced by WGA was accompanied by the phosphorylation of 40-kDa protein, which reached a peak within 2 min of platelet activation (fig.2A). In addition to 40-kDa protein, 20-kDa protein, a myosin light chain [20], was also phosphorylated in platelets stimulated by WGA, although the relative intensity of phosphorylation of 20-kDa protein was much smaller than that of 40-kDa protein (fig.2A). Following the peak of  $^{32}\text{P}$  incorporation, a dephosphorylation took place over the next 3–7 min (fig.2A). The phosphorylation of these two proteins proceeded in a dose-dependent manner (not shown). When platelets were stimulated by WGA, DG was rapidly produced accompanied by the decrease of PI (fig.2B), and these reactions were immediately followed by 40-kDa protein phosphorylation (fig.2A).

Both phosphorylation of these two proteins and [ $^{14}\text{C}$ ]serotonin release were prevented by a prior

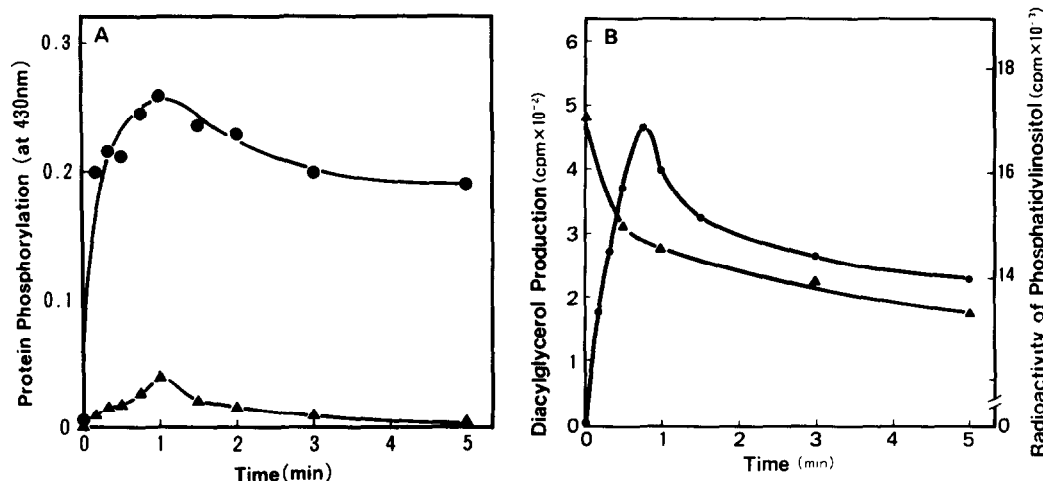


Fig.2. Time courses of platelet protein phosphorylation, diacylglycerol formation, and phosphatidylinositol breakdown in platelets stimulated by WGA (100  $\mu\text{g/ml}$ ). A, (●—●) 40-kDa protein phosphorylation; (▲—▲) 20-kDa protein phosphorylation. (B) (●—●) [ $^3\text{H}$ ]diacylglycerol formation; (▲—▲) [ $^3\text{H}$ ] phosphatidylinositol breakdown. The points represent mean values of duplicate samples.

Table 1

The inhibition of WGA-induced platelet protein phosphorylation and serotonin release

Addition	40-kDa protein phosphorylation ( <i>A</i> at 430 nm)	20-kDa protein phosphorylation ( <i>A</i> at 430 nm)	Serotonin release (cpm)
None	0.05	0.04	535
WGA	0.31	0.10	2607
WGA + W-7	0.08	0.06	1581
WGA + TMB-8	0.08	0.05	1242
WGA + dbcAMP	0.06	0.05	561

All values were means for 3 experiments. Platelet suspensions were incubated with W-7 (50  $\mu$ M), TMB-8 (200  $\mu$ M) or dibutyryl cyclic AMP (dbcAMP, 2 mM) at 37°C for 5 min and WGA (100  $\mu$ g/ml) was added. The incubation with WGA was carried out for 1 min to measure both platelet protein phosphorylation and serotonin release

treatment of platelets with a calmodulin inhibitor, W-7 (50  $\mu$ M), a calcium blocker, TMB-8 (200  $\mu$ M), or dibutyryl cyclic AMP (2 mM) (table 1).

In the presence of external calcium (1 mM), WGA (100  $\mu$ g/ml) evoked a rise in  $[Ca^{2+}]_i$  in a few seconds from the basal level to near 1  $\mu$ M. The rise was only short-lived and declined towards the resting level within a few minutes. The increase of  $[Ca^{2+}]_i$  with WGA proceeded in a dose-dependent manner (not shown). Fig. 3A shows a typical response to WGA (100  $\mu$ g/ml). The mean peak  $[Ca^{2+}]_i$  with 100  $\mu$ g/ml was  $910 \pm 82$  nM (mean  $\pm$  SD,  $n=5$ ). In the presence of 3 mM EGTA there was a much smaller increase in  $[Ca^{2+}]_i$  of a similar pattern and the mean peak  $[Ca^{2+}]_i$  with 100  $\mu$ g/ml was  $230 \pm 68$  nM (mean  $\pm$  SD,  $n=5$ ).

The membrane-bound  $Ca^{2+}$  was immediately decreased after addition of WGA as in the case of thrombin (fig.3B), suggesting that increased  $[Ca^{2+}]_i$  is partially due to the mobilization of membrane-bound  $Ca^{2+}$ . These changes of CTC fluorescence proceeded in a dose-dependent manner (not shown).

#### 4. DISCUSSION

WGA, one of the *N*-acetylglucosamine specific lectins [1–3], induced platelet agglutination and release reaction without cell lysis (fig.1A, B). A previous study [2] showed that WGA-induced platelet activation is inhibited by EDTA or PGE<sub>1</sub>, suggesting that WGA interacts with platelets as a

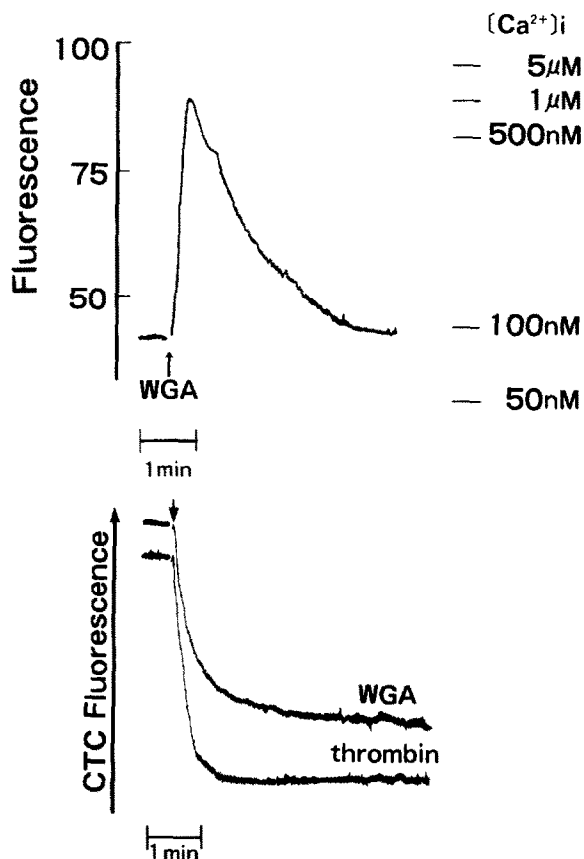


Fig.3. (A) The change of quin2 fluorescence in platelets stimulated by WGA (100  $\mu$ g/ml). (B) The change of CTC fluorescence. WGA (100  $\mu$ g/ml) or thrombin (0.5 U/ml) was added at the indicated point.

true aggregating agent such as thrombin or collagen. However, the precise mechanism of WGA-induced platelet activation remains obscure.

Here, we showed that WGA induced the phosphorylation of both 40-kDa and 20-kDa proteins in a parallel manner (fig.2A) and that, at least, the phosphorylation of 40-kDa protein was preceded by transient formation of endogenous DG accompanied by the decrease of PI (Fig.2B). These results seem to provide evidence that the receptor-mediated breakdown of inositol phospholipids induced by WGA is directly coupled to the activation of protein kinase C, which is responsible for the phosphorylation of 40-kDa protein [21]. 20-kDa protein was identified as myosin light chain [20], and a specific calmodulin-dependent protein kinase was proposed to be responsible for 20-kDa protein phosphorylation [14,22], which plays an important role in the release reaction such as serotonin release [23]. Kaibuchi et al. [24] showed that 40-kDa protein phosphorylation and  $\text{Ca}^{2+}$  mobilization acted synergistically to elicit serotonin release through experiments with synthetic diacylglycerol and  $\text{Ca}^{2+}$  ionophore. It is conceivable that WGA, like other stimulants such as thrombin [8–10] and platelet-activating factor [25], provoke inositol phospholipid turnover as well as  $\text{Ca}^{2+}$  mobilization as judged by both 20-kDa protein phosphorylation (Fig.2A) and the change of  $[\text{Ca}^{2+}]_i$  and membrane-bound  $\text{Ca}^{2+}$  measured with a new fluorescent indicator, quin2 and CTC, respectively (fig.3A,B). Moreover, W-7, TMB-8, or dibutyryl cyclic AMP prevented the phosphorylation of 40-kDa and 20-kDa proteins, and serotonin release (table 1), suggesting that  $\text{Ca}^{2+}$  mobilization plays an important role in WGA-induced platelet activation.

Here, we have shown for the first time that WGA induces not only cell agglutination, but also the receptor-mediated breakdown of inositol phospholipid followed by 40-kDa protein phosphorylation and release reaction. Ganguly and Fossett [6] showed that sialic acid played an essential role in platelet activation by WGA, and the extent of aggregation and secretion of the platelets under pathological conditions, compared to normals, might provide a rapid estimation of the sialic acid present on the surface of these cells. However, our results show that platelet activation abnormalities with WGA, whether aggregation or secretion,

are not always due to membrane abnormalities, because impaired  $\text{Ca}^{2+}$  mobilization in the platelets under pathological conditions may be responsible for reduced responsiveness to stimulants [26,27]. It remains to be determined whether WGA-induced platelet activation is reduced in the platelets which show impaired  $\text{Ca}^{2+}$ -mobilization, and if the content of sialic acid is responsible for  $\text{Ca}^{2+}$  mobilization in response to WGA.

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