Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 306-313

THE PLATELET PROTEIN PHOSPHORYLATION INDUCED BY A MONOCLONAL ANTIBODY AGAINST HUMAN PLATELETS (TP82)

Masaaki Higashihara[1], Hiroo Maeda[2], Yutaka Yatomi[1], Kyoya Takahata[3], Hiroshi Oka[1], and Shoji Kume[4]

[1]The First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

[2]The Blood Transfusion Service, University of Tokyo, Tokyo, Japan

[3]The Central Laboratory Center, Faculty of Medicine, University of Tokyo, Tokyo, Japan

[4]The Central Laboratory Center, Yamanashi Medical College, Yamanashi, Japan

Received October 4, 1985

SUMMARY: In human platelets, a monoclonal anti-human platelet antibody (TP82) induced platelet aggregation and release of granules (i.e., serotonin, platelet factor 4, N-acetyl-β-D-glucosaminidase). The release reaction occurred even in the absence of aggregation and was preceded by not only the protein phosphorylation, but the transient formation of endogenous diacylglycerol (DG). These results suggest that polyphosphoinositide breakdown plays an essential role in antibody-induced release of platelet granules.

INTRODUCTION: Previous studies showed that anti-human platelet antibody (IgG) found in patients with mutitransfusions (1,2) or anti-platelet heteroantibodies (3,4,5) caused platelet aggregation and release reaction. Recently, we (6) and others (7-9) reported that some kinds of monoclonal antibodies which reacted with human platelets, induced platelet aggregation and release of dense bodies. However, the precise mechanism of antibody-induced platelet activation remains in quite obscure. We in (6) demonstrated that a monoclonal anti-human platelet antibody,

Abbreviations: DG, diacylglycerol; TPA, 12-o-tetradecanoyl phorbol-13-acetate; NAG, N-acetyl-β-D-glucosaminidase; β-TG, β-thromboglobulin; PF4, platelet factor 4; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride.

0006-291X/85 $1.50

designated TP82, which reacted with 23-KDa membrane protein in human platelets, caused irreversible aggregation of human platelets accompanied by TXA_2 formation and the release of dense bodies (ATP and serotonin).

In this paper, we showed that TP82 antibody induced platelet protein phosphorylation and diacylglycerol (DG) formation. These results suggest that polyphosphoinositide breakdown plays an important role in TP82-induced platelet activation like the case of thrombin (10,11), collagen (12), or platelet acivating factor (PAF)(13).

Materials and Methods

TP82 antibody production and purification, platelet-rich plasma (PRP) preparation, washed platelets preparation were performed as previously described (6). Platelet aggregation and ATP release were measured on Chrono-Log lumiaggregometer with stirring (1,100 rpm)(6). Luciferin-luciferase and 12-o-tetradecanoyl phorbol-13-acetate (TPA) were purchased from Sigma. The release of serotonin was measured with platelets preloaded with [^{14}C] serotonin (Amersham) as described elesewhere (6). The release of platelet factor 4 (PF4) and β-thromboglobulin (β-TG) were measured using radioimmunoassay kits of Amersham and Abbott, respectively. N-acetyl-β-D-glucosaminidase (NAG) was measured according to the method of Noto et al. (14). Samples to assay PF4, β-TG and NAG were obtained by adding 0.05 mL of ice-cold stopping buffer (55 μM PGE_1, 55 mM EDTA, 220 μM indomethacin) to 0.5 mL of incubation sample and centrifuging at 2,000g for 20 min. The activity of β-TG, PF4 and NAG in the supernatant was expressed as a percentage of the total amount determined by solubilization with 1% (wt/vol) Triton X-100. Assay for platelet protein phosphorylation and analysis of DG were carried out as previously described (15). Carrier free ^{32}P and [^{3}H] arachidonic acid were purchased from New England Nuclear. Quin 2 loading and fluorescence measurements were carried out according to the method of Tien et al. (16). All other materials used were reagent grade.

Results and Discussion

TP82 antibody which reacts with 23-KDa membrane protein in human platelets, whether whole IgG or $F(ab')_2$ fragments, causes irreversible aggregation of human platelets accompanied by TXA_2 formation and the release of dense bodies (ATP and serotonin)(6). The release of dense bodies synchronized with platelet aggregation (Fig. 1A, Fig. 2 in ref. 6), and the pattern of release reaction

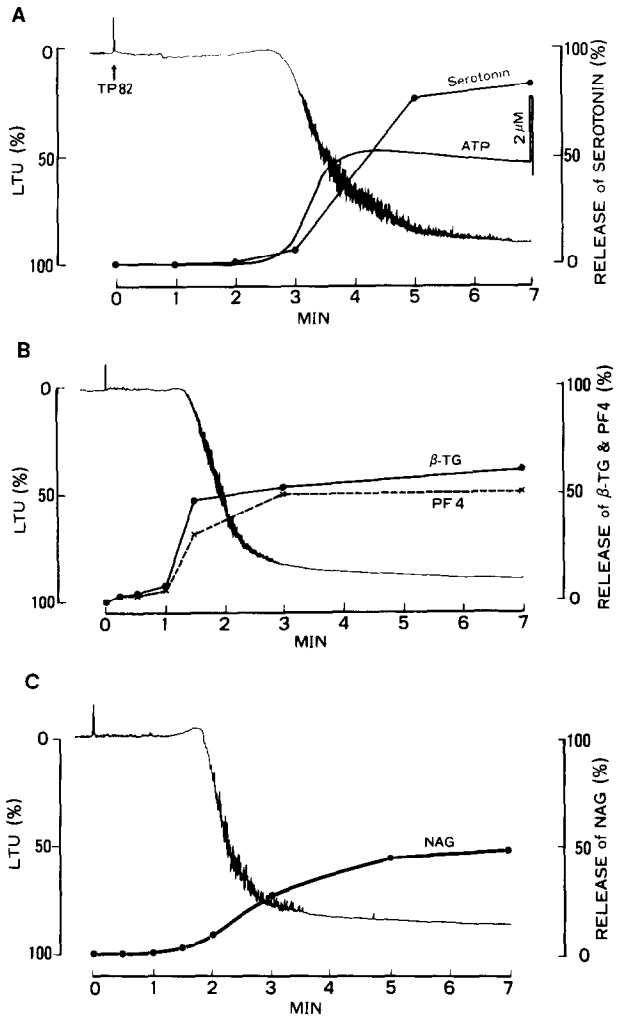

Fig. 1. TP82-induced release of platelet granules. A, ATP and serotonin; B, β-TG and PF4; C, NAG. TP82 (12 μg/mL) was added to PRP at the indicated point. PRP in each experiments (A,B,C) was obtained from different donors and there was a little difference in the aggregation pattern (length of the lag time or extent of % aggregation) among donors (6). The release reaction proceded in a dose-dependent manner (data not shown). The similar results were obtained in the experiments with washed platelets (data not shown). Platelet count was adjusted to 3×10^8/mL. The values were means of three experiments.

was same with the case of α-granules (β-TG and PF4, Fig. 1B) or lysosomal enzyme (NAG, Fig. 1C). In the absence of aggregation, these three types of granules were slowly released, and almost maximal secretion was obtained after 7 min (Fig. 2), suggesting that TP82-induced release reaction was essentially independent of

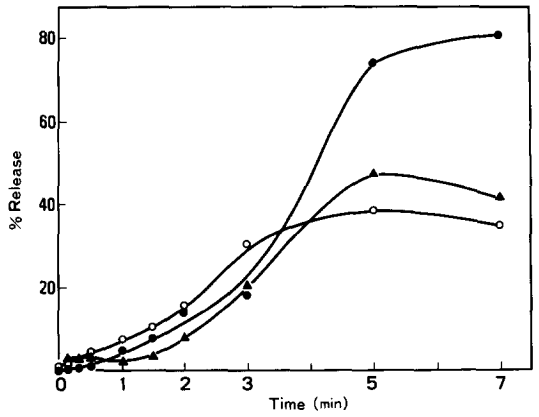

Fig. 2. TP82-induced release of granules in washed platelets without stirring. ●—●, serotonin; o—o, NAG; ▲—▲, β-TG. The platelet count was adjusted to $3x10^8$/mL. The concentration of TP82 was 12 μg/mL. The same pattern was obtained in PRP but % release of these granules was smaller than in washed platelets (data not shown). Control monoclonal antibody, 1C6-1A (anti-HLA, A,B,C common determinant)(6), caused no measurable release of these granules. Values were means of three experiments.

aggregation. The release of granules was not due to cell lysis because LDH release was very slight (6), and because electron microscopic examination showed that TP82 caused the centralization of granules and contractile gel formation with intact plasma membrane (data not shown), both of which were found in platelets stimulated by an aggregating agent such as thrombin (17).

Recently, synergistic effect of Ca^{2+} mobilization and the activation of Ca^{2+}-activated, phospholipid-dependent protein kinase C (C-kinase) plays an important role in the release of dense body (18) or lysosomal enzyme (19). It has been shown that 40-KDa protein in platelets is phosphorylated by C-kinase (20), which is activated by DG (21) or tumor-promoting phorbol ester TPA (22), and that 20-KDa protein, myosin light chain (23), is phosphorylated by Ca^{2+}-calmodulin dependent myosin light chain kinase (24). So we studied TP82-induced protein phosphorylation without stirring and the effects of inhibitors on this phosphorylation were evaluated. TP82 induced 20-KDa and 40-KDa protein phosphorylation in a parallel manner and maximal phosphorylation

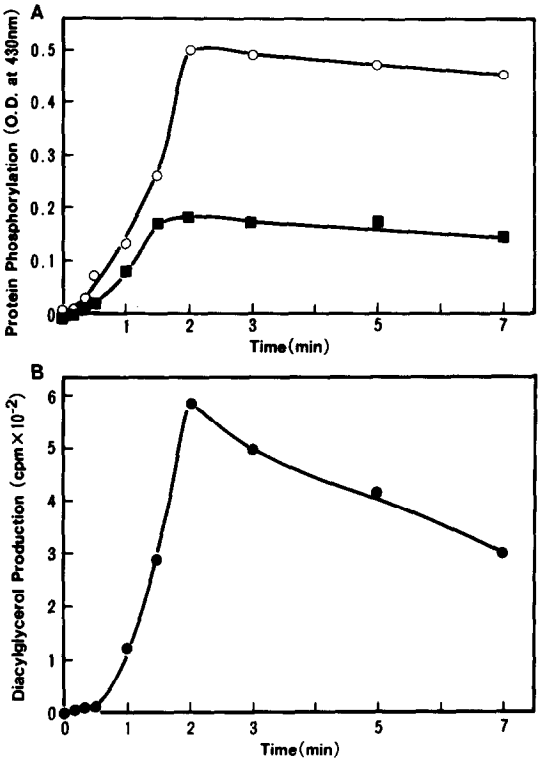

Fig. 3. The time course of TP82-induced platelet protein phosphorylation and diacylglycerol formation. A, the platelet protein phosphorylation (o——o, 40-KDa protein; ■——■, 20-KDa protein). B, [^{3}H] diacylglycerol formation. The ^{32}P- or ^{3}H-prelabeled platelet suspensions were incubated with TP82 (12 μg/mL) for indicated time and the relative intensity of phosphorylation was quantitated by measuring the absorbance at 430 nm as previously described (16). Radioactive diacylglycerole was separated and quantified by thin layer chromatography (16). The values were means of duplicate samples. TP82-induced protein phosphorylation proceded in a dose-dependent manner (data not shown).

was obtained in 2 min after addition of TP82 (Fig. 3A), and DG was rapidly and transiently produced (Fig. 3B). These results suggest that TP82 induces polyphosphoinositide breakdown followed by DG formation like the case of thrombin (11,12), collagen (12) or PAF (13). Moreover, W-7, TMB-8, PGE_1 or dibutyryl cyclic AMP (dbcAMP) fully prevented the phosphorylation of 40-KDa and 20-KDa protein, but aspirin, indomethacin or EGTA did not inhibit these protein phosphorylations (Fig. 4), suggesting that internal Ca^{2+} flux and calmudulin involve antibody-induced protein phosphorylation but that neither external Ca^{2+} nor TXA_2 plays a critical

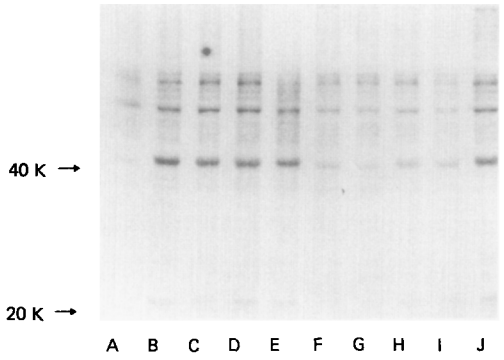

Fig. 4. Inhibition of TP82-induced protein phosphorylation by several inhibitors. Platelet suspensions were incubated with NaCl or inhibitors at 37°C for 5 min and then TP82 (12 μg/mL) was added (B-I). The incubation with TP82 was carried out for 2 min. A, control; B, NaCl; C, EGTA (2 mM); D, aspirin (100 μg/mL); E, indomethacin (20 μM); F, PGE_1 (2 μM); G, dbcAMP (2 mM); H, TMB-8 (0.5 mM); I, W-7 (100 μM); J, thrombin (0.1 U/mL). Thrombin was added to platelet suspension without inhibitors and the incubation was carried out for 1 min. The same results were obtained in another two separate experiments.

role in this effect. Although 20-KDa protein phosphorylation was occurred by TP82 and inhibited by agents that prevented Ca^{2+} flux, no measurable change in cytosolic free Ca^{2+} ($[Ca^{2+}]i$) was seen, in contrast to thrombin (Fig. 5). The same pattern was obtained with TPA (Fig. 5) as shown by others (25,26). It seems likely that the increase of $[Ca^{2+}]i$ prerequisite to synergistic effect on TP82-induced release reaction is so small, like the case of collagen (25), that it cannot be detected by using a new fluorescent calcium-indicator, quin 2 (16).

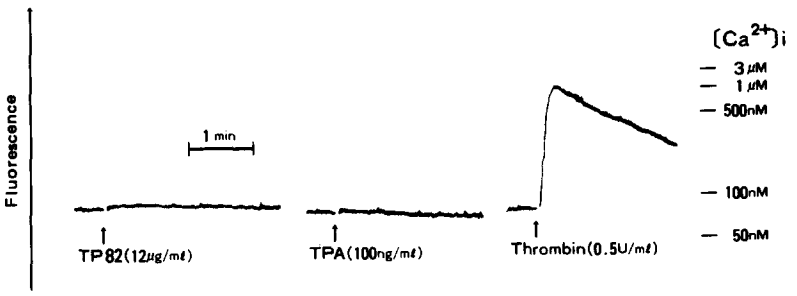

Fig. 5. Quin 2 fluorescence by TP82, TPA and thrombin. Quin 2 fluorescence was recorded at 37°C in Hitachi fluorescence spectrometer 650-60 without continous stirring (excitation at 339 nm, emission at 490 nm). External Ca^{2+} concentration was 1 mM.

Zahvi et al. (27) reported the first example of acquired "storage pool disease (28,29)" in a patient with nephritis, polyarthralgia, chondritis, thrombophlebitis, Raynaud's phenomenon and circulating anti-platelet antibodies. Weiss et al. (30) showed that platelet-associated IgG was increased in platelets from five patients including two with systemic lupus erythematosus and one with compensated chronic idiopathic thrombocytopenic purpura (ITP), and that in those platelets the content of granule substances were diminished. To be of interest, Woods et al. (31,32) showed that anti-human platelet monoclonal antibody against platelet glycoprotein Ib or glycoprotein IIb/IIIa showed decreased binding to platelets from some patients with ITP. We hereby propose that, if in vivo autoantibody to 23-KDa membrane protein is produced in the abnormal state, this antibody produces an aquired storage pool disease by the mechanism of antibody-induced activation, that is, antibody-stimulated polyphosphoinositide breakdown followed by the release of platelet granules. It must be proven (a) whether or not TP82 antigen (23-KDa membrane protein) is one of the membrane proteins which autoantibodies in ITP patients exhibit binding to; (b) whether or not there are any patients with ITP whose serum IgG causes platelet aggregation and release reaction in vitro.

References

1. De Gaetano, G., Vermylen, J., and Verstraete, M. (1970) Thromb. Diath. Haemorrh. 24, 419-431.
2. Tobelem, G., Levy-Toledano, S., Nurden, A.T., Degos, L., and Caen, J.P. (1979) Br. J. Haematol. 41, 427-436.
3. Colman, R.W., and Schreiber, A.D. (1976) J. Biol. Chem. 248, 2928-2936.
4. Colman, R.W., Kuchibhotla, J., and Schreiber, A.D. (1977) Blood 50, 575-585.
5. Favis, G.R., and Colman, R.W. (1980) Immunol. Commun. 9, 13-18.
6. Higashihara, M., Maeda, H., Shibata, Y., Kume, S., and Ohashi, T. (1985) Blood 65, 382-391.
7. Thiagarajan, P., Perussia, B., Marco, L.D., Wells, K., and Trinchierie, G. (1983) Am. J. Hematol. 14, 255-269.
8. Boucheix, C., Soria, C., Mirshahi, M., Soria, J., Perrot, J-Y., Fournier, N., Billard, M., and Rosenfeld, C. (1983) FEBS Lett. 161, 289-295.

9. Jennings, L.K., Phillips, D.R., and Walker, W.S. (1985) Blood 65, 1112-1119.
10. Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580-587.
11. Sano, K., Takai, Y., Yamanashi, J., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010-2013.
12. Haslam, R.J., Lynham, J.A., and Fox, J.E.B. (1979) Biochem. J. 178, 397-406.
13. Ieyasu, H., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1982) Biochem. Biophys. Res. Commun. 108, 1701-1704.
14. Noto, A., Ogawa, Y., Mori, S., Yoshioka, M., Kitakaze, T., Hori, T., Nakamura, M., and Miyake, T. (1983) Clin. Chem. 29, 1713-1716.
15. Higashihara, M., Takahata, K., Ohashi, T., Kariya, T., Kume, S., and Oka, H. (1985) FEBS Lett. 183, 433-438.
16. Tsien, R.Y., Pozzan, T., and Rink, T.J. (1982) J. Cell Biol. 90, 210-213.
17. Gerrard, J.M., and White, J.G. (1976) Pathology Annual, pp. 31-58, Appleton-Century-Crofts, New York.
18. Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701-6704.
19. Kajikawa, N., Kaibuchi, K., Matsubara, T., Kikkawa, U., Takai, Y., and Nishizuka, Y. (1983) Biochem. Biophys. Res. Commun. 116, 743-750.
20. Nishizuka, Y. (1984) Nature 308, 693-698.
21. Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K., and Nishizuka, Y.(1980) Biochem. Biophys. Res. Commun. 97, 309-317.
22. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
23. Adelstein, R.S., Conti, M.A., and Anderson, W. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3115-3119.
24. Dabrowska, R., and Hartshorne, D.J. (1978) Biochem. Biopys. Res. Commun. 85, 1352-1359.
25. Rink, T.J., Sanchez, A., and Hallam, T.J. (1983) Nature 305, 317-319.
26. MacIntyre, D.E., McNicol, A., and Drummond, A.H. (1985) FEBS Lett. 180, 160-164.
27. Zahavi, J., and Marder, V.J. (1974) Am. J. Med. 56, 883-890.
28. Weiss, H.J., and Rogers, J. (1972) Blood 39, 187-196.
29. Holmsen, H., and Weiss, H.J. (1972) Blood 39, 197-209.
30. Weiss, H.J., Rosove, M.H., Lages, B.A., and Kaplan, K.L. (1980) Am. J. Med. 69, 711-717.
31. Woods, V.L., Oh, E.H., Mason, D., and Mcmillan, R. (1984) Blood 63, 368-375.
32. Woods, V.L., Kurata, Y., Montgomery, R.R., Tani, P., Mason, D., Oh, E.H., and McMillan, R. (1984) Blood 64, 156-160.