Influence of Bay k 8644 on Aequorin-Loaded Human Platelets

Nobuyuki OGAWA and Hiroshi ONO*

Department of Pharmacology and Toxicology, Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257, Japan

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Abstract—In aequorin-loaded human platelets, Bay k 8644 (1–100 μ M) had no effect on the resting level of cytoplasmic Ca^{2+} and did not induce aggregation by itself. Though the rise of cytoplasmic Ca^{2+} and platelet aggregation induced by thrombin (0.1 U/ml) also was not inhibited by 1–10 μ M of Bay k 8644, at concentration of 3–10 μ M, it dose-dependently reduced those induced by collagen (5 μ g/ml). The present study shows that human platelets do not have dihydropyridine sensitive Ca channels.

It is well-known that Ca^{2+} plays an important role in platelet aggregation. Previous studies have shown that Ca antagonists such as verapamil, nifedipine and diltiazem inhibited platelet aggregation induced by several agonists (1–3). However, high concentrations of Ca antagonists are required to inhibit platelet aggregation in comparison to the concentrations exhibiting effects on cardiac muscle and vascular smooth muscle. Fritschka et al. (4) recently reported that thrombin-induced ^{45}Ca influx into platelets was reduced by nitrendipine and considered that this effect of nitrendipine was due to the inhibition of voltage-dependent Ca^{2+} influx. However, there has been no identification of Ca channels on the plasma membrane of platelets by the radioligand binding technique (5). If the inhibitory effects of Ca antagonists on platelet aggregation are caused by their Ca channel blocking action, a Ca channel activator, such as Bay k 8644, may induce platelet aggregation. Therefore, we have examined whether Bay k 8644 induces platelet aggregation and increases cytoplasmic Ca^{2+} concentration by using aequorin-loaded human platelets and have studied the effects of Bay k 8644 on the rise of cytoplasmic Ca^{2+} and aggregation of human platelets caused by thrombin or collagen.

Blood was collected from healthy male

volunteers and anticoagulated with 1/9 volume of 3.8% sodium citrate. The blood was centrifuged at $200\times g$ for 10 min at room temperature to separate the plasma containing platelets. Platelets were prepared by centrifuging the plasma containing prostaglandin E_1 (1 μ M) for 15 min at $800\times g$. Aequorin loading was performed according to Johnson et al. (6). After an initial wash with Hepes-Tyrode's buffer containing 10 mM EGTA and 1 μ M prostaglandin E_1 , platelets were incubated at 0°C in a loading solution containing 10 mM EGTA, 5 mM ATP, 1 μ M prostaglandin E_1 and aequorin for one hour. After loading, the platelets were recentrifuged and reincubated for one hour in recovery solution, containing 0.1 mM EGTA, 10 mM Mg^{2+} and 1 μ M prostaglandin E_1 . Platelets were centrifuged at $12000\times g$ for 12 sec and resuspended in a Hepes-Tyrode's buffer containing 1 mM Ca^{2+} and adjusted to 2×10^8 platelets/ml. The aequorin response and aggregation were simultaneously monitored by a Platelet Ionized Calcium Aggregometer (Chrono-Log).

Materials used in this study were obtained as follows: aequorin was purchased from Chrono-Log Co.; thrombin (human thrombin), from the Green Cross Co.; and collagen solution, from Hormon-Chemie München GmbH.

Data are presented as mean $\pm$ S.E. values. Differences of means were analyzed using the paired *t*-test and were considered significant

* To whom all correspondence should be addressed.

when $P < 0.05$.

Bay k 8644 had no effect on the resting level of cytoplasmic Ca^{2+} as monitored by the aequorin signal, nor did it induce aggregation by itself (Fig. 1). On the other hand, thrombin (0.1 U/ml) and collagen (5 $\mu\text{g}/\text{ml}$) obviously induced both responses (Fig. 1).

The effect of Bay k 8644 on cytoplasmic Ca^{2+} concentration and aggregation of platelets in response to thrombin and collagen is shown in Fig. 2. Preincubation with Bay k 8644 for 3 min did not influence the rise of cytoplasmic Ca^{2+} concentration and aggregation of platelets induced by thrombin (0.1 U/ml). However, 3–10 μM of Bay k

8644 dose-dependently reduced the rise of cytoplasmic Ca^{2+} concentration and aggregation induced by collagen (5 $\mu\text{g}/\text{ml}$).

We observed that Bay k 8644 did not influence the resting level of cytoplasmic Ca^{2+} concentration and not induce aggregation in human platelets in vitro. Doyle and Ruegg (7) also reported that Bay k 8644 did not affect the resting level of cytoplasmic Ca^{2+} in human platelets using the fluorescent calcium indicator Quin 2. Therefore, it is suggested that the platelet plasma membrane does not possess dihydropyridine-sensitive Ca channels. It is also reported that Ca channels on the platelet plasma membrane were not

Fig. 1. Typical recordings of aequorin luminescence indicating intracellular Ca^{2+} concentration and optical density indicating aggregation level during stimulation with thrombin, collagen and Bay k 8644.

Fig. 2. Effect of Bay k 8644 on aggregation and the peak intracellular Ca^{2+} concentration as indicated by aequorin in response to thrombin (0.1 U/ml, left side) and collagen (5 $\mu\text{g}/\text{ml}$, right side). Values reported are the mean $\pm$ S.E. of four separate experiments. * $P < 0.05$, ** $P < 0.01$.

identified by the radioligand binding technique using dihydropyridines (5). However, thrombin is known to induce platelet aggregation following Ca^{2+} influx. Recently, Zschauer et al. (8) reported that the human platelet membrane possessed thrombin-activated Ca channels which were not inhibited by a 1,4-dihydropyridine Ca channel blocker, nisoldipine, but were inhibited by inorganic Ca channel blockers such as Ni^{2+} , in an electrophysiological experiment.

Ware et al. (9) observed that aspirin treatment of human platelets reduced collagen-induced aggregation but not the thrombin-induced one. The present study also showed that Bay k 8644 inhibited collagen-induced aggregation but not the thrombin-induced one. Therefore, Bay k 8644 may influence the biosynthesis pathway of thromboxane A_2 induced by collagen. However, further studies are necessary to elucidate the mechanism of action of Bay k 8644 in platelet aggregation. In conclusion, the present data support the result that the human platelet plasma membrane does not possess dihydropyridine-

sensitive Ca channels.

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References

- 1 Ono, H. and Kimura, M.: Effect of Ca^{2+} -antagonistic vasodilators, diltiazem, nifedipine, perhexiline and verapamil, on platelet aggregation in vitro. *Arzneimittelforschung* **31**, 1131–1134 (1981)
- 2 Ware, J.A., Johnson, P.C., Smith, M. and Salzman, E.W.: Inhibition of human platelet aggregation and cytoplasmic calcium response by calcium antagonists: Studies with aequorin and Quin 2. *Circ. Res.* **59**, 39–42 (1986)
- 3 Kiyomoto, A., Sasaki, Y., Odawara, A. and Morita, T.: Inhibition of platelet aggregation by diltiazem. Comparison with verapamil and nifedipine and inhibitory potencies of diltiazem metabolites. *Circ. Res.* **52** Suppl. I, 115–119 (1983)
- 4 Fritschka, E., Kribben, A., Distler, A. and Philipp, T.: Inhibition of aggregation and calcium influx of human platelets by nitrendipine. *J. Cardiovasc. Pharmacol.* **9** Suppl. 4, S85–S89 (1987)
- 5 Glossmann, H., Ferry, D.R., Lübbecke, F., Mewes, R. and Hofmann, F.: Calcium channels: Direct identification with radioligand binding studies. *Trends Pharmacol. Sci.* **3**, 431–437 (1982)
- 6 Johnson, P.C., Ware, J.A., Cliveden, P.B., Smith, M., Dvorak, A.M. and Salzman, E.W.: Measurement of ionized calcium in blood platelets with the photoprotein aequorin: Comparison with Quin 2. *J. Biol. Chem.* **260**, 2069–2076 (1985)
- 7 Doyle, V.M. and Ruegg, U.T.: Lack of evidence for voltage dependent calcium channels on platelets. *Biochem. Biophys. Res. Commun.* **127**, 161–167 (1985)
- 8 Zschauer, A., VanBreemen, C., Buhler, F.R. and Nelson, M.T.: Calcium channels in thrombin-activated human platelet membrane. *Nature* **334**, 703–705 (1988)
- 9 Ware, J.A., Johnson, P.C., Smith, M. and Salzman, E.W.: Effect of common agonists on cytoplasmic ionized calcium concentration in platelets. Measurement with 2-methyl-6-methoxy-8-nitroquinoline (Quin 2) and aequorin. *J. Clin. Invest.* **77**, 878–886 (1986)