PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 393-395 (1983)

CALMODULIN ANTAGONIST W-7 INHIBITS AGGREGATION OF HUMAN PLATELETS INDUCED BY PLATELET ACTIVATING FACTOR

JOSEPH V. LEVY

Pharmacology Laboratories, Kuzell Institute
for Arthritis Research, Medical Research Institute,
Pacific Medical Center, San Francisco, California 94115

Abstract. Experiments were done to test the hypothesis that aggregation of human platelets induced by platelet activating factor (PAF) may be mediated by calmodulin-dependent processes. W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide], a potent calmodulin antagonist, caused dose-dependent inhibition of PAF induced aggregation of human platelets in vitro. The ED_{50} for W-7 was 51.5 ± 9.5 μM (mean ± SEM). This concentration is known to be platelet calmodulin-specific. These data are consistent with the hypothesis.

Introduction - Platelet activating factor (PAF or PAF-acether) is a phospholipid (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine), released from a variety of cells, which is capable of causing aggregation of platelets from certain species (1,2), including humans (2,3). PAF-induced aggregation appears to be independent of products of arachidonic acid. However, calcium plays a key role in mediating PAF effects (2). The exact mechanism of action of PAF remains to be determined.

In this report, experiments are described examining the hypothesis that PAF activation and aggregation of human platelets is calmodulin-dependent. Calmodulin is recognized as playing a vital role in platelet function (4), since it modulates diverse enzymes such as phosphodiesterase, ATPase, protein kinase and phospholipase A_2 (5).

Methods - Platelet rich plasma (PRP) was prepared from citrated whole blood obtained from normal human male volunteers (22-54 years). Blood was collected by venepuncture of an antecubital vein with a 19 gauge butterfly needle (Abbott Laboratories). Blood was allowed to flow into a plastic tube containing 3.8% sodium citrate (9 parts blood; 1 part citrate). Blood was centrifuged at room temperature at 900g for 10 minutes. The PRP obtained was placed into aggregometer tubes (0.45 ml) and allowed to stabilize for 30 minutes prior to study. PRP tubes were capped to minimize pH changes. Aggregation of PRP was measured with a Chronolog Aggregometer, stirring the PRP constantly at 37°C. Whole blood platelet counts were in the normal range (250,000-300,000 mm^3).

Platelet activating factor was obtained from Calbiochem (La Jolla) or from Dr. J. Godfroid (Paris). The material was dissolved in either 60% ethanol or in 80% chloroform and 20% methanol. In the volumes used (1-5 μl/0.45 ml PRP), the solvents did not promote detectable aggregation of the PRP.

The calmodulin antagonist W-7 [N-(6-aminohexyl)-5-chloro-1-Naphtalene sulfonamide] was purchased from Rikaken Co., Nagoya, Japan. This compound is a potent and specific calmodulin antagonist (4). It was dissolved in distilled water and kept at 4°C. Dilutions of the stock solution were made fresh daily. Exposure of the PRP to the solvent in volumes used in W-7 experiments (1-10 μl) neither produced detectable aggregation, nor did it alter the normal aggregation response to PAF. PRP samples were treated with W-7 for 5 minutes at 37°C in the aggregometer prior to addition of PAF. The effect of W-7 on PAF-induced aggregation was expressed as percent inhibition of the response to PAF obtained in the absence of the antagonist.

Results - In concentrations of 6.0 - 7.88 μM, PAF produced submaximal aggregation of normal PRP, compared to the consistent maximum response seen with

0037-9727/83 $1.50

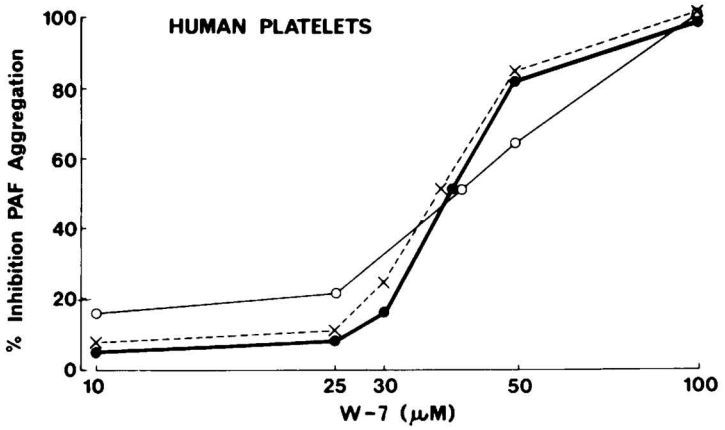

Fig. 1. Typical dose-response curves for inhibition of PAF-induced aggregation of human platelets in vitro produced by the calmodulin antagonist W-7. Each curve represents the response obtained from samples of each of three normal subjects.

3.4 μM ADP. This concentration of PAF is higher than that reported to cause near maximum aggregation of washed rabbit or guinea pig platelets in vitro (1,2) or human PRP tested under different conditions (3).

Pretreatment of the PRP with W-7 for 5 minutes prior to challenge with PAF produced a dose-dependent inhibition of the normal PAF-induced aggregation. Figure 1 shows three typical dose-response curves obtained on PRP samples from three subjects. Under the conditions of these experiments, the ED_{50} for this inhibitory effect of W-7 ranged from 37.5 to 82 μM. The mean (± SEM) value for five normal subjects tested was 51.5 ± 9.5 μM. Complete inhibition was noted in a concentration of 100 μM in the samples tested.

Discussion - The results indicate that PAF-induced aggregation of human PRP is inhibited by concentration of W-7 known to be specific for calmodulin interaction (4).

The ED_{50} for W-7 inhibition of human PRP aggregation induced by PAF is consistent with the notion that PAF action is dependent on calmodulin-mediated processes. It has been shown previously (4) that W-7 is an effective antagonist of human platelet calmodulin dependent processes in the concentration range examined in the present study. The drug also is capable of inhibiting platelet aggregation induced by thrombin, ADP and collagen (4).

W-7 is chemically unrelated to the local anesthetics and phenothiazines, which also can inhibit calmodulin-dependent processes (6,7). There is evidence that W-7 binds to the calcium modulator protein complex with high affinity (K_D= 11 μM) (8). Compared to other calmodulin-interacting drugs, W-7 appears to be the most potent in its effects on platelet aggregation. However, the fact that W-7 also can inhibit aggregation induced by a variety of stimulants (e.g., ADP, thrombin, collagen) (4) besides PAF suggests it is not acting as a specific "receptor" antagonist.

The results obtained in this study are consonant with the hypothesis that PAF aggregation of human platelets, in part, is calmodulin-dependent. This adds further evidence pointing to a critical role of calcium in mediating the effects of PAF on platelet function.

Acknowledgement - This investigation was supported by the Carrie Baum Browning and Hirst Trust Funds of the Kuzell Institute for Arthritis Research, and N.I.H. RR-05566.

References

1. Benveniste J, Henson P, Cochrane C. Leukocyte-dependent histamine release from rabbit platelets: the role of IgE, basophils and a platelet-activating factor. J Exp Med *136*:1356-1377. 1972.

2. Vargaftig B, Chignard M, Benveniste J, Lefort J, Wal F. Background and present status of research on platelet-activating factor (PAF-acether). Ann NY Acad Sci *370*:119-137, 1981.

3. Marcus A, Safier L, Ullman H, Wons K, Broekman M, Weksler B, Kaplan K. Effects of acetyl glyceryl ether phosphorylcholine on human platelet function in vitro. Blood *58*:1027-1031, 1981.

4. Nishikawa M, Hidaka H. Role of calmodulin in platelet aggregation. Structure-activity relationship of calmodulin antagonists. J Clin Invest *69*:1348-1355, 1982.

5. White G, Levine S, Steiner A. Platelet calcium-dependent proteins: identification and localization of the calcium-dependent regulator, calmodulin, in platelets. Am J Hematol *10*:359-367, 1981.

6. Volpi M, Shaafi R, Epstein P, Andrenyak D, Feinstein M. Antagonism of calmodulin by local anesthetics, mepacrine and propranolol. Ann NY Acad Sci *356*:441-442, 1980.

7. Weiss B, Prozialeck W, Cimino M, Barnette M, Wallace T. Pharmacological regulation of calmodulin. Ann NY Acad Sci *356*:319-345, 1980.

8. Hidaka H, Yamaki T, Naka M, Tanaka T, Hayashi H, Kobayashi R. Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. Mol Pharmacol *17*:66-72, 1980.