American Journal of Hematology 33:96–100 (1990)

Deficient Elevation of the Cytoplasmic Calcium Ion Concentration by Epinephrine in Epinephrine-Insensitive Platelets of Patients With Myeloproliferative Disorders

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Elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]i$) by epinephrine and epinephrine-induced inhibition of prostaglandin E_1 (PGE_1)-stimulated cyclic adenosine monophosphate (cAMP) accumulation were assessed in platelets from three groups of subjects: normal controls (NS, n = 11) and patients with myeloproliferative disorders whose platelets were either sensitive (ES, n = 9) or specifically insensitive (EI, n = 7) to the aggregatory effect of epinephrine. The inhibition by epinephrine of cAMP accumulation in the platelets exposed to 500 nM PGE_1 was not significantly different between the three groups. Therefore, despite the defective aggregation response to epinephrine, platelets from the EI group seemed to retain normal response, which was attained through α_2-adrenergic receptors, guanine nucleotide binding regulatory protein, and the adenylate cyclase system. However, in aequorin-loaded, washed platelets, the epinephrine-stimulated rise in $[Ca^{2+}]i$ showed significant decrease in the EI group compared with the other groups ($P < 0.01$). Thus the mechanism for the impaired aggregation response to epinephrine in platelets from the EI group could include the defect that exists in the pathway from receptor binding of epinephrine to the aggregation response through $[Ca^{2+}]i$ elevation.

Key words: platelet aggregation, cytoplasmic Ca^{2+} concentration, cyclic adenosine monophosphate

INTRODUCTION

Epinephrine interacts with platelets and induces platelet aggregation and secretion responses. Studies using the selective radioligand have shown that the epinephrine-induced platelet responses are mediated by α_2-adrenergic receptors on platelet membranes [1–4]. After receptor binding, epinephrine induces several events, including inhibition of adenylate cyclase activity [5–7], stimulation of Ca^{2+} uptake [8], elevation in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]i$) [9], expression of fibrinogen receptors [10], and alkalinization of cytoplasm [11]. However, precise mechanisms leading to platelet aggregation and secretion by epinephrine have not been well recognized.

It is well known that platelets from patients with myeloproliferative disorders (MPD) frequently show abnormal platelet functions, and, among these abnormalities, the defective response to epinephrine is most frequently encountered [12–14]. Decreased epinephrine responsiveness was reported to be associated with deficient [15,16] or normal [17] numbers of platelet-adrenergic receptors. Some investigators have reported normal coupling of α_2-adrenoceptor to adenylate cyclase in these epinephrine-insensitive platelets from MPD patients [18].

In this study, we examined another postreceptor event, $[Ca^{2+}]i$ elevation, using photoprotein aequorin after stimulation of platelets by epinephrine, and the result obtained was compared with epinephrine-induced platelet aggregability in each patient.

Received for publication April 25, 1989; accepted September 26, 1989.

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TABLE I. Patient and Control Groups

Group	n	Patients with[a] PV	ET	CML	Age (years)	Sex (M/F)
Epinephrine-insensitive	7	7			43–74	5/2
Epinephrine-sensitive	9	2	4	3	25–74	2/7
Control	11				25–54	10/1

[a]PV, polycythemia vera; ET, essential thrombocythemia; CML, chronic myeloid leukemia.

MATERIALS AND METHODS

Materials

Epinephrine was obtained from Daiichi Seiyaku Co. (Osaka, Japan). Prostaglandin E_1 (PGE_1) was a generous gift from Ono Pharmaceutical Co. (Osaka, Japan). Human fibrinogen was obtained from Midorijyuji Co. (Osaka, Japan). Sepharose CL-2B was purchased from Pharmacia Fine Chemicals Inc. (Piscataway, NJ). All other materials were obtained from the same sources as described previously [19].

Patients and Controls

Three groups of subjects were recruited for this study: normal controls (NC, n = 11) and MPD patients whose platelets had been shown to be sensitive to epinephrine (ES, n = 9) and those whose platelets were insensitive to epinephrine (EI, n = 7, see below and Table I).

Sample Preparation

Blood was routinely drawn from the antecubital vein using a 19-gauge needle into 10% volume of 3.8% sodium citrate as an anticoagulant, but the volume of citrate added to the patient's blood was adjusted according to each hematocrit value [20]. All tests on platelets were done when the patients and normal donors were free from drugs known to interfere with platelet functions and arachidonate metabolism for at least 2 weeks. All studies were done after informed consent was obtained. Platelet-rich plasma (PRP) and platelet-poor plasma were prepared by centrifugation as previously reported [19]. Aequorin-loaded, washed platelets were prepared and finally suspended in HEPES-Tyrode's buffer as previously reported [19].

Platelet Aggregation Study

Platelet aggregation in PRP at a density of 3×10^5 platelets/μl (PRP aggregation) was studied using a dual-channel aggregometer NKK hematracer 1 model PAT-2A (Niko Bioscience Co., Tokyo, Japan) as previously reported [19]. PRP aggregation was estimated by the use of maximal aggregation, which was defined as the maximal increase in light transmittance at or within 10 min after the addition of epinephrine to PRP. The light transmission of PRP and platelet-poor plasma was taken as 0% and 100%, respectively, and platelets were considered epinephrine insensitive when maximal PRP aggregation was below 3%. When platelets were insensitive to 5 μM epinephrine, PRP aggregation was reassessed by the use of 135 μM epinephrine. Platelet aggregation in washed platelet suspension was monitored using a platelet ionized calcium aggregometer (Chrono-Log Corp., Harverstone, PA), with simultaneous recording of the elevation of platelet $[Ca^{2+}]i$ as described previously [19]. PRP aggregation was repeated two to three times over a period of several months, and the responses to epinephrine were not significantly changed in all patients. PRP aggregation and washed platelet aggregation were studied within 3 and 5 hr, respectively, after venipuncture.

Measurement of Cyclic Adenosine Monophosphate (cAMP) Level

Aliquots (200 μl) of washed plateles suspended in HEPES-buffered saline [19] at a density of 3×10^5 platelets/μl were incubated at 37°C in glass cuvettes with continuous stirring for 30 sec with or without 500 nM PGE_1, followed by incubation for another 1 min in the presence or absence of 5 μM epinephrine. The samples were extracted with 6% trichloroacetic acid two times. The cAMP levels were measured using a [^{125}I]cAMP radioimmunoassay kit (Yamasa Soy Co., Tokyo, Japan).

Studies on $[Ca^{2+}]i$ Using Aequorin-Loaded Platelets

Platelet $[Ca^{2+}]i$ was determined by using photoprotein aequorin as previously reported [19] except that fibrinogen (100 μg/ml) was added together with 1 mM $CaCl_2$ 5 min prior to the $[Ca^{2+}]i$ measurement. The elevation of platelet $[Ca^{2+}]i$ induced by 5 μM epinephrine was measured with simultaneous recording of platelet aggregation as mentioned above. The value of $[Ca^{2+}]i$ was calculated according to Johnson et al. [21].

Statistical Analysis

Values for three groups in the text and in Table I are expressed as means ± SEM. The differences of means were assessed by Tukey's method [22].

RESULTS

Platelet Aggregation Study

Platelets from the EI group showed the defective aggregation response to 5 μM and 135 μM epinephrine in plasma, whereas maximal aggregation of aequorin-loaded, washed platelets by the same agonist was 3.0 ±

TABLE II. Effects of PGE_1 and Epinephrine on Platelet cAMP Levels

Group	Basal levels[a]	PGE_1-stimulated[b]	Inhibition by epinephrine[c]
Epinephrine-insensitive	11.1 ± 1.1[d]	355 ± 57	60.9 ± 5.4
Epinephrine-sensitive	14.5 ± 1.9	449 ± 58	53.2 ± 5.1
Control	12.2 ± 1.8	336 ± 49	71.3 ± 4.6

[a]pmoles/10^9 platelets.
[b]percent of basal levels.
[c]percent inhibition of PGE_1-elevated cAMP.
[d]Mean ± SEM (n = 7).

0.6% (M ± SEM, n = 4). On the other hand, platelets from the ES group and the NC group showed normal responses at the same condition similar to those of the EI group (data not shown).

Platelet cAMP Levels

Basal cAMP levels of platelets that had been incubated in the absence of stimuli were similar in NC, ES, and EI groups (Table II). Neither 500 nM PGE_1-induced elevation of platelet cAMP levels nor the inhibition of this elevation by 5 μM epinephrine was significantly different between these three groups (Table II).

Effect of Epinephrine on Platelet $[Ca^{2+}]i$

Platelets from the EI group showed decreased elevation of $[Ca^{2+}]i$ when stimulated with 5 μM epinephrine (Fig. 1). On the other hand, platelets from the ES group showed normal responses when the normal range of epinephrine-induced $[Ca^{2+}]i$ elevation was defined as mean ± 2 SD of the values obtained from normal subjects (Fig. 1), and the differences of $[Ca^{2+}]i$ levels between either of these groups and the EI group were significant ($P < 0.01$).

DISCUSSION

The defective aggregation response to epinephrine is well documented in some MPD patients, but the precise mechanism of this defect has remained unclear [12–14]. Some investigators examined directly the state of platelet α_2-adrenergic receptors using a variety of specific radioligands, including ^{3}H-dihydroergocryptine and ^{3}H-yohimbine. Kaywin et al. [15] and Pfeifer et al. [16] reported diminished platelet α_2-adrenergic receptors in epinephrine-insensitive MPD patients. Rao et al. [23] reported a familial platelet abnormality that showed selective defect of aggregation and secretion to epinephrine. The platelets from this family showed diminished α_2-adrenergic receptors. On the other hand, other investigators reported normal numbers of α_2-adrenergic receptors in a large number of such MPD patients [24]. Regarding the coupling of α_2-adrenergic receptors to adenylate cyclase, many investigators reported that there was no abnormality in epinephrine-insensitive platelets from MPD patients [18] and other subjects [23,25,26]. Our data on the epinephrine-induced inhibition of PGE_1-induced cAMP elevation in the platelets from both epinephrine-sensitive and -insensitive MPD patients as well as from normal subjects were in support of these reports.

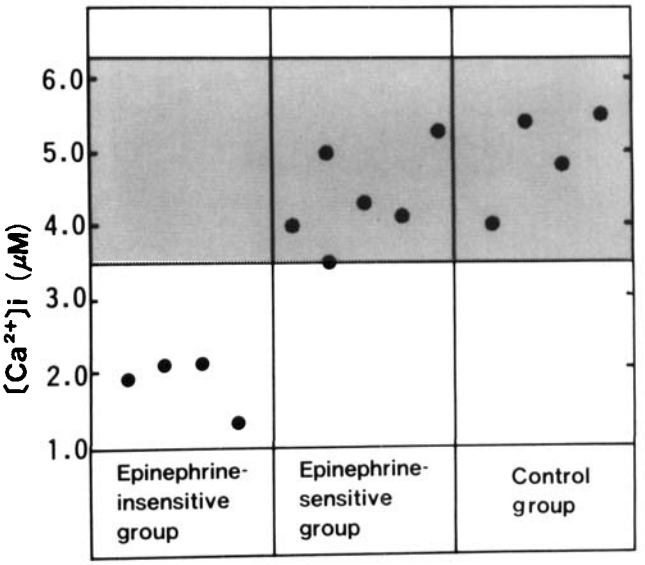

Fig. 1. Epinephrine-induced elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]i$) in epinephrine-insensitive and -sensitive platelets. The shaded area illustrates the normal range as defined by mean ± 2 SD of the values from normal subjects.

It is known that there is a receptor reserve in platelets for the inhibition of adenylate cyclase and that the occupancy of only about 10% of the α_2-adrenergic receptor by norepinephrine elicits half-maximal inhibition of this enzyme [27]. This coincides with the fact that in intact platelets yohimbine inhibits the action of epinephrine on adenylate cyclase at ten times lower concentration than that required for an equal degree of saturation of α_2-adrenergic receptors [28] and at nine times higher concentration than that required for the inhibition of epinephrine-induced aggregation [23]. It is clear from these facts that the receptor requirements for epinephrine-induced aggregation and for adenylate cyclase inhibition are different. However, it has not yet been made clear whether this difference depends on the difference of the number of receptors required for two events or whether this difference implies the existence of different types of receptors. On the other hand, it is clear that the inhibition of cAMP levels alone is not sufficient to elicit platelet aggregation by epinephrine and that other mechanisms exist for aggregation. Recently it has been reported that epinephrine stimulates Na^+/H^+ exchange and local

Ca^{2+} elevation in platelets and that these changes stimulate phospholipase A_2 [29,30]. Ware et al. [9] demonstrated an elevation of $[Ca^{2+}]i$ following exposure of platelets to epinephrine with the use of aequorin as a $[Ca^{2+}]i$ indicator. From these facts it seems possible that epinephrine-induced elevation of $[Ca^{2+}]i$ may be a necessary step included in the pathway through which epinephrine induces platelet aggregation. We demonstrated the deficient elevation of $[Ca^{2+}]i$ following exposure to epinephrine in platelets from patients with polycythemia vera who showed the selectively defective response to epinephrine. In the present study, we investigated platelets from patients with MPD including polycythemia vera, essential thrombocythemia, and chronic myeloid leukemia, but all the patients with epinephrine-insensitive platelets had polycythemia vera. Therefore, it remains to be elucidated whether the calcium defect we found here is a feature of polycythemia vera or whether it is a feature of epinephrine insensitivity.

Although a defect in the signal transduction pathway for calcium should result in a hemorrhagic tendency, patients with this defect did not always show this symptom. The reasons for the lack of this association are not clear. However, in that the platelets from the patients of the EI group showed the defective response only to epinephrine, other physiological agonists including thrombin, adenosine diphosphate, collagen, and thromboxane A_2 could induce normal platelet functions in the patients. This could compensate for the defective responses to epinephrine. Platelets from some MPD patients may have heterogeneous abnormalities finally leading to common expression, namely, defective aggregation to epinephrine. In one case this abnormality may be a decreased number of receptors, and in another case it may be abnormal receptor functions or abnormal postreceptor mechanisms. Further studies will be necessary to understand fully the mechanism of epinephrine-induced platelet aggregation, and for this purpose these platelets showing the impaired aggregation response to epinephrine will be useful and important materials.

ACKNOWLEDGMENTS

This research was supported in part by Grant-in-Aid for Scientific Research (#3657057) and by Grant-in-Aid for Scientific Research on Priority Areas (#63637007) from the Ministry of Education, Science and Culture of Japan.

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