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Different effects of two thromboxane A_2/prostaglandin H_2 receptor ligands, U46619 and S-145, on rabbit platelets

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Stimulation of rabbit platelets with U46619 induced platelet shape change, aggregation and secretion of ATP. However, S-145, which specifically binds to the thromboxane A_2/prostaglandin H_2 receptor like U46619, induced only shape change. Both compounds rapidly elevated cytoplasmic Ca^{2+} concentration although only U46619 evoked the formation of inositol phosphates. Chelating external Ca^{2+} with EGTA did not affect the S-145-induced platelet shape change while intracellular Ca^{2+} movement was severely reduced. These results suggest an essential role of phospholipase C in the induction of platelet aggregation and secretion and that some factor other than Ca^{2+} and phospholipase C participates in platelet shape change.

Thromboxane A_2; Ca^{2+}; Shape change; Phospholipase C; U46619; Inositol phosphate

1. INTRODUCTION

Platelets are activated by stimulation with many physiological agonists [1]. Platelet activation is thought to be promoted by the synergistic action of Ca^{2+} and protein kinase C [2–4]. A rise in cytosolic Ca^{2+} concentration is induced by inositol 1,4,5-trisphosphate (IP_3), a product of the breakdown of phosphatidylinositol 4,5-bisphosphate by phospholipase C. Diacylglycerol, another product of phospholipase C action, activates protein kinase C.

Three separate physiological responses, shape change, aggregation, and secretion, are induced as a result of platelet activation. However, the biochemical reaction essential to each physiological response has not been precisely investigated, since it is difficult to clearly separate the physiological responses, and the biological responses as well. Many agonists usually induce all the responses almost simultaneously.

U46619, an agonist of the thromboxane A_2 (TXA_2)/prostaglandin H_2 (PGH_2) receptor [5], also induces the three physiological responses. However, S-145 ((±)-5(*Z*)-7-(3-*endo*-phenylsulfonylamino[2.2.1]bicyclohept-2-*exo*-yl)heptenoic acid), a newly synthesized TXA_2/PGH_2 receptor antagonist with an affinity higher than U46619, which antagonizes the action of U46619 on vascular smooth muscle and platelets [6,7], induces only platelet shape change. Therefore, we thought that S-145 would be an ideal tool to use in studying the signal transduction of platelet shape change. In this study we investigated the responses of rabbit platelets stimulated with U46619 or S-145 and found that activation of phospholipase C was accompanied by induction of aggregation and secretion, but not by platelet shape change, which was induced without a significant increase of cytosolic Ca^{2+}.

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Abbreviations: TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2; IP_3, inositol 1,4,5-trisphosphate

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2. MATERIALS AND METHODS

U46619 was purchased from Upjohn Co., Kalamazoo. S-145 was synthesized at Shionogi Research Laboratories, Co., Ltd. *myo*-[^{3}H]Inositol was purchased from Amersham Japan, Tokyo. Fura2-(acetoxymethyl)ester was obtained from Dojin, Kumamoto, Japan.

Rabbit platelet-rich plasma was prepared from freshly drawn blood and mixed with PGE_1 (0.5 μg/ml). Platelets were sedimented by centrifugation at 1200 × *g* for 15 min and resuspended at 2 × 10^9 cells/ml in a resuspension buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 3.8 mM NaH_2PO_4, 3.8 mM Hepes, 5.6 mM glucose and 0.035% bovine serum albumin, pH 7.35). If necessary, platelets were incubated with 1 μM fura2-AM or 50 μCi/ml *myo*-[^{3}H]inositol for 1 or 2 h at room temperature in the presence of 0.5 μg/ml PGE_1. Platelets were sedimented onto 40% bovine serum albumin, isolated with a column of Sepharose 2B, and resuspended in the resuspension buffer at 5 × 10^8 cells/ml. $CaCl_2$ (1 mM) or EGTA (2.5 mM) was added to the platelets 2 min before stimulation. Secretion of ATP was monitored by a PICA aggregometer (Chrono-log) using a luciferase-luciferin reagent, Chronolume (Chrono-log). The fluorescence signal of fura2 was monitored while stirring at 37°C with a CAF-100 Ca^{2+} analyzer (Japan Spectroscopic Co., Ltd, Tokyo). The Ca^{2+} concentration was calculated as described elsewhere [8]. To the [^{3}H]inositol-labeled platelets, 15 mM LiCl was added 30 min before stimulation. [^{3}H]Inositol phosphates were extracted and separated as described by Berridge et al. [9].

3. RESULTS AND DISCUSSION

As shown in fig.1, U46619 dose-dependently induced shape change, aggregation and secretion of ATP. However, S-145 induced only shape change even at the highest concentration (1 μM). This difference in response should not have been caused by the difference between binding affinities of the compounds to TXA_2/PGH_2 receptor, since a binding study with radiolabeled U46619 has revealed that the affinity of S-145 to the receptor of rabbit platelet is higher than that of U46619 [6].

Fig.2 shows intracellular Ca^{2+} mobilization during stimulation with U46619 and S-145. Both U46619 and S-145 rapidly elevated cytoplasmic Ca^{2+} concentration, which reached a maximum within 10 s. S-145-induced Ca^{2+} elevation was rapidly decreased to the resting level while Ca^{2+} concentration maintained a somewhat higher value than the resting level when platelets were stimulated with U46619. In order to determine whether Ca^{2+} elevation is essential for inducing shape change, the response of platelets to S-145 was examined in the absence of extracellular Ca^{2+}. As shown in table 1, depletion of Ca^{2+} did not affect

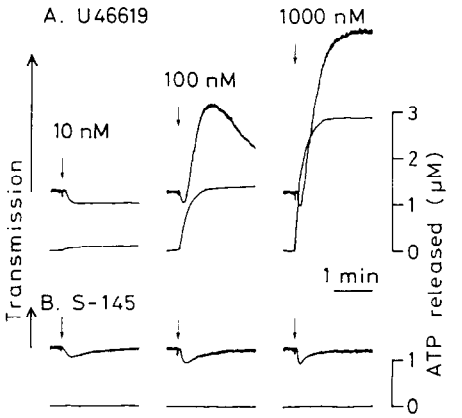

Fig.1. Shape change, aggregation and secretion of ATP. U46619 (A) or S-145 (B) was added at the arrow. Increase and decrease of light transmission represent shape change and aggregation, respectively. ATP release was monitored with PICA aggregometer (Chrono-log).

S-145-induced shape change. However, in the absence of external Ca^{2+}, S-145 induced only a slight increase of cytoplasmic Ca^{2+} which seemed to be too small to induce Ca^{2+}-dependent responses. This result suggests that a rise in Ca^{2+} is not necessary for shape change.

As phospholipase C is thought to have a central role in platelet activation, we studied the phospholipase C action during stimulation with U46619 and S-145 by measuring the increase of [^{3}H]inositol phosphates in [^{3}H]inositol-labeled platelets. Fig.3 shows inositol phosphate formation during stimulation with 1 μM U46619 and 1 μM S-145. A significant increase of inositol phosphates was observed when platelets were stimulated with U46619. However, S-145 did not change the level of inositol phosphates. This finding indicates that phospholipase C acted during stimulation with U46619 but not during stimulation with S-145.

Platelet shape change is associated with many cellular processes including repositioning of the equatorial band of microtubules, polymerization of actin, phosphorylation of myosin light chains, formation of actomyosin, centralization of secretory granules, and formation of filopodia and pseudopodia [10–13]. Movement of the cytoskeleton is considered to be involved in these responses. However, the factor triggering the response of the cytoskeleton has not been as-

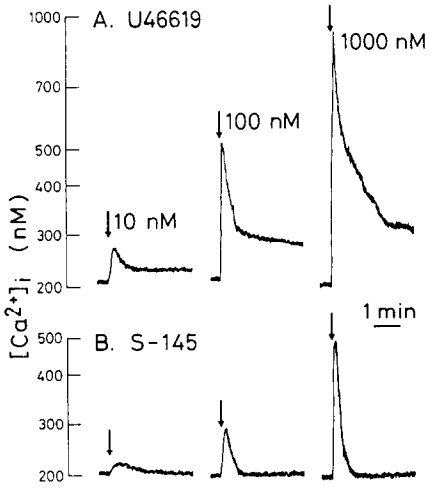

Fig.2. Change in cytosolic Ca^{2+} concentration. Fura2-loaded platelets were stimulated with U46619 (A) or S-145 (B). The fluorescence ratio, obtained by dividing the fluorescence at 340 nm by that at 380 nm, was determined with CAF-100 Ca^{2+} analyzer. The emission wavelength was 500 nm.

certained. Ca^{2+} is one of the candidates for the trigger. However, our results indicate that shape change can be induced without Ca^{2+} elevation. Simpson et al. [14] also reported that a low dose of U44069 induces shape change of human platelets without Ca^{2+} elevation. It has been suggested that either diacylglycerol or phosphatidylinositol 4,5-bisphosphate may play a part in forming a nucleation site for the polymerization of actin [15,16]. However, stimulation of inositol lipid turnover is not induced by S-145. Although the putative alternative signal transduction mechanism remains unclear, the interaction between the receptor and the cytoskeleton would be a very interesting subject for further study.

Table 1

Effect of extracellular Ca^{2+} on S-145-induced Ca^{2+} mobilization and shape change

	$\Delta[Ca^{2+}]_i$ (nM)	Shape change (%)
A. 1 mM Ca^{2+}	227 ± 33	100 ± 9
B. No Ca^{2+}	17 ± 3	99 ± 10

Platelets were stimulated with 1 μM S-145 in the presence of 1 mM Ca^{2+} (A) or in the presence of 2.5 mM EGTA with no added Ca^{2+} (B). $\Delta[Ca^{2+}]_i$ is the measured increment over the resting level. Shape change is measured as the % of the maximal increment in transmission. The data are mean ± SD for five experiments

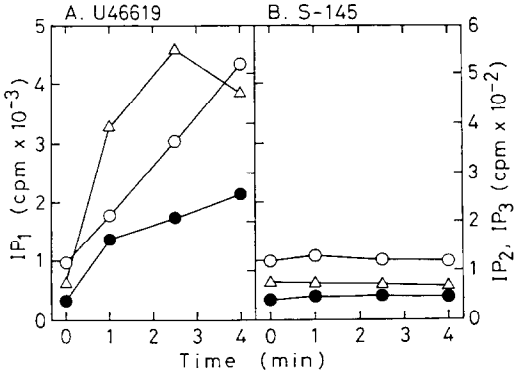

Fig.3. Formation of inositol phosphates. [^{3}H]Inositol-labeled platelets were stimulated with 1 μM U46619 (A) or 1 μM S-145 (B). Radioactivity of inositol 1-monophosphate (IP_1, ○), inositol 1,4-bisphosphate (IP_2, △) and inositol 1,4,5-trisphosphate (IP_3, ●) is represented.

IP_3 is thought to release Ca^{2+} from the intracellular Ca^{2+} store [3]. In this study, however, S-145 induced Ca^{2+} mobilization without increase of IP_3. This finding suggests the presence of some factor other than IP_3 which induces Ca^{2+} mobilization. The result that depletion of external Ca^{2+} severely reduced Ca^{2+} mobilization suggests that influx of external Ca^{2+} may be involved in S-145-induced Ca^{2+} mobilization. However, the release of Ca^{2+} from the intracellular store must occur since S-145 causes a slight increase in Ca^{2+} in the absence of external Ca^{2+}. It has been reported that stimulation of rat platelets with U46619 and stimulation of human platelets with ADP also raises Ca^{2+} without IP_3 formation [17,18], but no precise mechanism has been offered.

We have reported earlier that only shape change is induced when rat platelets are stimulated with U46619 while aggregation and secretion are induced by the synergistic action of U46619 and collagen, and that induction of aggregation and secretion is accompanied by activation of phospholipase C [17,19,20]. The results of the present study also suggest an important role of phospholipase C in the induction of aggregation and secretion. Clearly, Ca^{2+} mobilization is not a sufficient signal for aggregation and secretion. During stimulation with U46619, IP_3 formed by the action of phospholipase C may reinforce Ca^{2+}

movement. U46619 induced a higher maximum Ca^{2+} and a more sustained level of Ca^{2+} than S-145, which may have led to aggregation and secretion. However, the maximum Ca^{2+} concentration is unlikely to be related to the occurrence of aggregation and secretion, since 1 μM S-145, which induced nearly the same level of peak Ca^{2+} as 100 nM U46619, induced only shape change, whereas 100 nM U46619 also induced aggregation and secretion. In U46619 stimulation of platelets, diacylglycerol, an activator of protein kinase C, must be generated via phospholipase C. Some reports have indicated that protein kinase C suppresses platelet function [21–23]. However, protein kinase C probably also has a role in platelet activation [2]. Therefore, in addition to the reinforced Ca^{2+} mobilization, activation of protein kinase C may be essential to U46619-induced aggregation and secretion.

Why the response induced by S-145 differs from that induced by U46619 is not yet clear. S-145 induces Ca^{2+} mobilization, and U46619 induces the phospholipase C response in addition to Ca^{2+} mobilization. More precise analyses of the interaction between the receptor and these compounds may provide the answers.

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