Possible Involvement of Cytoskeleton in Collagen-stimulated Activation of Phospholipases in Human Platelets*

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The action of phospholipases A2 and C in the course of collagen-stimulated platelet activation and the effect of cytochalasins on the responses were studied. Stimulation of human platelets with collagen was accompanied by aggregation, Ca2+ mobilization, inositol phosphate formation, and arachidonic acid release. However, in the presence of a cyclooxygenase inhibitor or a thromboxane A2 (TXA2) receptor antagonist, collagen induced only weak arachidonic acid release and weak inositol phosphate formation. The TXA2 mimetic agonist U46619 induced all the responses except for arachidonic acid release, which was induced by synergistic action of collagen and U46619. The result that U46619 did not induce arachidonic acid release despite the activation of phospholipase C suggested that arachidonic acid was not released via phospholipase C but by phospholipase A2. These findings suggested that collagen initially induced weak activation of phospholipases A2 and C and that further activation of phospholipase C as well as Ca2+ mobilization and aggregation were induced by TXA2, whereas further activation of phospholipase A2 required the synergistic action of collagen and TXA2. Platelets pretreated with cytochalasins did not respond to collagen. Further analysis revealed that the initial activation of phospholipases A2 and C was specifically inhibited by cytochalasins, but the responses induced by U46619 or a synergistic action of collagen and U46619 were not inhibited. Therefore, we proposed that interaction of collagen receptor with actin filaments might have some roles in the collagen-induced initial activation of phospholipases.

Phospholipases A2 and C play important roles in platelet activation. Phospholipase A2 acts on phospholipids and liberates arachidonic acid (1–4). The cyclooxygenase pathway converts arachidonic acid into thromboxane A2 (TXA2) which is a potent inducer of platelet aggregation as well as vascular contraction (5–9). On the other hand, phospholipase C acts on inositol phospholipids (10). Degradation of phosphatidylinositol 4,5-bisphosphate by phospholipase C results in the formation of two secondary messengers, diacylglycerol and inositol 1,4,5-trisphosphate (11, 12). Moreover, it is postulated that diacylglycerol is further hydrolyzed by diacylglycerol and monoacylglycerol lipases to release arachidonic acid (13–15). However, it is not precisely understood how phospholipases A2 and C are regulated in the course of platelet activation.

Collagen-induced platelet aggregation is essentially dependent on endogenously generated TXA2, since it is inhibited by cyclooxygenase inhibitors and TXA2/prostaglandin H2 (PGH2) receptor antagonists (16–19). Using rat platelets, we have clarified that phospholipase A2 action is an initial, critical event in collagen-induced platelet activation and that synergistic action of collagen and TXA2 is necessary to induce phospholipase C action and further activation of phospholipase A2 (20–23). Therefore, collagen-stimulated platelets might be suitable for studying the regulation of phospholipases A2 and C. However, detailed analysis of responses of collagen-stimulated human platelets had not been done. In this study, we separated the collagen-induced responses of human platelets into two phases, TXA2-independent and TXA2-dependent responses by means of a cyclooxygenase inhibitor and a TXA2/PGH2 receptor antagonist and investigated the actions of phospholipases A2 and C in the respective phases.

To obtain clues to the regulation of these enzymes, we studied the effects of cytochalasins on collagen-induced responses. Cytochalasins prevent assembly of actin and actin-binding protein bundles in platelets (24–26). Actin filaments construct the platelet cytoskeleton together with other components such as actin-binding protein and microtubules (27). Recently, some glycoproteins (GPs) on platelet plasma membranes have been found to be linked to actin filaments (28, 29). A candidate for a receptor of collagen is involved in the GPs (30, 31). However, the role of the linkage of the GPs with actin filaments is not yet known. The present study is the first to demonstrate that the actin filaments may be involved in receptor-mediated signal transduction in platelets.

EXPERIMENTAL PROCEDURES

Materials—SQ29,548 was synthesized at Shionogi Research Laboratories. Collagen (type IV, soluble), thrombin, indomethacin, cytochalasins B, D, and E, and prostaglandin E1 (PGE1) were obtained from Sigma. [5,6,8,9,11,12,14,15-3H]arachidonic acid (180–240 Ci/ mmol) and TXB1.3H radioimmunoassay kits were from Du Pont-New England Nuclear, myc-2-[3H]inositol (10–20 Ci/mmole) was from Amersham Corp., U46619 was from Upjohn, and FurA2-AM was purchased from Dojin, Japan. Structures of SQ29,548 and U46619 are shown in Fig. 1.

Preparation of Human Platelets—Platelets were isolated from the blood of healthy human donors who had not taken medication for at least the previous 2 weeks. The blood was anticoagulated with 0.15 volumes of acid citrate dextrose (85 mM trisodium citrate, 70 mM citric acid, and 110 mM dextrose) and 0.5 µg/ml PGE1, and centrifuged at 160 × g for 10 min. The platelet-rich plasma was then centrifuged for 15 min at 1,200 × g, and the platelets were resuspended in the appropriate volume of resuspension buffer as described below. The resuspension buffer, adjusted to pH 7.35, contained 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl2, 3.8 mM Na2HPO4, 3.8 mM Hepes, 5.6
mm dextrose, 0.0385% bovine serum albumin. After labeling, platelets were sedimented onto 40% bovine serum albumin, isolated by gel filtration with a column of Sepharose 2B, and suspended in the resuspension buffer at 2.5 x 10^9 cells/ml. CaCl_2 (1 mM) was added to the platelets 2 min before stimulation.

**Measurement of Intracellular Ca^{2+} Concentration**—Platelets were suspended in the resuspension buffer containing 0.5 µg/ml PGE_2 at 1 x 10^9 cells/ml and incubated with 1 µM Fura2-AM for 30 min at room temperature. Platelets were isolated and resuspended as described above. The fluorescence ratio, obtained by dividing the fluorescence at 340 nm by that at 380 nm, was determined using a CAF-100 Ca^{2+} analyzer (Japan Spectroscopic Co., Ltd., Japan) while stirring at 37°C. The emission wavelength was 500 nm. Changes in the fluorescence ratio were calibrated to changes in intracellular Ca^{2+} levels using the method of Grynkiewicz et al. (32).

**Measurement of Platelet Aggregation**—Platelets were suspended in an NKK HEMA Tracer 1 (Nikon Bioscience Co., Ltd., Japan) and aggregation of platelets was followed continuously in it.

**Measurement of Inositol Phospholipid Hydrolysis**—Platelets were suspended in the resuspension buffer containing 0.5 µg/ml PGE_2 at 2 x 10^9 cells/ml and incubated with 100 µCi/ml [3H]inositol for 2 h at room temperature. Platelets were isolated and resuspended as described above. LiCl (15 mM) was added 30 min before stimulation. [3H]inositol-labeled platelets were stimulated as indicated in the figures, and the reactions were stopped by addition of an equal volume of an 15% trichloroacetic acid. The acid-soluble inositol phosphates were separated by anion exchange chromatography as described by Berridge et al. (33).

**Measurement of Arachidonic Acid Release**—Platelets were suspended in the resuspension buffer containing 0.5 µg/ml PGE_2 at 2 x 10^9 cells/ml and incubated with 20 µCi/ml [14C]arachidonic acid for 2 h at room temperature. Platelets were isolated and resuspended as described above and stimulated as indicated in the figure. The reactions were stopped by addition of volumes of chloroform/methanol (1:2, v/v), and lipids were extracted by the method of Bligh and Dyer (34). [14C]Labeled eicosanoids were separated by thin layer chromatography according to the method of Bertele et al. (55). The areas corresponding to arachidonic acid, TXB_2, hydroxyeicosatetraenoic acid, and hydroxyeicosanoids were scraped off and the radioactivity measured. Arachidonic acid metabolites other than these eicosanoids were scarcely detected.

**Measurement of TXA_2 Formation**—Platelets were stimulated as indicated in the figure. The reactions were stopped by addition of an equal volume of ice-cold 10 mM EGTA and 50 µM indomethacin. Platelets were precipitated and TXB_2, a stable metabolite of TXA_2, in the supernatant was measured by TXB_2 radioimmunoassay kits.

**RESULTS**

**TXA_2 Dependence of Collagen-induced Aggregation and Ca^{2+} Mobilization**—As shown in Fig. 2A, collagen (10 µg/ml) induced aggregation of human platelets after a lag period of about 1 min. The threshold concentration of collagen for the induction of full aggregation was 5–10 µg/ml. Indomethacin (10 µM), an inhibitor of cyclooxygenase, inhibited the collagen-induced aggregation. Furthermore, 1 µM SQ29,548, which is a specific antagonist of TXA_2/GPIIb receptor (36) also completely blocked the platelet response to collagen.

Using Fura2-loaded platelets, the collagen-induced elevation of cytoplasmic Ca^{2+} concentration was observed (Fig. 2B). Ca^{2+} mobilization was not detected during the lag period. Along with the shape change and aggregation, Ca^{2+} concentration increased and then decreased. The elevation of Ca^{2+} was also completely inhibited by indomethacin and SQ29,548.

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formation. A cyclooxygenase inhibitor, indomethacin (10 μM), completely blocked it. SQ29,548 (1 μM) also inhibited the TXA₂ formation, although unlike indomethacin, it could not interfere with the pathway of TXA₂ synthesis (36). Similar results were obtained on arachidonic acid release as shown in Fig. 4B. Collagen-induced arachidonic acid release represented by [³H]eicosanoid formation was greatly reduced by inhibiting TXA₂ formation with indomethacin and also by a blockade of TXA₂ binding to the receptor with SQ29,548, although these compounds did not directly affect the arachidonic acid release (21, 36). These compounds inhibited the collagen-induced responses by about 80%, indicating that about 20% of arachidonic acid release and TXA₂ formation was induced by the action of collagen alone and that the action of TXA₂ is necessary to induce further arachidonic acid release and TXA₂ formation.

Responses of Human Platelets Stimulated with U46619—In order to find whether TXA₂ would induce the responses inhibited by indomethacin and SQ29,548, responses of platelets stimulated with U46619, an agonist of TXA₂/PGH₂ receptor (37), were studied. As shown in Fig. 5A, U46619 dose-dependently induced aggregation of human platelets. Increase of intracellular Ca²⁺ concentration was also observed upon addition of U46619 (Fig. 5B). It reached maximum within 10 s and rapidly decreased. As shown in Fig. 6, U46619 induced inositol phosphate formation. U46619 at 1000 nM induced almost the same level of inositol monophosphate formation as collagen and induced more than 60% increase of inositol bisphosphate and inositol trisphosphate. These results indicated the aggregation, Ca²⁺ mobilization, and activation of phospholipase C could be induced by the action of TXA₂. However, as shown in Fig. 7, U46619 did not induce arachidonic acid release. TXA₂ formation, as measured by radioimmunoassay, was not observed by the stimulation with U46619 (data not shown).

Synergistic Action of Collagen and TXA₂ on Platelet Responses—Since a synergistic action of collagen and TXA₂ had been revealed to be important for full activation of rat platelets, mobilization of phospholipases in platelets was investigated. The platelets were stimulated for 3 min with 10 μg/ml collagen (0), various concentrations of U46619 (O), or U46619 plus 10 μg/ml collagen (●). [³H]inositol phosphate formation is represented as described in Fig. 3. The resting levels of radioactivity in inositol monophosphate, inositol bisphosphate, and inositol trisphosphate in the absence of indomethacin were 360 ± 41 dpm, 81 ± 36 dpm, and 45 ± 12 dpm, respectively.
lets (20-22), we supposed that similar synergistic action might be necessary for inducing full arachidonic acid release in human platelets. The synergistic action was studied by stimulating platelets with collagen plus U46619 in the presence of indomethacin (Fig. 7). Indomethacin was used to prevent endogenous TXA2 generation. In the presence of indomethacin, collagen induced only a small amount of arachidonic acid release. However, further addition of U46619 induced arachidonic acid release in a dose-dependent manner, indicating that collagen and U46619 synergistically induced arachidonic acid release.

In addition to arachidonic acid release, the formation of inositol phosphates was also enhanced by the synergistic action (Fig. 6), although U46619 alone could induce the response. The effect of the synergistic action was remarkable on the formation of inositol bisphosphate and inositol triphosphate but not on the formation of inositol monophosphate. Kaibuchi et al. (38) also reported the synergistic action of collagen and a TXA2 analogue on phosphatidic acid formation.

Furthermore, the synergistic action was also observed in Ca2+ mobilization (Fig. 8). Stimulation of platelets with collagen plus U46619 resulted in the appearance of a second Ca2+ peak after the rapid, first Ca2+ peak which could be induced by U46619 alone. The appearance of the second Ca2+ peak has also been observed in rat platelets by the synergistic action of collagen and U46619 (20, 21).

Effect of Cytochalasin B on Collagen-induced Responses of Human Platelets—As shown in Fig. 9, we found that 10 μM cytochalasin B completely inhibited collagen-induced platelet aggregation and Ca2+ mobilization. Furthermore, cytochalasin B completely inhibited collagen-induced arachidonic acid release (Fig. 10A, I) and inositol phosphate formation (Fig. 11).}

![Fig. 8. Ca2+ mobilization induced by collagen plus U46619.](image)

Indomethacin (10 μM) was added to Fura2-loaded platelets 3 min before stimulation. The platelets were stimulated with 100 or 1000 nM U46619 plus 10 μg/ml collagen at the time indicated by the arrow. Ca2+ mobilization was monitored as described in Fig. 2.

![Fig. 9. Effect of cytochalasin B on collagen-induced platelet aggregation (A) and Ca2+ mobilization (B).](image)

Vehicle (a) or 10 μM cytochalasin B (b) was added to platelets 4 min before stimulation. Washed human platelets (A) or Fura2-loaded platelets (B) were stimulated with collagen at the time indicated by the arrow. Aggregation and Ca2+ mobilization were monitored as described in Fig. 2.

![Fig. 10. Effect of cytochalasin B on arachidonic acid release.](image)

Vehicle (a) or 10 μM cytochalasin B (b) was added to [3H] arachidonic acid-labeled platelets 4 min before stimulation. Vehicle (A-I and B) or 10 μM indomethacin (A-II, III, and IV) was added to the platelets 3 min before stimulation. The platelets were stimulated for 3 min with 10 μg/ml collagen (A-I and II), 1 μM U46619 (A-III), 10 μg/ml collagen plus 1 μM U46619 (A-IV) or 0.5 units/ml thrombin (B). [3H]Eicosanoid formation is represented as described in Fig. 4. The resting level of radioactivity in eicosanoid in the absence of indomethacin and cytochalasin B was 6940 ± 90 dpm, which was not significantly affected by cytochalasin B and/or indomethacin.

![Fig. 11. Effect of cytochalasin B on inositol phosphate formation.](image)

Vehicle (a) or 10 μM cytochalasin B (b) was added to [3H] inositol-labeled platelets 4 min before stimulation. Vehicle (I) or 10 μM indomethacin, (II, III, and IV) was added to the platelets 3 min before stimulation. The platelets were stimulated for 3 min with 10 μg/ml collagen (I and II), 1 μM U46619 (III), or 10 μg/ml collagen plus 1 μM U46619 (IV). [3H]IPs represents the increase of the total of inositol monophosphate, inositol bisphosphate, and inositol trisphosphate over the resting level, 503 ± 19 dpm. Cytochalasin B and/or indomethacin did not significantly change the resting level. Data are mean ± S.E. (n = 3).

D. Cytochalasins D and E also gave the same results as cytochalasin B in the experiments represented in Figs. 9–12 (data not shown).

Effect of Cytochalasin B on the Responses of Human Platelets Stimulated by U46619 and Thrombin—In order to find whether the inhibitory effect of cytochalasins would be common among different stimuli, the effect of cytochalasin B on thrombin- and U46619-induced responses of platelets was examined. Fig. 10B shows the effect of cytochalasin B on thrombin-induced arachidonic acid release. Cytochalasin B did not inhibit the response. As shown in Fig. 11, III, U46619-induced inositol phosphate formation was not inhibited. Moreover, U46619-induced Ca2+ mobilization was not affected by cytochalasin B (Fig. 12). However, 10 μM cytochalasin B completely blocked the thrombin- and U46619-induced platelet shape change (data not shown), for which polymerization...
crease was almost the same as that of the response for factor of collagen alone, which were independent of TXA2. The responses induced by cytochalasin B were only the responses induced by the action of endogenously generated TXA2. The responses induced by collagen in the absence of indomethacin, the responses induced by collagen alone, 2) the responses induced by U46619, and 3) the responses induced by the synergistic action of collagen and U46619. As U46619 alone could not induce arachidonic acid release, the arachidonic acid release represented in Fig. 10A, II, and Fig. 11, II, arachidonic acid release and inositol phosphate formation induced by the action of collagen alone were inhibited by cytochalasin B. When platelets were stimulated with collagen plus U46619 in the presence of indomethacin, the total response consisted of: 1) the responses induced by collagen alone, 2) the responses induced by U46619 alone, and 3) the responses induced by the synergistic action of collagen and U46619. As U46619 alone could not induce arachidonic acid release, the arachidonic acid release represented in Fig. 10A, IV, was a combination of factors 1 and 3. Cytochalasin B slightly decreased the response, and the decrease was almost the same as that of the response for factor 1 shown in Fig. 10A, II. Inositol phosphate formation induced by collagen plus U46619 in the presence of indomethacin is represented in Fig. 11, IV. The response was factor 1 plus factor 2 plus factor 3. Like arachidonic acid release, the decrease by cytochalasin B was almost the same as that of factor 1 which is represented in Fig. 11, II. The factor 2 response has been shown to not be inhibited by cytochalasin B. These findings indicated that the responses inhibited by cytochalasin B were only the responses induced by the action of collagen alone, which were independent of TXA2. The responses induced by U46619 alone and the responses induced by the synergistic action of collagen and U46619 were not inhibited, indicating that cytochalasin B did not impair the interaction of U46619 and collagen with platelets. The reason the responses induced by collagen in the absence of indomethacin were completely inhibited by cytochalasin B (Fig. 10A, I and Fig. 11, I) can be explained well from our results that all subsequent reactions depended on the TXA2, initially generated by the action of collagen alone, which was susceptible to cytochalasin B.

**Discussion**

Collagen-induced platelet activation is greatly dependent on endogenously generated TXA2. The responses of collagen-stimulated platelets can be separated into two categories, TXA2-independent and TXA2-dependent responses. First, collagen induces the TXA2-independent responses including initial TXA2 generation. Next, the TXA2-dependent responses are successively induced. We refer to the TXA2-independent process as the “first-phase” responses and the TXA2-dependent one as the “second-phase” responses. The first-phase responses are observed when TXA2 formation is inhibited by indomethacin or when binding of TXA2 to the receptor is prevented by a receptor antagonist SQ29,548. In the presence of these compounds, collagen induced release of arachidonic acid, formation of TXA2 (only when SQ29,548 was used), and formation of inositol phosphates without Ca2+ mobilization and aggregation. However, these responses were very weak in comparison to the responses observed in the absence of indomethacin and SQ29,548. Therefore, the greater part of the collagen-induced responses seems to be induced by the action of TXA2.

It has been postulated that arachidonic acid is liberated by a combination of phospholipase C (10) and diacylglycerol and monoacylglycerol lipase activities (13–15) and by phospholipase A2 activity (1–4). However, the finding that activation of phospholipase C by U46619 was not accompanied by arachidonic acid release suggests that the former pathway does not actually function in human platelets, and arachidonic acid is mainly released by the latter pathway, i.e. phospholipase A2. We have obtained the same results with rabbit platelets. The importance of phospholipase A2 in arachidonic acid release is also suggested by the finding that an inhibitor of diacylglycerol lipase does not inhibit arachidonic acid (39). Degradation of several phospholipid classes by phospholipase A2 in collagen-stimulated human platelets has been confirmed by Takamura et al. (40) and Pollock et al. (41). Our results also suggest that phospholipase A2 and phospholipase C are independently controlled.

In order to study the synergistic action of collagen and TXA2 in the second-phase responses, platelets were stimulated with collagen plus U46619 in the presence of indomethacin. For the second-phase arachidonic acid release, the synergistic action was essential as shown in Fig. 7. Moreover, U46619-induced inositol phosphate formation was also enhanced by the synergistic action of collagen and U46619. Enhanced formation of inositol triphosphate may induce the second Ca2+ peak as shown in Fig. 8 by releasing Ca2+ from intracellular Ca2+ store (11).

From these findings, we summarized collagen-induced signal transduction as occurring as follows. In the first-phase, collagen slightly activates phospholipases A2 and C. Activation of phospholipase A2 results in formation of a small amount of TXA2. Despite the activation of phospholipase C, Ca2+ mobilization is not detected, probably because of insufficient formation of inositol triphosphate. In the second phase, phospholipase C is further activated by the action of TXA2. However, for the further activation of phospholipase A2, the synergistic action of TXA2 and collagen is necessary.

Although the importance of phospholipases A2 and C is widely accepted, it is not understood how receptor occupation is able to activate these enzymes. Recent publications have suggested the involvement of some factors such as GTP-binding proteins (39, 42–46), Na+/H+ exchange (47–51) and lipocortin (caldaparin) (52–55) in the activation mechanism. The present study is the first to demonstrate the involvement of actin filaments in collagen-stimulated activation of these enzymes.

Actin is a major protein in platelets and is one of the components of the platelet cytoskeleton (27). The relationship

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2. T. Nakano, K. Hanasaki, S. Matsumoto, and H. Arita, submitted for publication.
between the cytoskeleton and platelet shape change is well accepted (56-59). On the other hand, the interaction of cyto
tin and focal adhesions with the actin cytoskeleton is suggested to be the main site of interaction. Although the findings
of Fox et al. (70) have been confirmed by several investigators, the exact mechanism remains to be elucidated.

The regulation of phospholipase A2 activity by cytochalasin B is specific to the collagen receptor. Based on these
findings, we have proposed that cytochalasin B inhibits the activity of phospholipase A2 by an interaction with the actin
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