Enhancement of collagen-induced phosphoinositide turnover by thromboxane A_2 analogue through Ca^{2+} mobilization in human platelets

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In human washed platelets, collagen-induced phosphoinositide turnover was inhibited by indomethacin, an inhibitor of thromboxane A_2 (TXA_2) formation, particularly at lower doses of collagen. This inhibition was counteracted by the addition of 9,11-epithio-11,12-methano-TXA_2 (STA_2), a stable analogue of TXA_2 as well as by the Ca^{2+} ionophore A23187. STA_2 and A23187 did not stimulate phosphoinositide turnover markedly, but significantly increased cytoplasmic free Ca^{2+} concentrations. The actions of STA_2 were blocked by 13-azaprostanoic acid, a TXA_2 receptor antagonist. These results suggest that TXA_2 is generated during the action of collagen and increases cytoplasmic free Ca^{2+} which then stimulates phosphoinositide turnover in cooperation with collagen.

Platelet *Collagen* *Thromboxane* *Phosphoinositide* *Ca^{2+}* *Protein phosphorylation*

1. INTRODUCTION

Collagen induces both secretion and aggregation in human platelets [1]. These reactions induced by collagen are inhibited by indomethacin or aspirin which are known to inhibit collagen-induced generation of PGG_2, PGH_2 and TXA_2 [2,3]. These arachidonic acid metabolites have been shown to induce secretion and aggregation [2,3]. Based on these observations, it has been suggested that PGH_2 and TXA_2 may play a role of crucial importance in the action of collagen [2–6]. Another line of evidence indicates that collagen induces phosphoinositide turnover and Ca^{2+} mobilization, which are involved in the secretory processes through the activation of protein kinase C and calmodulin, respectively [6–8]. Rittenhouse and Allen [4] have shown that PGH_2 and its stable analogue U46619 induce phosphoinositide turnover to a small extent but counteract the inhibition by aspirin of collagen-induced phosphoinositide turnover.

A preceding report from our laboratory has described that STA_2, a stable analogue of TXA_2, increases cytoplasmic free Ca^{2+} concentrations [9]. During the course of studies on the mode of action of STA_2, we have clarified that STA_2 by itself does not induce phosphoinositide turnover to a great extent but enhances this reaction induced by collagen in the platelets pretreated with indomethacin. Moreover, evidence has been obtained that this action of STA_2 is replaced by the Ca^{2+} ionophore A23187. This paper describes a possible function of Ca^{2+} in the action of STA_2 which enhances collagen-induced phosphoinositide turnover. Here, elevation of cytoplasmic free Ca^{2+} concentrations was estimated by measuring the phosphorylation of MLC, since this protein phosphorylation is triggered by Ca^{2+} in proportion

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Abbreviations: PG, prostaglandin; TX, thromboxane; STA_2, 9,11-epithio-11,12-methanothromboxane A_2; MLC, myosin light chain; PA, phosphatidic acid; IP_3, inositol 1,4,5-trisphosphate

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to its increase through the activation of Ca^{2+}-activated, calmodulin-dependent protein kinase [10,11].

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

STA_2 and 13-azaprostanoic acid were donated by Ono Pharmaceutical Co. Collagen and thrombin were the products of Hormon-Chemie and Mochida Pharmaceutical, respectively. TXB_2 was obtained from Funakoshi Pharmaceutical. Carrier-free $^{32}P_i$ and [^{3}H]arachidonic acid (78.2 Ci/mmol) were purchased from Japan Radioisotope Association and New England Nuclear, respectively. Other materials and chemicals were obtained from commercial sources.

2.2. *Preparation of radioactive platelets*

Human washed platelets were prepared by the method of Mustard et al. [12]. Platelets were labeled with $^{32}P_i$ or [^{3}H]arachidonic acid as described [6]. The radioactive washed platelets were finally suspended at 6×10^8 cells/ml in a Tyrode's solution containing 128 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $MgCl_2$ and 5.5 mM glucose.

2.3. *Assay for phosphoinositide turnover*

Washed platelets prelabeled with $^{32}P_i$ were stirred at 37°C in an aggregometer, and various drugs and stimuli were added as indicated in each experiment. The radioactive lipids were directly extracted from the platelets by the method of Bligh and Dyer [13] and subjected to thin-layer chromatography as in [8]. The areas corresponding to PA were scraped off and the radioactivity was determined.

2.4. *Assay for MLC phosphorylation*

Washed platelets prelabeled with $^{32}P_i$ were incubated with various drugs and stimuli as described above. Radioactive platelet proteins were directly subjected to SDS slab gel electrophoresis, stained, dried on filter paper, and then exposed to an X-ray film to prepare an autoradiograph as described [6]. Electrophoresis was carried out by the method of Laemmli [14]. The relative intensity of each band was quantitated by densitometric tracing of the autoradiograph using a Shimadzu model CS-910 dual-wavelength chromatogram scanner.

2.5. *Analysis of TXB_2 formation*

Washed platelets prelabeled with [^{3}H]arachidonic acid were incubated with various drugs and stimuli as described above, and the reaction was terminated by the addition of formic acid. Radioactive arachidonic acid metabolites were analyzed by the method of Needleman et al. [15]. TXB_2 was identified by an authentic marker and its radioactivity was determined.

3. RESULTS

As shown in fig.1, collagen stimulated ^{32}P incorporation into PA and MLC phosphorylation in a parallel manner. These reactions induced by collagen were markedly inhibited by preincubation of platelets with indomethacin. Such inhibitory effects of indomethacin were more prominent at

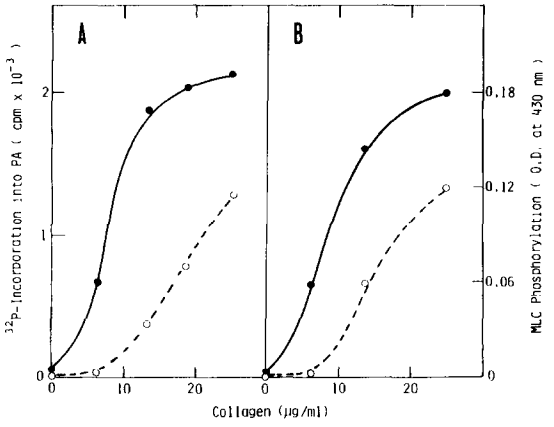

Fig.1. Inhibition by indomethacin of collagen-induced ^{32}P incorporation into PA and MLC phosphorylation in platelets. The platelets prelabeled with $^{32}P_i$ were incubated with indomethacin (5 μg/ml) for 2 min at 37°C, and then stimulated by various doses of collagen for 1.5 min at 37°C as indicated. Other details are described in section 2. The basal levels of ^{32}P incorporation into PA and MLC phosphorylation were 320 cpm and 0.04 *A* units at 430 nm, respectively. These values were subtracted from experimental values. Results are presented as the average of an experiment performed in triplicate. (A) ^{32}P incorporation into PA, (B) MLC phosphorylation. (●——●) In the absence of indomethacin, (○---○) in the presence of indomethacin.

lower doses of collagen. Under similar conditions, indomethacin completely blocked the generation of TXA_2 as measured by the formation of TXB_2, a stable inactive metabolite of TXA_2 (not shown). These results are in good agreement with earlier observations [4–6] and suggest that arachidonic acid metabolites such as TXA_2 may be involved in collagen-induced ^{32}P incorporation into PA and MLC phosphorylation.

Table 1 shows that STA_2 elicited sufficiently MLC phosphorylation but stimulated ^{32}P incorporation into PA to a small extent. However, this TXA_2 analogue overcame the inhibitory effect of indomethacin upon collagen-induced ^{32}P incorporation into PA as shown in table 1. Moreover, the simultaneous addition of STA_2 and collagen to the platelets pretreated with indomethacin stimulated ^{32}P incorporation into PA significantly more than the theoretical summation of this reaction which was induced by collagen plus indomethacin and STA_2 plus indomethacin. The potentiating effect of STA_2 was more prominent at lower doses of collagen. STA_2 and collagen stimulated only additively ^{32}P incorporation into PA in the platelets which were not treated with indomethacin. This might be due to the ability of collagen which by itself induced arachidonic acid mobilization and produced endogenous TXA_2. These results are consistent with the earlier observations made with PGH_2 and U46619 [4]. Under the same conditions, STA_2 also reversed the inhibi-

Table 1

Counteraction by STA_2 of inhibitory effects of indomethacin on collagen-induced ^{32}P incorporation into PA and MLC phosphorylation

Additions	^{32}P incorporation into PA (cpm)	MLC phosphorylation (*A* at 430 nm)
None	0	0.00
STA_2	690	0.12
STA_2 + indomethacin	610	0.12
Indomethacin	10	0.00
Collagen (12.5 μg/ml)	1950	0.15
+ indomethacin	350	0.06
+ indomethacin + STA_2	2260	0.16
+ STA_2	2510	0.18
Collagen (25 μg/ml)	2230	0.18
+ indomethacin	1290	0.12
+ indomethacin + STA_2	3180	0.18
+ STA_2	3290	0.18

Platelets prelabeled with $^{32}P_i$ were incubated with indomethacin (5 μg/ml) for 2 min at 37°C, and then stimulated by collagen and STA_2 (6 nM) for 1.5 min at 37°C as indicated. Other details are described in section 2. The basal levels of ^{32}P incorporation into PA and MLC phosphorylation were 320 cpm and 0.04 *A* units at 430 nm, respectively. These values were subtracted from experimental values. Results are presented as the average of an experiment performed in triplicate

Table 2

Counteraction by A23187 of inhibitory effects of indomethacin on collagen-induced ^{32}P incorporation into PA and MLC phosphorylation

Additions	^{32}P incorporation into PA (cpm)	MLC phosphorylation (*A* at 430 nm)
None	0	0.00
A23187	130	0.19
A23187 + indomethacin	90	0.19
Indomethacin	10	0.00
Collagen (12.5 μg/ml)	1950	0.15
+ indomethacin	350	0.06
+ indomethacin + A23187	1790	0.19
+ A23187	1970	0.19
Collagen (25 μg/ml)	2230	0.18
+ indomethacin	1290	0.12
+ indomethacin + A23187	2150	0.19
+ A23187	2250	0.19

Platelets prelabeled with $^{32}P_i$ were incubated with indomethacin (5 μg/ml) for 2 min at 37°C, and then stimulated by collagen and A23187 (0.6 μM) for 1.5 min at 37°C as indicated. Other details are described in section 2. The basal levels of ^{32}P incorporation into PA and MLC phosphorylation were 320 cpm and 0.04 *A* units at 430 nm, respectively. These values were subtracted from experimental values. Results are presented as the average of an experiment performed in triplicate

tion by indomethacin of collagen-induced MLC phosphorylation, but did not show synergistic effects on this phosphorylation. These effects of STA_2 were blocked by 13-azaprostanoic acid, which is an antagonist for a TXA_2 receptor [16] (not shown).

In another set of experiments, we examined whether the Ca^{2+} ionophore A23187 is able to substitute for STA_2 in the actions described above, since it has been described that A23187 increases cytoplasmic free Ca^{2+} concentrations and induces much less phosphoinositide turnover than other agonists such as thrombin [17]. Consistent with this observation, A23187 by itself did not stimulate ^{32}P incorporation into PA but fully induced MLC phosphorylation as shown in table 2. Moreover, A23187 counteracted the inhibition by indomethacin of collagen-induced ^{32}P incorporation into PA and MLC phosphorylation. As in the case of STA_2, A23187 and collagen synergistically enhanced ^{32}P incorporation into PA in the platelets pretreated with indomethacin. However, A23187 and collagen stimulated this reaction only additively in the platelets which were not treated with indomethacin. Under the same conditions, A23187 reversed the inhibition by indomethacin of collagen-induced MLC phosphorylation, but did not show synergistic effects on this phosphorylation.

4. DISCUSSION

This paper clearly demonstrates that STA_2 increases cytoplasmic free Ca^{2+}, as estimated by measuring the phosphorylation of MLC, without marked induction of phosphoinositide turnover, but significantly enhances this reaction induced by the agonist in the platelets pretreated with indomethacin. Evidence is also presented that these actions of STA_2 are observed with Ca^{2+} ionophore A23187. These results strongly suggest that these agents enhance collagen-induced phosphoinositide turnover through Ca^{2+} mobilization. Since the actions of STA_2 are inhibited by an antagonist for the TXA_2 receptor, they are mediated through the TXA_2 receptor. Presumably, TXA_2 is generated during the action of collagen, increases cytoplasmic free Ca^{2+}, and finally stimulates collagen-initiated phosphoinositide turnover. It is evident from our results as well as earlier observations [4,17] that Ca^{2+} mobilization alone is not sufficient and the occupation of the receptor with collagen is absolutely necessary for the induction of phosphoinositide turnover.

It has been described that thrombin-induced phosphoinositide turnover is initiated by the hydrolysis of phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate by the action of phospholipase C, resulting in the production of diacylglycerol and respective phosphorylated inositols [4,6,18–22]. Recent analysis has revealed that thrombin-induced IP_3 production is independent of Ca^{2+} mobilization and IP_3 serves as a messenger for Ca^{2+} mobilization from the dense tubular system to the cytoplasm [23]. Evidence is also available that thrombin-induced hydrolysis of phosphatidylinositol is stimulated by Ca^{2+} [11,24]. Although it has not yet been demonstrated whether collagen and TXA_2 may produce IP_3, it is conceivable that TXA_2 may enhance collagen-induced hydrolysis of phosphatidylinositol and phosphatidylinositol 4-phosphate through Ca^{2+} mobilization and thereby stimulate phosphoinositide turnover. The modes of action of the collagen receptor and Ca^{2+} on the activation of phospholipase C are now under investigation in our laboratory.

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