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Does protein kinase C activation mediate thrombin-induced arachidonate release in human platelets?

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Thrombin stimulated rapid formation of diacylglycerol, inositol 1,4,5-trisphosphate (IP_3) and thromboxane B_2 (TXB_2) in human platelets. Formation of diacylglycerol and IP_3 appeared to precede that of TXB_2. Activation of protein kinase C by diacylglycerol combining with Ca^{+2} mobilization by IP_3 has been implicated in mediating arachidonate release. However, addition of the protein kinase C inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) to platelet suspension did not inhibit thrombin-stimulated arachidonate release and TXB_2 synthesis, whereas addition of the Ca^{+2} antagonist, 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) or the calmodulin antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) abolished arachidonate release. The correlation of IP_3 production with arachidonate release on increasing the concentrations of thrombin was further examined. IP_3 production reached near maximum at 0.2 U/ml, whereas TXB_2 synthesis continued to increase at 1 U/ml. These results suggest that protein kinase C activation may not mediate arachidonate release and that Ca^{+2} mobilization by IP_3 may only partially account for arachidonate release in platelets stimulated with relatively high concentrations of thrombin.

Introduction

Thrombin is known to stimulate secretion of granular contents and to cause arachidonate release and thromboxane synthesis in human platelets [1,2]. Mechanisms that lead to platelet secretion and thromboxane synthesis are not fully understood. Recent evidence indicates that thrombin induces rapid phosphodiesteratic cleavage of PIP_2 into two putative second messengers, IP_3 and diacylglycerol [3,4]. IP_3 has been shown to mobilize intracellular calcium [5] and to stimulate thromboxane synthesis in permeabilized platelets [6], whereas diacylglycerol can activate protein kinase C [7]. Studies on the role of protein kinase C in platelet responses have been facilitated by the use of PMA which binds to and activates protein kinase C [8]. Pretreatment of platelets with PMA impairs thrombin-induced generation of IP_3 and diacylglycerol [9,10] and of arachidonate release [11,12] suggesting that protein kinase C plays a

Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; TXB_2, thromboxane B_2; PMA, 4β-phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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role in negative feedback regulation. However, whether protein kinase C is involved in the initial mediation of arachidonate release is not clear.

Previously, it was proposed that protein kinase C might catalyze phosphorylation and inactivation of lipocortin resulting in stimulation of phospholipase A_2 and release of arachidonate [13,14]. Recent studies on phospholipase A_2 inhibitory activity of lipocortin have revealed that inhibition by lipocortin is due to substrate depletion not direct interaction with phospholipase A_2, since lipocortin was found to be a phospholipid-binding protein [15]. This finding raises the question of whether lipocortin and protein kinase C are involved in triggering arachidonate release.

We have employed a potent inhibitor of protein kinase C, H-7 [16], to examine whether this inhibitor affects thrombin-induced arachidonate release and thromboxane synthesis. We have also utilized calcium antagonists to probe into the role of calcium in arachidonate release. Our results indicate that calcium mobilization is primarily responsible for thrombin-induced arachidonate release and thromboxane synthesis.

Materials and Methods

Materials. Human thrombin (3000 NIH units/mg), phorbol 12-myristate-13-acetate (PMA), 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), calcium ionophore (A23187), desipramine, and Dowex 1-X8 (Cl^- form) were obtained from Sigma Chemical Co. Thromboxane B_2 (TXB_2) was supplied by the Upjohn Company. [2-^{3}H]Inositol was purchased from the American Radiolabeled Chemicals, Inc.

Preparation and incubation of platelet suspension. Washed human platelets were prepared as previously described [17]. An aliquot of the platelet suspension ($(2.5–5) \cdot 10^8$ platelets) was used in various incubations at a final volume of 1 ml of Ca^{+2}/Mg^{+2}-free Tyrode's buffer. Incubations were carried out at 37 °C for the indicated length of time and terminated by the addition of 0.03 ml of 1 M HCl. Platelets were then pelleted by centrifugation at $1900 \times g$ for 10 min. The supernatant was neutralized by the addition of 1 M Tris base and assayed for TXB_2 immunoreactivity. Radioimmunoassay ot TXB_2 was carried out as previously described [18].

Pretreatment of platelets with PMA, TMB-8, H-7 or W-7. The above platelet suspension was preincubated at 37 °C with 1 μM PMA (3 min) or TMB-8 (5 min) before initiating platelet activation with thrombin. If H-7 or W-7 was used, the platelet suspension was preincubated with these agents for 3 min before incubation with PMA (3 min).

Stimulation of [2-^{3}H]inositol-labeled platelets and isolation of inositol-labeled metabolites. Platelets were labeled with [2-^{3}H]inositol as described previously [17]. The labeled platelets were suspended in Ca^{+2}/Mg^{+2}-free Tyrode's buffer at a concentration of $(3–5) \cdot 10^8$ platelets per ml. 1 ml of this labeled platelets was stimulated with thrombin for the indicated length of time at 37 °C. The reaction was terminated by chloroform/methanol/HCl (50 : 100 : 1, v/v) and the mixture was fractionated and assayed as described previously [17]. Briefly, 100 μl of the $CHCl_3$ phase was removed and assayed for TXB_2 immunoreactivity. The neutralized aqueous phase was chromatographed on a Dowex 1-X8 column, the [2-^{3}H]inositol trisphosphate fraction (including the IP_3 isomer) was collected in 2 ml of 0.5 M HCl and the radioactivity was determined by liquid scintillation counting.

Results

Thrombin stimulated rapid formation of IP_3 and TXB_2 as shown in Fig. 1. The kinetics of IP_3 formation and degradation coincided with that of diacylglycerol synthesis and catabolism as reported earlier [19]. This is consistent with the fact that phosphatidylinositol 4,5-bisphosphate undergoes receptor-mediated hydrolysis into diacylglycerol and IP_3, which are known to be further metabolized [20–23]. Formation of IP_3 and diacylglycerol appeared to precede that of TXB_2. If, indeed, thrombin triggers the release of arachidonic acid and synthesis of TXB_2 through IP_3-mediated Ca^{+2} mobilization and diacylglycerol-induced protein kinase C activation, inhibition of either action by the respective inhibitors should attenuate arachidonate release. The intracellular Ca^{+2} antagonist TMB-8 [24] effectively inhibited

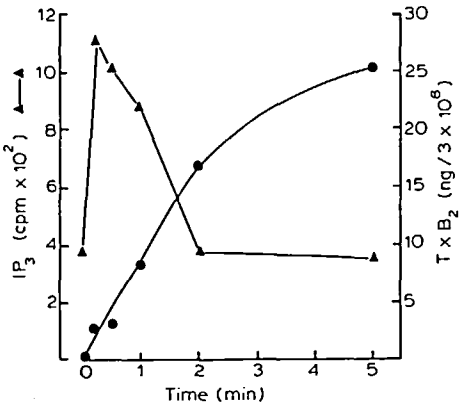

Fig. 1. The time course of thrombin-induced synthesis of inositol 1,4,5-trisphosphate (IP_3) and TXB_2. Inositol labeled platelets ($5 \cdot 10^8$/ml) were prepared and stimulated with thrombin (1 U/ml) as described in the Materials and Methods. Samples were taken at the indicated time and assayed for IP_3 and TXB_2. The values indicated are the averages of two determinations.

thrombin-induced TXB_2 synthesis at 100 μM as shown in Fig. 2. Similarly, the calmodulin antagonist W-7 [25] completely inhibited thrombin-induced TXB_2 synthesis at 10 μM as shown in the same figure. Another calmodulin antagonist, desipramine [26], also inhibited thrombin-induced TXB_2 synthesis, almost completely, at the same

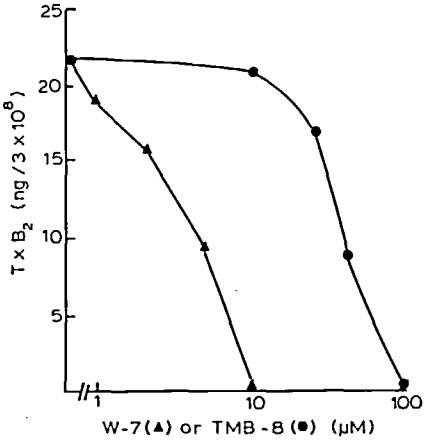

Fig. 2. Effect of TMB-8 and W-7 on thrombin-induced TXB_2 synthesis. A platelet suspension ($3 \cdot 10^8$/ml) was pretreated with the indicated concentrations of TMB-8 or W-7 before activation with thrombin (1 U/ml). The reaction was terminated after 5 min of activation and TXB_2 was assayed as described in the Materials and Methods. The values indicated are the averages of two determinations.

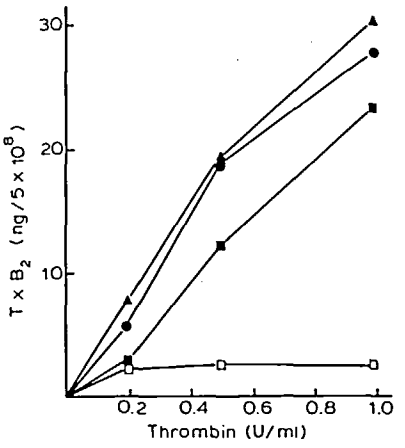

Fig. 3. Effect of H-7 and PMA on the thrombin-induced TXB_2 synthesis. A platelet suspension ($5 \cdot 10^8$/ml) was pretreated with PMA (1 μM) or H-7 (100 μM) for 3 min before activation with the indicated concentrations of thrombin. The reaction was terminated after 5 min of activation and TXB_2 was assayed as described in the Materials and Methods. The values indicated are averages of two determinations. ●, thrombin alone; ▲, thrombin + H-7; □, thrombin + PMA; ■, thrombin + PMA + H-7.

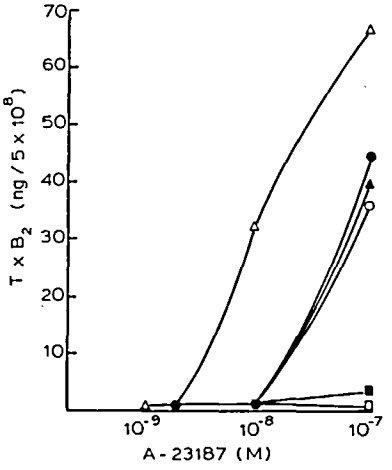

Fig. 4. Effect of PMA, H-7 and W-7 on the A23187-induced TXB_2 synthesis. A platelet suspension ($5 \cdot 10^8$/ml) was pretreated with PMA (1 μM), H-7 (100 μM), W-7 (10 μM), PMA + H-7 or PMA + W-7 as described in the Materials and Methods before activation with the indicated concentrations of A23187. The reaction was terminated after 5 min of activation and TXB_2 was assayed as described. The values indicated are the averages of two determinations. ○, A23187 alone; △, A23187 + PMA; □, A23187 + W-7; ●, A23187 + H-7; ▲, A23187 + PMA + H-7; ■, A23187 + PMA + W-7.

concentration (data not shown). Neither TMB-8 [24] nor W-7 (data not shown) affected arachidonate conversion into TXB_2, indicating that they inhibited arachidonate release from the membrane phospholipids.

H-7, a potent protein kinase C inhibitor [16], did not appear to inhibit thrombin induced TXB_2 synthesis at concentrations as high as 100 μM as indicated in Fig. 3. This concentration of H-7 apparently was able to antagonize the action of protein kinase C as demonstrated by the fact that H-7 was able to reverse PMA-mediated inhibition of thrombin-induced TXB_2 synthesis as is also shown in Fig. 3. Similarly, H-7 also inhibited the PMA-mediated synergistic effect on calcium ionophore A23187-induced TXB_2 synthesis as shown in Fig. 4. However, H-7 did not affect A23187-stimulated TXB_2 synthesis, indicating that protein kinase C was not involved in A23187-induced arachidonate release and metabolism.

If IP_3-mediated release of Ca^{+2} is primarily responsible for the thrombin-induced release of arachidonic acid and synthesis of TXB_2, production of IP_3 and release of arachidonic acid should be correlated. When inositol-labeled platelets were stimulated by increasing concentrations of thrombin, accumulation of IP_3 at 30 s approached a maximum at 0.2 U/ml, whereas synthesis of TXB_2 continued to increase steadily up to 1 U/ml as shown in Fig. 5. These data suggest that thrombin-induced IP_3 formation may not be fully responsible for triggering arachidonate release and thromboxane synthesis.

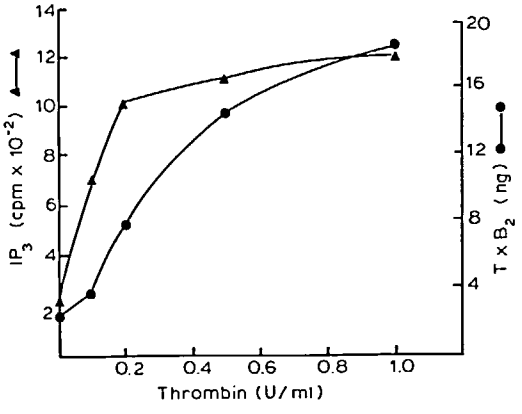

Fig. 5. Correlation of IP_3 production with TXB_2 synthesis in human platelets stimulated with different concentrations of thrombin. Inositol-labeled platelets ($3.6 \cdot 10^8$/ml) were prepared and stimulated with the indicated concentrations of thrombin for 30 s as described in the Materials and Methods. The platelets were processed and assayed for IP_3 and TXB_2 as described.

Discussion

IP_3 and diacylglycerol are generally considered as second messengers for a variety of hormones, neurotransmitters and growth factors in many cell types including platelets [8]. These two signals are rapidly formed in thrombin-stimulated platelets and are thought to mediate platelet secretion and arachidonate release [8]. This proposition stems from the facts that IP_3 can mobilize Ca^{+2} from the intracellular stores [5] and diacylglycerol is a protein kinase C activator [7]. Ca^{+2} mobilization combining with protein kinase C activation may trigger the total response in arachidonate release. Actually, how these two actions may work in concert leading to arachidonate release is not clear. It is reasonable to assume that Ca^{+2} mobilization, in addition to other effects, may activate phospholipase A_2 which catalyzes Ca^{+2}-mediated deacylation of phospholipids and releases arachidonic acid. However, a mechanism by which protein kinase C may participate in arachidonate release remains unknown. Ca^{+2} mobilization induced by A23187 stimulated thromboxane synthesis. Further addition of PMA enhanced thromboxane production, although PMA did not stimulate thromboxane synthesis by itself. This synergism is particularly evident at low A23187 concentrations [11]. PMA did not affect arachidonic acid metabolism to TXB_2 as shown by exogenous addition of [1-^{14}C]arachidonic acid (data not shown). This indicates that the potentiating effect of PMA is located at the stage of arachidonate release. H-7 abolished the effect of PMA, but not that of A23187, suggesting that Ca^{+2} mobilization alone could stimulate arachidonate release. Similarly, H-7 did not affect thrombin-induced arachidonate release and thromboxane synthesis, but attenuated the inhibitory effect of PMA, indicating that protein kinase C activation was not involved in mediating thrombin-induced arachidonate release, but participated in feedback regulation. Although

H-7 may inhibit other protein kinases at a higher concentration, its use in the current system at the concentration indicated does not alter the conclusion.

Thrombin stimulates the production of IP_3 which induces Ca^{+2} mobilization and, consequently, may be responsible for arachidonate release. Ca^{+2} mobilization appears to be essential in arachidonate release, since the intracellular Ca^{+2} antagonist TMB-8 and the calmodulin antagonists W-7 and desipramine can completely inhibit arachidonate release. The concurrent synthesis of diacylglycerol and, thereby, protein kinase C activation may be primarily involved in negative feedback control of the thrombin-induced effects, as suggested in other studies [9,10].

IP_3-induced Ca^{+2} mobilization, however, may not be totally responsible for thrombin-induced arachidonate release, particularly at high concentrations of thrombin. This proposition is derived from the fact that IP_3 production approached a maximum at 0.2 U/ml, whereas arachidonate release and thromboxane synthesis continued to elevate on increasing the concentration of thrombin. This suggests that there is an alternative mechanism for thrombin-induced Ca^{+2} mobilization or a separate pathway for thrombin-stimulated arachidonate release independent of IP_3 production. This possibility is supported by the finding that inhibition of Na^+/H^+ exchange reduces Ca^{+2} mobilization without affecting the initial cleavage of PIP_2 in thrombin-stimulated platelets [27]. Na^+/H^+ exchange may, in some way, affect Ca^{+2} mobilization and arachidonate release as suggested by Sweat et al. [28]. Epinephrine and ADP also appear able to mobilize membrane-bound Ca^{+2}, independent of phospholipase C activation [29,30]. Therefore, not only may diacylglycerol-induced protein kinase C activation not mediate the thrombin-stimulated arachidonate release, but the IP_3-induced Ca^{+2} mobilization may account for only part of the arachidonate release in platelets stimulated with relatively high concentrations of thrombin. It may well be that stimulation of thrombin receptor may be coupled to phospholipase A_2 activation via a distinct G-protein which is also responsible for mobilizing membrane-bound Ca^{+2}. The existence of a separate G-protein in human platelets for phospholipase A_2 and phospholipase C activation has been suggested [17,31].

It has been suggested that thrombin may stimulate at least two distinct G-proteins in human platelets [32,33]. This is based on the observation that thrombin stimulates GTPase activity and inhibits adenylate cyclase activity and that pertussis toxin largely abolishes thrombin-induced adenylate cyclase inhibition, but decreases thrombin-stimulated GTPase activity by only about 30%. One of the G-proteins involved may be related to G_i, since treatment of platelets with thrombin inhibits the pertussis toxin-induced ADP-ribosylation of a 41 kDa protein [34,35]. The identity of the other G-protein remains to be defined.

Whether or not protein kinase C activation may mediate arachidonate release induced by other platelet agonists remains to be determined. The mechanism by which protein kinase C activation may enhance A23187-induced arachidonate release also requires elucidation. These studies are currently in progress in our laboratory.

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