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NO DIRECT CORRELATION BETWEEN Ca^{2+} MOBILIZATION AND DISSOCIATION OF G_i DURING PLATELET PHOSPHOLIPASE A_2 ACTIVATION

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SUMMARY. Stimulation of human platelets with thrombin is accompanied by activation of both phospholipases C and A_2. These have been considered to be sequential events, with phospholipase A_2 activation resulting from the prior hydrolysis of inositol phospholipids and mobilization of intracellular Ca^{2+} stores. However, our and other laboratories have recently questioned this proposal, and we now present further evidence that these enzymes may be activated by separate mechanisms during thrombin stimulation. Alpha-thrombin induced the rapid hydrolysis of inositol phospholipids, and formation of inositol trisphosphate and phosphatidic acid. This was paralleled by mobilization of Ca^{2+} from internal stores. These responses were blocked by about 50% by prostacyclin. In contrast, the liberation of arachidonic acid induced by alpha-thrombin was totally inhibited by prostacyclin. The less-effective agonists, platelet activating factor (PAF) and gamma-thrombin also both stimulated phospholipase C, but whereas PAF evoked a rapid and transient response, that of gamma-thrombin was delayed and more sustained. The abilities of these agonists to induce the release of Ca^{2+} stores closely paralleled phospholipase C activation. However, the maximal intracellular Ca^{2+} concentrations achieved by these two agents were the same. Despite this, gamma-thrombin and not PAF, was able to release a small amount of arachidonic acid. When alpha-thrombin stimulation of platelets was preceded by epinephrine, there was a potentiation of phospholipase C activation, Ca^{2+} mobilization and aggregation. The same was true for gamma-thrombin and PAF. However, unlike alpha-thrombin, the gamma-thrombin-stimulated arachidonic acid release was not potentiated by epinephrine, but rather somewhat reduced. These results suggested that phospholipase C and phospholipase A_2 were separable events in activated platelets. The mechanism by which alpha-thrombin stimulated phospholipase A_2 did not appear to be through dissociation of the inhibitory GTP-binding protein, G_i, since gamma-thrombin decreased the pertussis toxin-induced ADP-ribosylation of the 41 kDa protein as much as did alpha-thrombin, but was a much less effective agent than alpha-thrombin at inducing arachidonic acid liberation.

Arachidonic acid liberation in the platelet is predominantly under the control of phospholipases A_2, and less so on diglyceride lipase (1). The importance of this enzyme in activation of platelet responses ranges from a

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small contribution in the case of thrombin (2) to being totally necessary for agents such as epinephrine (3) and collagen (2).

Over past years phospholipase A_2 has been thought to be regulated by the intracellular Ca^{2+} concentration (4). The rise in the cytosolic Ca^{2+} concentration is now considered to be regulated by phospholipase C activity and formation of inositol-1,4,5-trisphosphate (5). In support of a causal relationship between the two enzyme activities, it was shown that exogenously added inositol-1,4,5-trisphosphate was able to induce aggregation and arachidonate formation in permeabilized platelets (6,7). However, more recent evidence has not supported the view that the ability of agonists to stimulate phospholipase A_2 resides in the resulting Ca^{2+} level (8).

GTP analogs are capable of stimulating phospholipase A_2 in mast cells (9). Other work on thyroid cells has shown phospholipase A_2 to be pertussis toxin sensitive, implicating the inhibitory GTP-binding protein, G_i, in enzyme stimulation (10). Further, in outer rod segments the β/γ dimer of G_i was found to activate phospholipase A_2 (11). Thus, a possible Ca^{2+}-independent link between receptor occupation and phospholipase A_2 activation was shown. Whether G_i is a physiological mediator of phospholipase A_2 is not known, nor have the effects of the β/γ dimer been explored in other cells.

We have examined the possible role of G_i in platelet phospholipase A_2 activation by thrombin, as well as the contribution of the intracellular Ca^{2+} level in this response. Our data suggest that neither event is the sole controller of arachidonic acid liberation in the thrombin-activated platelet.

MATERIALS AND METHODS

Materials. Alpha- and gamma-thrombins were kindly provided by Dr. John Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, NY. (+)-Epinephrine and platelet activating factor were purchased from Sigma Chemical Co., [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (80-135 Ci/mmol) and myo-[2-^{3}H]inositol (10-20 Ci/mmol) were from Amersham,

adenylate-[^{32}P]NAD (1000 Ci/mmol) from New England Nuclear, pertussis toxin from List Biochemicals, and Indo-1 AM from Behring Diagnostics.

Methods

Preparation of Human Platelets. Platelets were isolated from blood of healthy human donors who had not taken medication for at least the previous two weeks. The blood was anticoagulated with 3.8% trisodium citrate (6 ml in 60 ml blood) and centrifuged for 20 min at 200 g. The platelet-rich plasma was then centrifuged for 20 min at 800 g and the platelets resuspended in the appropriate volume of buffer or platelet-poor plasma, as described below. The buffers contained (mM): NaCl, 138; KCl, 2.9; Hepes, 20; NaH_2PO_4, 3.3; $MgCl_2$ 1.0; glucose 1 mM. They were maintained at 37°C, except for the studies of Ca^{2+} mobilization (25°C), and adjusted to pH 7.4. During periods of radioactive labelling the buffer was modified to contain 1 mM aspirin for 20 min prior to experimentation and, except when cell permeabilization was required, were suspended in the presence of an ADP scavenger [apyrase (0.6 ADP'ase units/ml)]. Prior to centrifugation and during periods of labelling, prostacyclin (100 ng/ml) was added to platelets to inhibit aggregation.

Measurement of Platelet Aggregation. Aggregation of platelets in Hepes buffer in response to agonists was followed continuously in a Chronolog aggregometer.

Measurement of Inositol Phospholipid Hydrolysis. Platelets from 200 ml of blood were resuspended in 1 ml of EGTA-containing buffer and 1 ml of [^{3}H]inositol (0.5 mCi) was added. After incubation for 3 h to label inositol phospholipids, platelets were diluted to 30 ml with buffer, centrifuged for 10 min and resuspended in final buffer without EGTA. Aliquots (0.5 ml) were incubated with or without agonist and, after the times indicated in the figure, the reactions were stopped by addition of 1.8 ml $CHCl_3/CH_3OH/HCl$ (100:200:2). The phases were then separated by addition of 0.6 ml each of $CHCl_3$ and H_2O and centrifugation. The water-soluble inositol phosphates produced were separated by anion exchange chromatography, as described previously (12).

Measurement of Arachidonic Acid Liberation. Platelets from 200 ml of blood were isolated and resuspended in 5 ml of platelet-poor plasma and incubated for 90 min with 50 µCi of [^{3}H]arachidonic acid. After labelling, platelets were diluted to 30 ml with platelet-poor plasma, centrifuged at 800 g for 15 min, and resuspended in 10 ml of buffer containing EGTA. This wash procedure was repeated and the platelets were finally resuspended in buffer without EGTA (10-20 ml). Aliquots (0.5 ml) of platelets were pre-incubated for 1 min with the cyclooxygenase/lipoxygenase inhibitor, BW755C (100 µM), and then the agonist or buffer was added. Samples were taken at appropriate times for the determination of liberated arachidonic acid, with reactions being terminated and phases separated as described for inositol phosphates. The organic phase was collected, the lipids dried under vacuum at room temperature, and arachidonic acid and phosphatidic acid were separated by thin layer chromatography (Whatman LK6D). The solvent system was the upper phase of an ethyl acetate/isooctane/acetic acid/water (90:50:20:100) solvent system containing 1% acetic acid. Radioactive species were localized by autoradiography and the corresponding areas on the plates were scraped and counted in a scintillation counter.

Measurement of Intracellular Ca2+ Concentration. Platelets from 100 ml of blood were incubated in plasma containing 5 µM Indo-1 AM for 45 min. The cells were then centrifuged and resuspended in buffer containing EGTA and 1 mM aspirin. After 20 min, the cells were again centrifuged and suspended in buffer without EGTA. Changes in the intracellular Ca^{2+} concentration of aliquots (2 ml) were monitored continuously using a fluorescence spectrophotometer (Perkin Elmer) with excitation and emission wavelengths of 340 and 390 nm, respectively. Changes in the fluorescence were calibrated to changes in cellular Ca^{2+} levels as previously described (13).

Measurement of Pertussis Toxin-Induced ADP-Ribosylation. Platelets from 50 ml of blood were isolated and suspended in 2 ml buffer. Platelet

samples (70 µl) were added to microcentrifuge tubes, containing 5 µl of agonist and 2 min later the 'ADP-ribosylation cocktail' was added. The cocktail contained (final concentration):saponin (20 µg/ml), cold NAD (20 µM), adenylate-[^{32}P]NAD (2 µCi/ml), pertussis toxin (7 µg/ml; preactivated with 1.1 mM dithiothreitol for 30 min at 37°C), EDTA (1 mM), thymidine (10 m), GTP (17 µM) and diethylenetriaminepentaacetic acid (2 µM). The incubation was for 20 min and was halted by addition of SDS-PAGE sample buffer (100 µl). Proteins were separated using SDS-PAGE (14), and the ADP-ribosylated bands were visualized by autoradiography.

RESULTS

Stimulation of human platelets with either alpha- (10 nM) or gamma- (50 nM) thrombin induced aggregation (15,16), inositol phosphate formation (Fig. 1) and Ca^{2+} mobilization from intracellular stores (Fig. 2). In each case, the response to gamma-thrombin was slower than that elicited by alpha-thrombin, and there was a characteristic delay between adding the

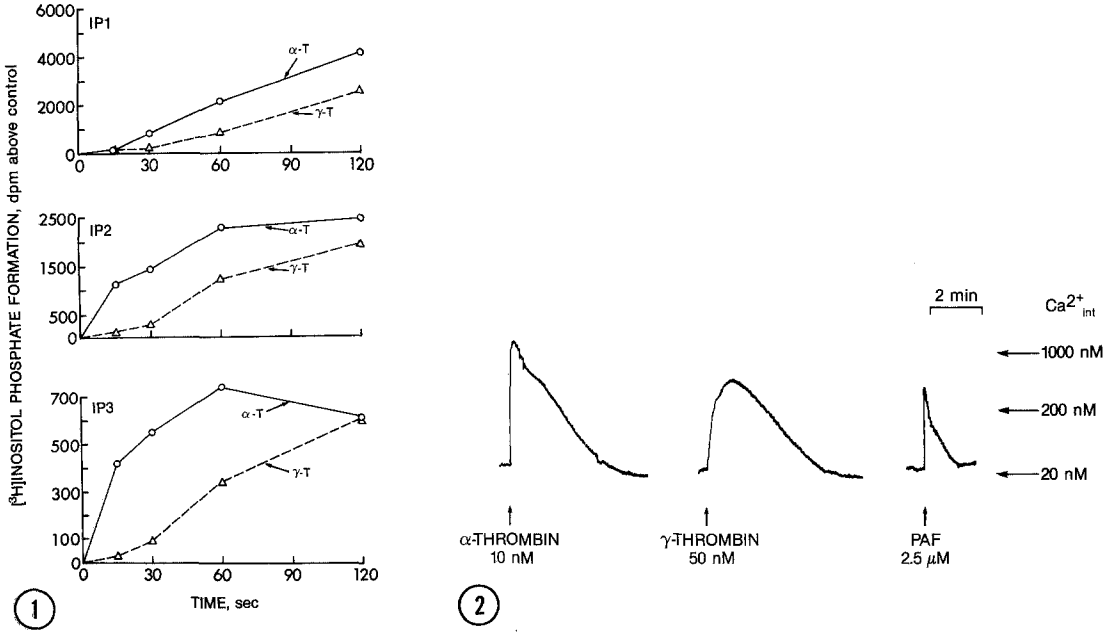

Fig. 1 Alpha- and gamma-thrombin stimulation of inositol phosphate formation. Platelets were labelled with [^{3}H]inositol and then stimulated with either alpha-thrombin (10 nM) or gamma-thrombin (50 nM) for the indicated times. The water-soluble inositol phosphates were then separated on Dowex anion exchange columns.

Fig. 2 Mobilization of intracellular Ca^{2+} stores by alpha- and gamma-thrombin and platelet activating factor. Platelets were loaded with the fluorescent Ca^{2+} indicator, Indo-1 AM and the ability of alpha-thrombin (α-T), gamma-thrombin (γ-T) and platelet activating factor (PAF) to mobilize cellular Ca^{2+} stores was measured in a fluorimeter.

agent and development of the subsequent response. The onset of alpha- and gamma-thrombin-induced Ca^{2+} mobilization and formation of inositol trisphosphate closely correlated (Figs. 1 and 2).

Although the responses of aggregation and inositol phosphate formation produced by gamma-thrombin were slower than that seen in response to alpha-thrombin, they eventually reached the same value (Fig. 1 and Ref. 15). In contrast, the mobilization of intracellular Ca^{2+} stores was always only about one third of that produced by alpha-thrombin (Fig. 2). PAF (0.1-2.5 μM) produced a rapid release of intracellular Ca^{2+}, the maximum level of which was the same as that evoked by gamma-thrombin (Fig. 2). The PAF response, however, was of much shorter duration (Fig. 2).

Alpha-thrombin (10 nM) was a strong releaser of esterified arachidonic acid (Figs. 3 and 4). Gamma-thrombin (50 nM) was not as effective (Fig. 3),

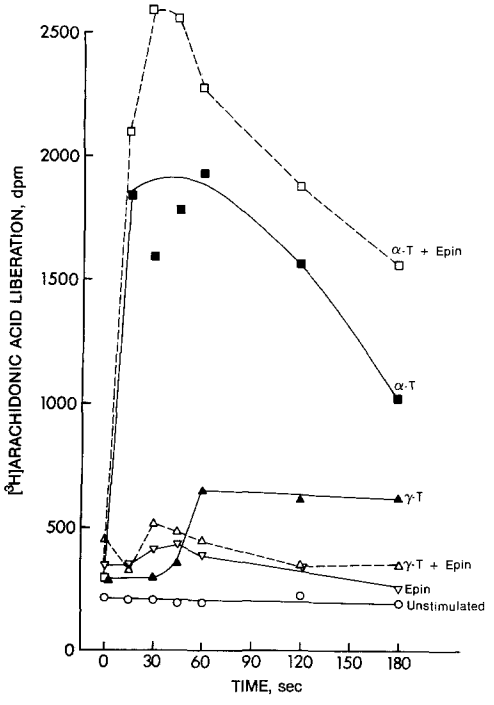

Fig. 3 Arachidonic acid liberation from platelets in response to alpha- and gamma-thrombin with or without epinephrine. Platelets were prelabelled with [^{3}H]arachidonic acid and stimulated for indicated times with alpha-thrombin (α-T; 10 nM), gamma-thrombin (γ-T; 50 nM) either alone or in combination with epinephrine (EPIN; 100 μM). The released arachidonic acid was resolved using thin layer chromatography.

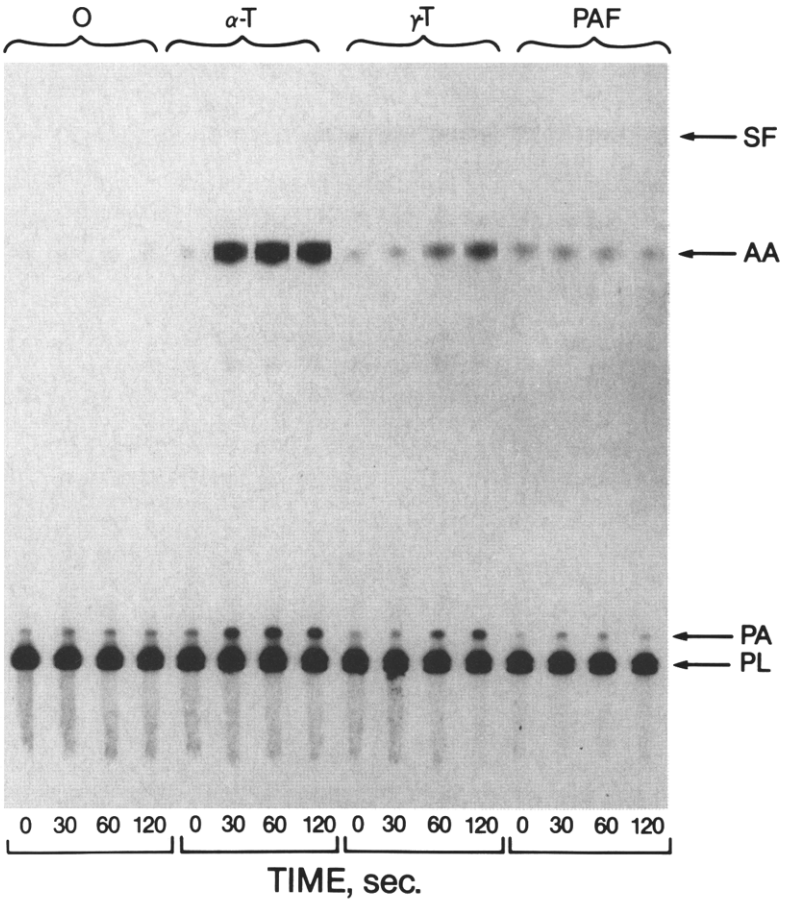

Fig. 4 Thin layer chromatography of arachidonic acid release induced by alpha- and gamma thrombin and platelet activating factor. [3H]Arachidonic acid-labelled platelets were stimulated with either alpha-thrombin (α-T; 10 nM), gamma-thrombin (γ-T; 50 nM) or platelet activating factor (PAF; 1 μM) for the indicated times. The cyclooxygenase/lipoxygenase inhibitor, BW755C (100 μM), was included to inhibit metabolism of arachidonic acid by these pathways. The esterified arachidonic acid release was separated on thin layer chromatography, and the plate subject to autoradiography.

and PAF even less so (Fig. 4). This was despite the observation that gamma-thrombin could also stimulate inositol trisphosphate and phosphatidic acid production to levels which were similar to those seen in response to alpha-thrombin (Figs. 1 and 4, Ref. 16). In addition, prostacyclin (100 ng/ml) totally inhibited alpha-thrombin-stimulated arachidonic acid liberation, but only reduced phosphatidic acid formation by about 30% (Fig. 5). The phosphatidic acid levels were restored in the presence of

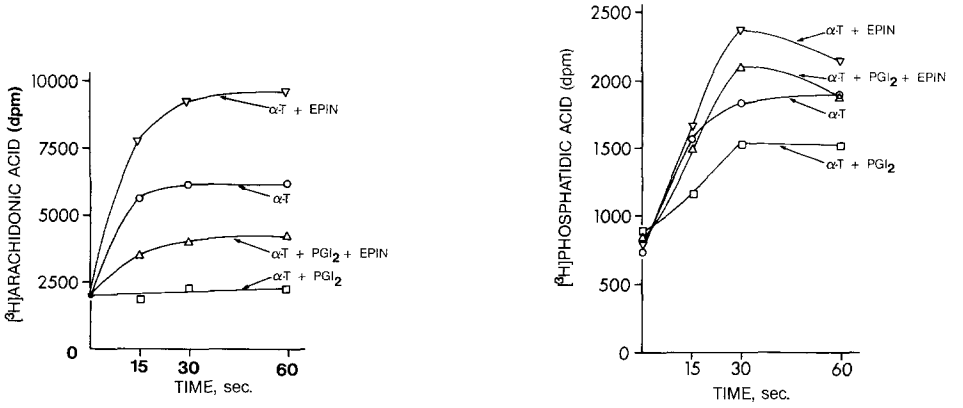

Fig. 5 Time courses of arachidonic and phosphatidic acid release in response to alpha-thrombin in the presence or absence of prostacyclin and epinephrine. The ability of a preincubation of platelets for 1 min with prostacyclin (PGI_2; 100 ng/ml) or 15 sec with epinephrine (EPIN, 100 μM) to modify the alpha-thrombin induced formation of arachidonic acid and phosphatidic acid was measured. Arachidonic acid and phosphatidic acid were separated on the same thin layer chromatography system, the radioactivity localized by autoradiography, and the spots scraped and counted.

prostacyclin when the alpha-thrombin stimulation was preceded by epinephrine (100 μM), although epinephrine alone had no effect (Fig. 5). The same was true for alpha-thrombin-stimulated aggregation, and 40 and 20 kDa protein phosphorylation (17). However, arachidonic acid release was only restored by 50% by pre-incubating platelets with epinephrine (Fig. 5).

In the presence of epinephrine, alpha-thrombin was a more potent agonist for stimulation of aggregation (15,16), Ca^{2+} mobilization (17) and release of arachidonic acid (Fig. 3). Gamma-thrombin-induced responses were also potentiated by epinephrine (Ref. 15 and results not presented), except that of arachidonic acid release, which was consistently reduced (Fig. 3).

PAF (0.1-2.5 μM) did not evoke any observable arachidonic acid liberation in our system (Fig. 4), although a small increase in phosphatidic acid (Fig. 4) and inositol trisphosphate (not shown) was observed.

Pertussis toxin catalyzed the ADP-ribosylation of a 41 kDa protein. This was almost totally inhibited by pretreatment of platelets for 2 min with either alpha-thrombin (10 nM) or gamma-thrombin (50 nM) (Fig. 6).

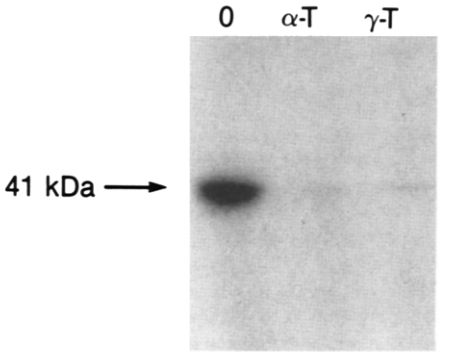

Fig. 6 The effect of alpha- and gamma-thrombin on pertussis toxin-induced ADP-ribosylation of G_i. Platelets were treated with or without either alpha- (10 nM) or gamma- (50 nM) thrombin for 2 min and then permeabilized with saponin. An ADP-ribosylation cocktail containing [^{32}P]-NAD and pertussis toxin was then added, and the cells left for a further 20 min. Proteins were then separated on SDS-PAGE and the gel subject to autoradiography.

DISCUSSION

The control of phospholipase A_2 in agonist-activated platelets has been thought to result from the stimulated rise in the cytosolic Ca^{2+} concentration (4). Since the level of Ca^{2+} appears to be regulated by agonist-induced phospholipase C activity (5)], it is often thought that these are sequential events in the activated platelet (18,19).

We have previously examined the role of protein kinase C and lipocortin in phospholipase A_2 activation (15), and found that there was little correlation. Since protein kinase C is controlled by inositol phospholipid hydrolysis and generation of diacylglycerol, the same system which delivers the Ca^{2+}-mobilizing messenger, inositol-1,4,5-trisphosphate, we wanted to see if this arm of the hormonal signal could account for the platelet phospholipase A_2 activity. This approach gave us further insights into the role of phospholipase C in activation of phospholipase A_2.

Several results suggested that the cytosolic Ca^{2+} level was not the major controller of arachidonic acid liberation. Firstly, gamma-thrombin was a poor stimulus of arachidonic acid release. When platelets were preincubated with epinephrine, there was enhanced Ca^{2+} mobilization and protein kinase C activation in response to gamma-thrombin, but the peak

release of arachidonic acid was consistently reduced. Secondly, although PAF was an even less effective agonist than gamma-thrombin for release of arachidonic acid, it was able to elevate the cytosolic Ca^{2+} level to a maximum which was the same as that found for gamma-thrombin. Thirdly, although prostacyclin inhibited alpha-thrombin-activated phospholipase C by only about 30%, phospholipase A_2 was totally blocked by this treatment. Epinephrine, under these conditions, totally restored phosphatidic acid production, but that of phospholipase A_2 by only 50%.

This lack of correlation between the ability of agonists to stimulate phospholipase C, and the subsequent events of Ca^{2+} mobilization and protein kinase C activation, with that of phospholipase A_2 suggested that another messenger system was involved. The inhibitory GTP-binding protein, G_i, is known to be stimulated in thrombin-activated human platelets, and Okano *et al.* (9) have found that GTP analogs will stimulate arachidonic acid release from permeabilized cells. In addition, in some cells pertussis toxin can inhibit phospholipase A_2 and addition of β/γ subunits of G_i was stimulatory (10,11). Therefore, these studies implied that a GTP-binding protein may be involved in platelet phospholipase A_2 activation, and in some cells that G_i may be the responsible GTP-binding protein.

We have examined the possible involvment of G_i in platelet phospholipase A_2 activation by comparing the effects of alpha- and gamma-thrombin on pertussis toxin-induced ADP-ribosylation. The ability of an agonist to decrease ADP-ribosylation by pertussis toxin is taken as a measure of the dissociation of the α subunit of G_i from the β/γ complex (20). Despite gamma-thrombin being a weak agonist in terms of its ability to stimulate phospholipase A_2, it reduced pertussis toxin-induced ADP-ribosylation of G_i as much as did alpha-thrombin. The time course of this decrease in ADP-ribosylation correlated well with that of phospholipcase C activation (17). We conclude that both thrombin species are effectively coupled to G_i and that thrombin stimulation of phospholipase A_2 in the platelet is not via G_i. Nor does it appear that the β/γ subunit of G_i stimulates this enzyme.

Our studies do not rule out the possibility that in the alpha-thrombin-stimulated platelet there is a concerted action of Ca^{2+}, protein kinase C and G_i dissociation in controlling phospholipase A_2. However, we can rule out the existence of a single controlling factor of arachidonic acid liberation in this cell.

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