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Modulation of Thrombin-Stimulated Lipid Responses in Cultured Fibroblasts. Evidence for Two Coupling Mechanisms†

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Received June 6, 1986; Revised Manuscript Received January 8, 1987

ABSTRACT: Treatment of cultured fibroblasts with thrombin results in the stimulation of cell division and lipid metabolism. Proteolytically active α-thrombin rapidly stimulates (a) release of arachidonic acid, (b) generation of inositol phosphates, and (c) increase in cellular diacylglycerol levels. Pretreatment of the fibroblasts with chymotrypsin before α-thrombin prevented the first two responses, (a) and (b), and reduced response c. Treatment of fibroblasts with γ-thrombin, a proteolytic derivative of α-thrombin, produced a response indistinguishable from the α-thrombin treatment when preceded by chymotrypsin. These data support a model, similar to one for platelets [McGowan, E. B., & Detwiler, T. C. (1986) *J. Biol. Chem. 261*, 739–746], that fibroblasts possess two coupling mechanisms for the stimulation of lipid metabolism by thrombin. Similar to platelets, one mechanism, R1, mediates the stimulated release of arachidonic acid and is capable of activating N_i, a GTP-binding protein. R1 is inactivated by chymotrypsin and does not respond to γ-thrombin. The other mechanism, R2, responds to γ-thrombin and is not inactivated by chymotrypsin. In contrast to the mechanisms proposed for platelets, we demonstrate that the phospholipase C responsible for the hydrolysis of phosphoinositides is not activated by R2 but is activated via R1. Importantly, stimulation of either mechanism results in the elevation of cellular diacylglycerol. This indicates that the stimulated elevation of diacylglycerol, or those events dependent upon the elevation of diacylglycerol, is not a reliable indicator for establishing the hydrolysis of phosphoinositides. Furthermore, studies with islet activating protein demonstrate that while a N_i-like protein(s) does (do) not appear to be involved in the stimulated hydrolysis of phosphoinositides, this protein does appear to be involved in at least part of the thrombin-stimulated release of arachidonic acid. A N_i-like protein(s) may be involved in the metabolism of stimuated diacylglycerol.

Addition of catalytically active α-thrombin to certain fibroblast-like cells results in the stimulation of cell division (Chen & Buchanan, 1975; Pohjanpelto, 1978; Carney et al., 1978) and cellular lipid metabolism (Hong & Levine, 1976; Carney et al., 1985; Raben & Cunningham, 1985; Murayama & Ui, 1985). The stimulated lipid metabolism has been strongly implicated in mitogenesis by thrombin (Carney et al., 1985; Raben & Cunningham, 1985) and other growth-promoting agents (Fisher & Mueller, 1968; Cunningham, 1972; Hoffman et al., 1980; Sawyer & Cohen, 1981; Habenicht et al., 1981; Macphee et al., 1984; Berridge et al., 1984; Dawson et al., 1983; Diringer & Friis, 1971; Ristow et al., 1980; Hiu & Harmony, 1980; Hasegawa-Sasaki & Sasaki, 1982; Vicentini & Villereal, 1984). α-Thrombin similarly stimulates lipid metabolism in platelets (Agaroff et al., 1983; Billah & Lapetina, 1982; Kawahara et al., 1980; Sano et al., 1983; Ieyasu et al., 1982; Rittenhouse-Simmons, 1979; Bell & Majerus, 1980; Takai et al., 1982; Lapetina & Cuatrecasas, 1979). Addition of catalytically active α-thrombin to platelets results in a rapid release of inositol phosphates and arachidonic acid and the generation of elevated levels of diacylglycerol (Agaroff et al., 1983; Billah & Lapetina, 1982; Kawahara et al., 1980; Sano et al., 1983; Ieyasu et al., 1982; Rittenhouse-Simmons, 1979; Bell & Majerus, 1980; Takai et al., 1982; Lapetina & Cuatrecasas, 1979). The detailed mechanism by which thrombin-mediated proteolysis is coupled to stimulated lipid metabolism in either fibroblasts or platelets remains to be established.

Detwiler and co-workers have demonstrated that pretreatment of platelets with chymotrypsin, which does not activate platelets, modifies the platelet response to α-thrombin (Tam et al., 1980; McGowan et al., 1983; McGowan & Detwiler, 1986). Interestingly, γ-thrombin, a proteolytic derivative of α-thrombin, elicited platelet responses which were nearly identical with those stimulated by α-thrombin on chymotrypsin-treated platelets (McGowan & Detwiler, 1986). In

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0006-2960/87/0426-2759$01.50/0

view of these data, McGowan and Detwiler suggest that two coupling mechanisms exist for thrombin-stimulated lipid metabolism. One of these mechanisms, R1, is inactivated by chymotrypsin and does not respond to γ-thrombin; the other mechanism, R2, is insensitive to chymotrypsin and responds to γ-thrombin. Both mechanisms respond to α-thrombin. These investigators further suggest that the GTP-binding protein designated as N_i mediates the responses activated via R1 and augments the responses activated via R2.

In view of the above points, we have examined the effect of chymotrypsin, γ-thrombin, and also islet activating protein (IAP)[1] on the α-thrombin-induced lipid metabolism in a subclone of Chinese hamster embryo fibroblasts called IIC9 cells. We conclude from our present data that at least two coupling mechanisms, R1 and R2, exist for the stimulation of lipid metabolism by α-thrombin in fibroblasts and present a model for this.

Experimental Procedures

Materials. Bovine serum albumin (BSA) (RIA grade fraction V), essentially fatty acid free BSA, bovine brain phospholipids, and TLCK–chymotrypsin were purchased from Sigma; phosphate-free DMEM containing 25 mM Hepes was obtained from Irvine Scientific; all other media components were from Gibco. Plastic cell culture dishes were from Falcon plastics. Radioisotopes were purchased from New England Nuclear. The specific activities were as follows: [^{3}H]arachidonic acid, 60–100 Ci/mmol; [^{3}H]glycerol, 5–10 Ci/mmol; [^{3}H]myoinositol, 10–20 Ci/mmol. Human transferrin was purchased from Calbiochem. Highly purified human α-thrombin, 3000 NIH units/mg (clotting activity), and γ-thrombin, 1.5 NIH units/mg (clotting activity), were generous gifts from Dr. J. W. Fenton, II (Fenton et al., 1977). γ-Thrombin possessed 88% of the α-thrombin esterolytic activity as measured with [^{3}H]BAEE as the substrate (Tollefsen et al., 1983). IAP was purchased from List Biological Laboratories, Inc. TLC plates were purchased from Analtech. TLC plates were preactivated by prerunning acetone through the plates and heating them at 100 °C for 10 min. The chloroform and methanol used for chromatography were redistilled before use. Bio-Rad AG1-X8 anion-exchange resin (formate form) was obtained from Bio-Rad. Hydrofluor liquid scintillation counting cocktail was purchased from National Diagnostics.

Cells and Cell Culture. The IIC9 Chinese hamster embryonic fibroblasts were grown and maintained as previously described in α-MEM/Ham&s F12 (1:1) supplemented with 5% fetal calf serum (Raben & Cunningham, 1985; Low et al., 1982). They were serum-starved as previously described with the exception that insulin was omitted (Raben & Cunningham, 1985). The serum-free media used in these studies were (1) DMEM buffered with 20 mM Hepes, pH 7.4, and supplemented with 5 μg/mL human transferrin and 1 mg/mL BSA and (2) inositol-free medium containing the following: Earle's balanced salt solution, α-MEM amino acids, 25 mM Hepes, pH 7.4, DMEM vitamins with the exception of inositol, 0.1 mg/L ($Fe(NO_3)_3 \cdot 9H_2O$, 2 g/L glucose, 1 mg/mL BSA, and 5 μg/mL human transferrin. For each experiment, cells were plated at 0.5×10^4 cells/cm^2 and cultured for 3 days at which point they were subconfluent at $(4–5) \times 10^4$ cells/cm^2. The cultures were then switched to serum-free medium.

Arachidonic Acid Release Assay. Cells in 35-mm culture dishes, which had been serum-starved for 24 h in the Hepes-buffered DMEM described above, were washed 2–3 times with Hepes-buffered DMEM containing 1 mg/mL fatty acid free BSA and 5 μg/mL human transferrin. They were then incubated in this medium supplemented with 0.5 μCi/mL [^{3}H]arachidonic acid for 24 h at 37 °C. The cultures were then washed 2–3 times and incubated for another 30 min in the fatty acid free BSA-containing medium to remove any arachidonic acid which had not been incorporated by the cells. The cultures were then washed once more, and the experiments were performed as described in the figure legends. To determine the amounts of arachidonic acid and metabolites that were released by the cells, the medium was removed from each culture and centrifuged at 150*g* for 5 min to remove any cells which may have detached from the dish. An aliquot of this medium was removed and assayed for radioactivity in a liquid scintillation counter using 10 mL of Hydrofluor counting cocktail.

Inositol Phosphate Assay. Cells in 60-mm culture dishes were washed 3 times with 5 mL of the inositol-free medium described above. The cultures were the incubated with this meidum supplemented with 5 μCi/mL [^{3}H]myoinositol for 24 h at 37 °C. The [^{3}H]myoinositol had been passed through a Bio-Rad AG1-X8 resin (formate form) and eluted with double-distilled water before use. At the end of the 24-h incubation, the cells were washed with serum-free Hepes-buffered DMEM, and the experiments were performed in this medium. Incubations were terminated by aspirating the medium and immediately adding 2.5 mL/dish acidified methanol (methanol/concentrated HCl 50:0.3 v/v). The cells were scraped into this solution, and the cell suspension was then added to 2.5 mL of chloroform in polypropylene tubes. After incubation at room temperature for 30 min, 0.75 mL of 5 mM EDTA in 1 N HCl was added to each tube, and the tubes were centrifuged at 100*g* for 10 min. The upper aqueous phase was removed, and the lower organic phase was extracted again with 0.5 mL of the upper phase from a chloroform/methanol/50 mM cyclohexane-1,2-diaminetetraacetic acid solution (16:8:5 v/v) (Litosch et al., 1985). The tubes were centrifuged again at 100*g* for 10 min, and the upper phase from this wash was combined with the previous upper phase. The combined upper phases were then dried under nitrogen. To analyze the inositol phosphates, the dried upper phases were resuspended in 10 mL of 10 mM imidazole, pH 7.0. Inositol phosphates were chromatographed on Bio-Rad AG1-X8 anion-exchange resin (formate form) as described by Downes and Michell (1981). The amount of radioactivity in eluates was determined in a liquid scintillaton counter using 10 mL of Hydrofluor counting cocktail.

Diacylglycerol Assay. Cells in 60-mm culture dishes were washed 3 times with 5 mL of the Hepes-buffered DMEM described above and incubated in this medium supplemented with 1 μCi/mL [^{3}H]glycerol for 48 h at 37 °C. The cultures were then wahsed once with the Hepes-buffered DMEM, and experiments were conducted in this medium. Levels of diacylglycerol were determined by aspirating the culture medium and quickly washing the cultures 3 times with PBS supplemented with 1 mg/mL BSA; 5 mL of trichloroacetic acid at 4 °C was added, and the dishes were incubated for 30 min at 4 °C. The dishes were then washed once with distilled water

[1] Abbreviations: IAP, islet activating protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TLCK–chymotrypsin, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone modified chymotrypsin; BAEE, benzoyl-L-arginine ethyl ester; EDTA, (ethylenedinitrilo)tetraacetic acid; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TLC, thin-layer chromatography; IP1, inositol 1-phosphate; IP2, inositol 1,4-bisphosphate; IP3, inositol 1,4,5-trisphosphate; NAD, nicotinamide adenine dinucleotide; RIA, radioimmunoassay; HPLC, high-pressure liquid chromatography.

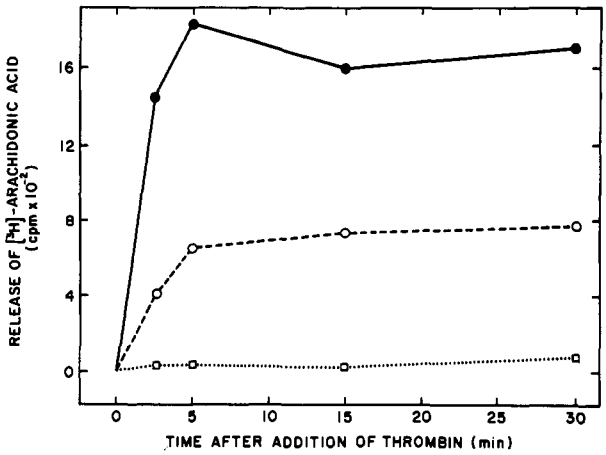

FIGURE 1: Effect of chymotrypsin and IAP on α-thrombin-stimulated release of arachidonic acid. 35-mm cultures of IIC9 cells were radiolabeled to steady state with [^{3}H]arachidonic acid, and the release of tritiated material stimulated by adding 14 pM α-thrombin at time zero was assayed at the indicated times as described under Experimental Procedures. The release was determined in thrombin-treated cultures (●) or in cultures which had been treated with 8 nM chymotrypsin at 37 °C for 5 min and washed 3 times prior to addition of α-thrombin (□). Other cultures received 200 ng/mL IAP during the last 3 h of [^{3}H]arachidonic acid labeling (○). IAP was present throughout the remainder of the experiment. Values presented are averages of duplicate cultures and represent the amount of material specifically released by α-thrombin. Control cultures released approximately 400 cpm/plate. Duplicate values did not vary more than 5%.

at 4 °C; each dish then received 0.4 mL of water. The cells were then scraped and added to 0.5 mL of chloroform. Each dish was then washed with 1 mL of methanol which was added to the water/chloroform mixture; 20 μg of 1,2-diolein was added to protect against nonspecific losses, and the lipids were then extracted essentially as described by Bligh and Dyer (1959) and dried under nitrogen. Neutral lipids were separated from polar lipids by resuspending the dried lipids from the Bligh–Dyer extraction in 0.5 mL of chloroform. The lipids were then applied to a 0.3-mL column of silicic acid, and the neutral lipids were eluted with an additional 3 mL of chloroform. To quantitate the diacylglycerol levels, the neutral lipids were dried under nitrogen and chromatographed on silica gel G plates employing benzene/ethyl ether/ethanol/15 M ammonia (50:40:2:0.1 v/v). The diacylglycerol spot was identified by iodine vapor using 1,2-diolein as the known standard. The diacylglycerol spots were scraped and placed into scintillation counting vials containing 0.2 mL of water and 10 mL of Hydrofluor counting cocktail; the amount of radioactivity in diacylglycerol was then quantitated.

Protein Determination. Cultures for protein determination were wahsed 10 times with PBS and solubilized with 2% CHAPS at 37 °C. Protein concentration was determined by the method of Kalb and Bernlohr (1977).

Results

Modulation of α-Thrombin-Stimulated Lipid Responses by Chymotrypsin

Arachidonic Acid Release. Treatment of IIC9 cells with 14 pM α-thrombin stimulated a rapid release of arachidonic acid (Figure 1). Pretreatment of the cells with chymotrypsin (8 nM for 5 min at 37 °C) was sufficient to inactivate this response for at least 30 min.

Release of Inositol Phosphates. Addition of α-thrombin to platelets (Agaroff et al., 1983) and fibroblasts (Carney et al., 1985) stimulates a rapid release of inositol phosphates. α-Thrombin also stimulated a rapid release of inositol phosphates from IIC9 cells which had been prelabeled with [^{3}H]myoinositol (Figure 2). It caused a 5-fold increase in labeled IP3 within 10 s, a 6-fold increase in IP2 within 20 s, and a 2–3-fold increase in IP1 within 40 s. Upon further incubation, these elevated levels returned to values which were 1.5–2-fold higher than the levels observed in control, unstimulated, cultures. Pretreatment of IIC9 cells with 8 nM chymotrypsin for 5 min at 37 °C eliminated the induced release of inositol phosphates observed within 1 min after the addition of α-thrombin (Figure 2). A 1.5–2-fold increase in IP2 and IP3 levels was observed 5–15 min after the addition of α-thrombin to chymotrypsin-treated cells. Further incubation at 37 °C for 30 min did not result in any significant increase in inositol phosphate levels (data not shown). Chymotrypsin alone did not stimulate the release of these inositol phosphates (data not shown).

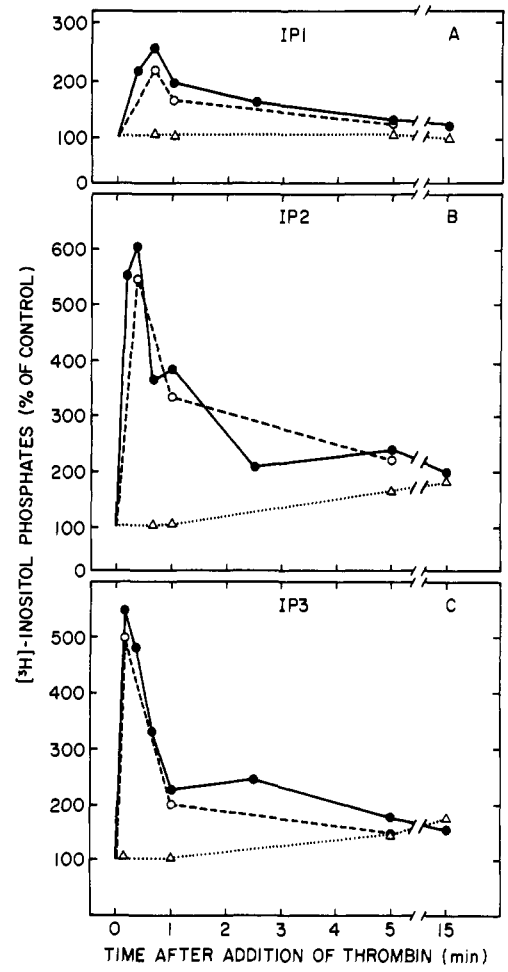

FIGURE 2: Effect of chymotrypsin and IAP on α-thrombin-stimulated increases in inositol phosphates. 60-mm cultures of IIC9 cells were radiolabeled with [^{3}H]myoinositol, and the generation of inositol phosphates stimulated by adding 14 pM α-thrombin at time zero was determined as described under Experimental Procedures. The inositol phosphate levels were determined in thrombin-treated cultures (●) or in cultures which had been treated with 8 nM chymotrypsin for 5 min at 37 °C and washed 3 times before the addition of α-thrombin (△). Other cultures received 200 ng/mL IAP during the last 3 h of labeling with [^{3}H]myoinositol (○). Values are the average of duplicate cultures and represent the fold increase over control, unstimulated, cultures. Values of duplicate cultures did not vary more than 15%. (A) IP1, control value = 10 000 ± 1200 cpm/plate; (B) IP2, control value = 800 ± 110 cpm/plate; (C) IP3, control value = 900 ± 125 cpm/plate.

Diacylglycerol Levels. α-Thrombin generates a transient increase in the level of diacylglycerol in platelets (Rittenhouse-Simmons, 1979). α-Thrombin also stimulated a tran-

Table I: Stimulated Lipid Responses in IIC9 Cells[a]

response	% α-thrombin response					
	α-Th	Ch–α-Th	γ-Th	Ch–γ-Th	IAP–Th	NaF
IP3	100 (6700 ± 60)	0	0	0	90 ± 10	60 ± 11
IP2	100 (5200 ± 100)	0	0	0	85 ± 11	45 ± 8
IP1	100 (28200 ± 1500)	0	0	0	75 ± 15	50 ± 12
DAG	100 [(5.8 ± 0.2) × 10^3]	45 ± 5	50 ± 8	40 ± 10	155 ± 7	ND[b]
AA	100 (1300 ± 50)	0	0	0	40 ± 5	60 ± 10

[a] Generation of inositol phosphates, diacylglycerol (DAG), and arachidonic acid (AA) was assayed as described under Experimental Procedures. Chymotrypsin (Ch) and IAP treatments were performed as described in the legends to Figures 1–3. Inositol phosphates were measured at 10 s (IP3), 20 s (IP2), and 40 s (IP1) after the addition of 28 pM α-thrombin (α-Th) or 1.4 nM γ-thrombin (γ-Th). The NaF values were obtained after an 8-min incubation (inositol phosphates) or a 10-min incubation (arachidonic acid) with 50 mM NaF supplemented with 10 μM $AlCl_3$. Control, unstimulated, values were as follows: IP1 = 11 600 ± 1300 cpm/plate; IP2 = 1100 ± 75 cpm/plate; IP3 = 1200 ± 20 cpm/plate; arachidonic acid release = 450 ± 15 cpm/plate; diacylglycerol level = (3.2 ± 0.3) × 10^3 cpm/mg of protein. Numbers in parentheses represent the values obtained with 28 pM α-thrombin. [b] Not determined.

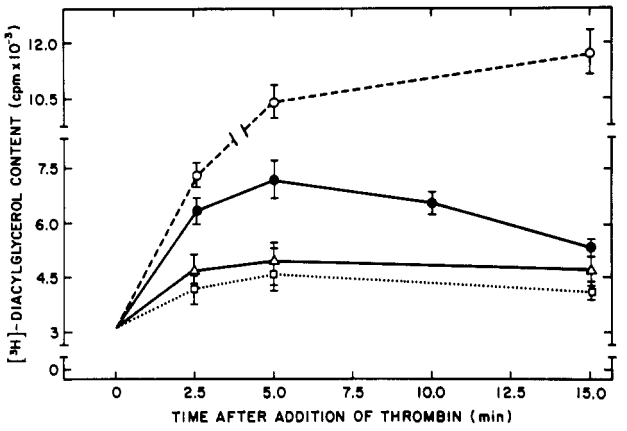

FIGURE 3: Time course of alterations in cellular diacylglycerol levels. 60-mm cultures of IIC9 cells were radiolabeled with [^{3}H]glycerol as described under Experimental Procedures. Cultures then received 28 pM α-thrombin (●) or 1.4 nM γ-thrombin (△). Some cultures were pretreated with 8 nM chymotrypsin for 5 min at 37 °C and washed 3 times before the addition of α-thrombin (□). Other cultures received 200 ng/mL IAP during the last 3 h of labeling with [^{3}H]-glycerol and throughout the remainder of the experiment (○). Zero time represents the time of addition of thrombin. Values represent the average of duplicate cultures. Error bars represent the standard error.

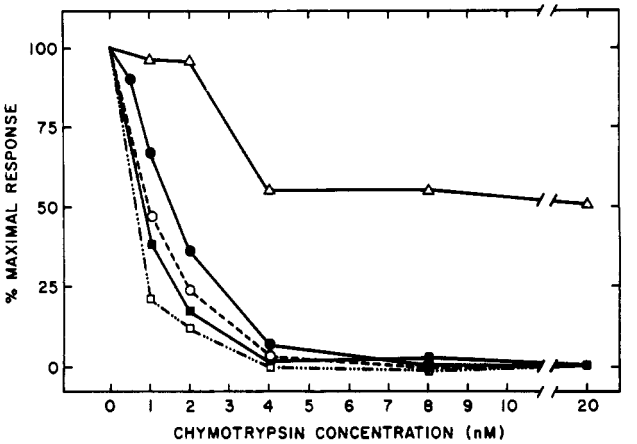

FIGURE 4: Dose dependence of the chymotrypsin-induced inhibition of α-thrombin stimulated lipid responses. α-Thrombin stimulation of arachidonic acid release (●), generation of IP1 (□), λπ2 (■), and IP3 (○), and elevation of cellular diacylglycerol levels (△) were assayed as described under Experimental Procedures. Arachidonic acid releases and diacylglycerol levels were determined 5 min after the addition of 14 pM α-thrombin. Inositol phosphates were measured 10 s (IP3), 20 s (IP2), and 40 s (IP1) after the addition of 14 pM α-thrombin. Cultures were pretreated with chymotrypsin or IAP as described in the legend to Figure 2. Control, unstimulated values were as follows: IP1 = 16 000 ± 2000 cpm/plate; IP2 = 1100 ± 150 cpm/plate; IP3 = 1200 ± 200 cpm/plate; arachidonic acid release = 250 ± 20 cpm/plate; and diacylglycerol level = (4.1 ± 0.5) × 10^3 cpm/mg of protein. All values represent averages of duplicate cultures and did not vary more than 5% (arachidonic acid) or 15% (diacylglycerol and inositol phosphates).

sient elevation of diacylglycerol in IIC9 cells (Figure 3). Within 5 min of thrombin addition, the level of diacylglycerol was elevated approximately 2-fold and later returned to values near the control level.

According to the current hypothesis, the generation and metabolism of diacylglycerol are associated with the generation of inositol phosphates and arachidonic acid. Since chymotrypsin pretreatment inactivated the ability of IIC9 cells to release inositol phosphates and arachidonic acid in response to α-thrombin (Figures 1 and 2), we examined the effect of chymotrypsin on α-thrombin stimulation of diacylglycerol levels. Pretreatment of IIC9 cells with 8 nM chymotrypsin for 5 min at 37 °C reduced the stimulated level of diacylglycerol by approximately 50% (Figures 3 and 4). Longer incubations with chymotrypsin (10–15 min) did not further reduce the stimulated level of diacylglycerol (data not shown).

Dose Dependence of Inhibition of α-Thrombin-Stimulated Responses by Chymotrypsin. Chymotrypsin pretreatment of IIC9 cells completely eliminated the α-thrombin-stimulated release of arachidonic acid (Figure 1 and Table I). Pretreatment of these cells with chymotrypsin also eliminated the stimulated generation of inositol phosphates (Figure 2) but only reduced the stimulated level of diacylglycerol (Figure 3). To further evaluate this difference, we examined the doses of chymotrypsin that inhibited the α-thrombin-stimulated responses.

Inhibition of stimulated arachidonic acid and inositol phosphate generation was observed at 1.0 nM chymotrypsin and was essentially complete at 4 nM (Figure 4). In contrast, chymotrypsin-induced reduction of stimulated diacylglycerol levels required concentrations above 2 nM; maximum reduction was never greater than 50–60%, even in cultures exposed to 20 nM chymotrypsin (Figure 4). Chymotrypsin which was catalytically inactive from derivatization with diisopropyl phosphate was ineffective in reducing any of the α-thrombin-stimulated responses (data not shown).

Effect of γ-Thrombin on Cellular Lipid Metabolism

McGowan and Detwiler (1986) reported that platelets respond to γ-thrombin in a manner which resembled the responses generated by α-thrombin on chymotrypsin-treated platelets. In view of this observation, we evaluated the γ-thrombin-stimulated responses in IIC9 cells.

Arachidonic Acid Release. γ-Thrombin did not stimulate release of arachidonic acid from IIC9 cells even at a concentration which was 300-fold higher than the concentration of α-thrombin required to maximally stimulate the release of arachidonic acid (Table I).

Inositol Phosphate Levels. γ-Thrombin did not stimulate a rapid release of inositol phosphates from IIC9 cells (Table I). No increase in inositol phosphate levels was detected during a 15-min incubation of these cells at 37 °C with 50 nM γ-thrombin (data not shown).

Diacylglycerol Levels. Althouth γ-thrombin did not stimulate release of arachidonic acid from platelets, it did stimulate activities which were dependent upon elevated diacylglycerol levels (McGowan & Detwiler, 1986). While it was suggested that this was due to a stimulated turnover of polyphosphoinositides in platelets (McGowan & Detwiler, 1986), we failed to detect γ-thrombin-stimulated increases in inositol phosphates in IIC9 cells (Table I). We therefore determined the effect of γ-thrombin on diacyglycerol levels in IIC9 cells. γ-Thrombin elevated diacylglycerol levels in IIC9 cells to approximately 40–50% of the level stimulated by α-thrombin (Figure 3). This level was comparable to that elicited by α-thrombin in chymotrypsin-treated IIC9 cells. In view of this, we determined the chymotrypsin sensitivity of the γ-thrombin-induced diacylglycerol elevation. The data in Table I indicate that while chymotrypsin treatment lowered the IIC9 response to α-thrombin, it did not affect the elevation of diacylglycerol stimulated by γ-thrombin.

Effect of IAP on α-Thrombin-Stimulated Lipid Metabolism

IAP-catalyzed ADP-ribosylation of N_i-like proteins renders these proteins inactive in their ability to mediate receptor-linked functions (Murayama et al., 1983; Murayama & Ui, 1983, 1984; Kurose et al., 1983; Kurose & Ui, 1983). IAP catalyzed the incorporation of [^{32}P]P_i from [α-^{32}P]NAD into a 41K protein in isolated IIC9 plasma membranes (data not shown). This indicates ADP-ribosylation of the α-subunit of an N_i-like protein. Therefore, if an N_i-like protein(s) was (were) involved in a particular α-thrombin-stimulated response, preincubation of the cells with IAP should inhibit the α-thrombin stimulation of that response.

α-Thrombin-Stimulated Release of Arachidonic Acid. Fluoride stimulated a large release of arachidonic acid from IIC9 cells (Table I). This is consistent with the hypothesis that GTP-binding proteins are involved in the stimulated release of this lipid. IAP inhibited thrombin-stimulated release of arachidonic acid from IIC9 cells (Figures 1 and 5 and Table I). Pretreatment of IIC9 cells with 200 ng/mL IAP for 3 h at 37 °C reduced the thrombin-stimulated release of arachidonic acid by approximately 60% (Figure 1 and Table I). IAP alone did not stimulate release of arachidonic acid (data not shown). As shown in Figure 5, maximal inhibition of arachidonic acid release was obtained with 5 ng/mL IAP. Longer incubations (4–5 h) with 200 ng/mL IAP did not result in an increase in the inhibition of the thrombin-stimulated release of arachidonic acid (Data not shown). It is important to note that the concentration of thrombin used in this experiment was 10-fold higher than that required for maximal stimulated release.

α-Thrombin-Stimulated Release of Inositol Phosphates. Pretreatment of IIC9 cells with IAP did not significantly affect the thrombin-stimulated release of inositol phosphates (Figure 2 and Table I). This was observed even when the cells were incubated for 3 h at 37 °C with concentrations of IAP which were 40-fold higher than the concentration required to maximally reduce thrombin-stimulated arachidonic acid release (Figures 1 and 5). It should be noted that the data in Table I indicate that fluoride stimulated an increase in inositol phosphate levels within 8 min at 37 °C. These data implicate the involvement of an IAP-sensitive GTP-binding protein(s) in the stimulated hydrolysis of phosphoinositides in these cells.

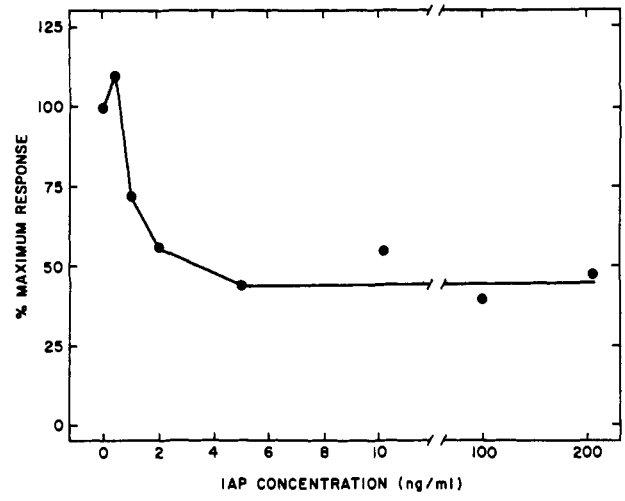

FIGURE 5: Dose dependence of IAP inhibition of α-thrombin-stimulated release of arachidonic acid. 35-mm cultures of IIC9 cells were radiolabeled with [^{3}H]arachidonic acid as described under Experimental Procedures. Cultures were treated with the indicated concentrations of IAP, and arachidonic acid release 5 min after the addition of 1.4 nM α-thrombin was assayed as described in the legend to Figure 1. Cultures not receiving IAP released 300 ± 25 cpm/plate without thrombin and 750 ± 100 cpm/plate 5 min after addition of 1.4 nM α-thrombin (maximum response). Values presented represent averages of duplicate cultures and did not vary more than 5%.

α-Thrombin-Stimulated Diacylglycerol Levels. One of the proposed mechanisms for the stimulated release of arachidonic acid involves the hydrolysis of arachidonic acid from diacylglycerol via diacylglycerol lipase (Bell et al., 1979; Prescott & Majerus, 1983; Irvine, 1982). The diacylglycerol used in this reaction is supposedly derived from the phospholipidase C mediated hydrolysis of phosphoinositides (Majerus et al., 1984; Bell et al., 1979; Nishizuka, 1984a,b). IAP did not significantly alter the thrombin-stimulated generation of inositol phosphates while it reduced the thrombin-stimulated release of arachidonic acid. Therefore, we examined the effect of IAP on thrombin-stimulated diacylglycerol levels. IAP did not prevent the α-thrombin-stimulated increase in diacylglycerol observed 5 min after the addition of thrombin (Figure 3). However, pretreatment of IIC9 cells for 3 h at 37 °C with IAP (200 ng/mL) prevented the subsequent decrease in the stimulated diacylglyerol levels observed upon further incubation of the cells at 37 °C (Figure 3). IAP did not significantly alter the basal, unstimulated, diacylglyerol levels during the incubation at 37 °C (data not shown).

Discussion

Evidence for Two Coupling Mechanisms in Thrombin Stimulation of Lipid Metabolism. The chymotrypsin sensitivity of the various α-thrombin-stimulated lipid responses suggests the existence of two coupling mechanisms. If one mechanism were responsible for the thrombin-stimulated responses, chymotrypsin should affect all the responses in the same dose-dependent manner. However, the data in Figure 4 clearly indicate that while chymotrypsin inactivates α-thrombin-stimulated generation of inositol phosphates and arachidonic acid release in the same dose-dependent manner, the α-thrombin-stimulated increase in diacylglycerol displays a much different sensitivity toward chymotrypsin.

Further support that two coupling mechanisms exist for thrombin-stimulated lipid metabolism is demonstrated by the ability to elevate cellular diacylglycerol levels without phospholipase C mediated hydrolysis of the phosphoinositides. Cellular diacylglycerol levels were elevated after a 2.5-min

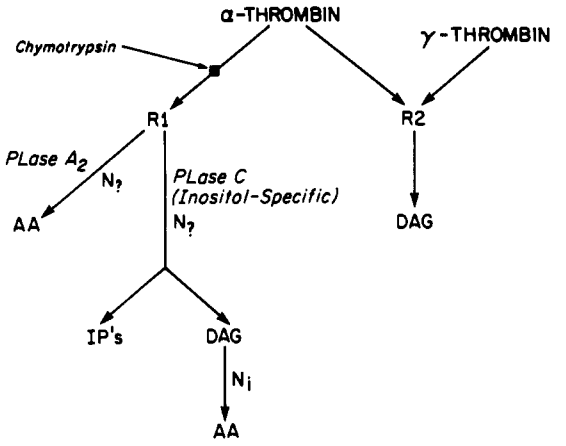

FIGURE 6: Hypothetical model for thrombin-stimulated lipid responses in fibroblasts. The model is presented under Discussion. Abbreviations: AA, arachidonic acid; IP's, inositol phosphates; DAG, diacylglycerol; N_i, GTP-binding protein.

incubation at 37 °C with γ-thrombin, or with α-thrombin on chymotrypsin-treated cells. Increases in inositol phosphates and arachidonic acid release were not observed in response to α-thrombin on chymotrypsin-treated cells during this time (Figures 1 and 2). γ-Thrombin also failed to stimulate increases in inositol phosphates or arachidonic acid release (Table I). The most likely interpretation of these data is that the diacylglycerol generated by γ-thrombin or by α-thrombin on chymotrypsin-treated cells is not derived from hydrolysis of phosphoinositides. This is consistent with the observation of Bocckino et al. (1985). These investigators demonstrated by HPLC analysis that vasopressin stimulates the formation of at least two molecular species of diacylglycerol in rat hepatocytes. They propose that one species is not derived from phosphoinositides. The possibility exists that α-thrombin also stimulates at least two molecular species of diacylglycerol while γ-thrombin stimulates only one of these species.

Involvement of a N_i-like Protein(s) in α-Thrombin-Stimulated Lipid Responses. GTB-binding proteins have been implicated in agonist-stimulated lipid metabolism (Okajima & Ui, 1984; Haslam & Davidson, 1984a,b; Molski et al., 1984; Murayama & Ui, 1985; Cockroft & Comperts, 1985; Brandt et al., 1985). Since fluoride, a known activator of GTP-binding proteins (Sternweis et al., 1981; Bokoch et al., 1983), stimulates increases in inositol phosphates and free arachidonic acid (Table I), we suggest that these proteins are also involved in the stimulated releases of these compounds from IIC9 cells. One of these binding proteins, N_i, is inactivated by IAP-catalyzed ADP-ribosylation (Murayama et al., 1983; Murayama & Ui, 1983, 1984; Kurose et al., 1983; Kurose & Ui, 1983). It has been demonstrated that in certain cell types, IAP prevents stimulated releases of arachidonic acid and inositol phosphates, suggesting a role for a N_i-like protein(s) in these responses (Murayama & Ui, 1985; Gomperts, 1983; Nakamura & Ui, 1974, 1985; Okajima & Ui, 1984; Bokoch & Gilman, 1984; Ohta et al., 1985). However, we observed that IAP did not significantly affect thrombin-stimulated increases of inositol phosphates in IIC9 cells. This is similar to the observations of Murayama and Ui that thrombin does not affect thrombin-stimulated release of inositol-containing compounds into the culture medium of Swiss 3T3 cells (Murayama & Ui, 1985). While Murayama and Ui also demonstrated that IAP prevented thrombin-stimulated release of arachidonic acid, we found that IAP prevented only part of this stimulated release from IIC9 cells (Figures 1 and 5 and Table I).

The mechanism by which IAP affects arachidonic acid release has not been determined. Arachidonic acid is liberated from phospholipids via phospholipase A2 mediated hydrolysis or by phospholipase C mediated hydrolysis and subsequent hydrolysis of the resulting diacylglycerol (Shoene & Iacono, 1975; Russell & Deykin, 1976; Bills et al., 1977; Blackwell et al., 1977; Bell et al., 1979; Irvine, 1982). Since IAP appears to prevent the degradation of the diacylglycerol whose release is stimulated by α-thrombin (Figure 4), it is tempting to suggest that a N_i-like protein(s) is (are) involved with the stimulated metabolism of this lipid. Further analysis is required to answer this question.

One possible explanation for the effects of IAP on lipid metabolism is that inactivation of N_i-like proteins results in the elevation of cAMP which prevents part of the thrombin-stimulated release of arachidonic acid. However, this interpretation is inconsistent with the inability of 1 mM dibutyrl-cAMP to reduce thrombin-stimulated release of arachidonic acid during the following 5-min incubations: control, unstimulated value = 450 cpm/plate; 28 pM α-thrombin = 1350 cpm/plate; 28 pM α-thrombin + 1 mM dibutryl-cAMP = 1525 cpm/plate.

Model for Thrombin-Stimulated Lipid Metabolism in Fibroblasts. A likely interpretation of our data is illustrated in the model shown in Figure 6. Similar to the model proposed by McGowan and Detwiler (1986) or platelets, IIC9 fibroblasts contain two coupling mechanisms, R1 and R2, for the stimulation of lipid metabolism by α-thrombin. R1 mediates the stimulated release of arachidonic acid and involves the activation of N_i; R1 is inactivated by chymotrypsin and does not respond to γ-thrombin. Another similarity to the platelet model is that R2 is not sensitive to chymotrypsin and does respond to γ-thrombin. However, there are two important differences between the platelet and fibroblast model. First, in fibroblasts, the R1-mediated mechanism is coupled to the hydrolysis of polyphosphoinositides while the R2-mediated mechanism does not stimulate hydrolysis of polyphosphoinositides via phospholipase C. Second, in the fibroblast model, both R1 and R2 result in the elevation of cellular diacylglycerol. In platelets, only the R1-mediated model would be expected to elevate cellular diacylglycerol levels. As McGowan and Detwiler (1986) noted for platelets, R1 and R2 may be separate proteins, a single protein with two different activities, or a single protein that interacts wtih two different effectors.

ACKNOWLEDGMENTS

We thank Sheree Hiramoto and Drs. James Thompson, David Farrell, William Van Nostrand, and Steven Wagner for helpful discussions. We thank Laura Cho and Alice Lau for technical assistance. We thank Dr. Joseph Baldassare for helpful discussions and for assaying the ability of IAP to ribosylate IIC9 membrane proteins.

Registry No. IP1, 573-35-3; IP2, 47055-78-7; IP3, 88269-39-0; IP, 105182-27-2; AA, 506-32-1; thrombin, 9002-04-4.

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