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Evidence for a GTP-binding protein coupling thrombin receptor to PIP_2-phospholipase C in membranes of hamster fibroblasts

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Two different methods were used to study directly α-thrombin modulation of polyphosphoinositide breakdown in membranes prepared from Chinese hamster lung (CHL) fibroblasts. In the first one we labelled the lipid pool by incubating the intact cells with *myo*-[^{3}H]inositol prior to membrane isolation; in the other we used exogenous [^{3}H]PIP_2 with phosphatidylethanolamine (1:10) added as liposomes to freshly isolated membranes. A Ca^{2+}-dependent PIP_2 and PIP phospholipase C activity was characterized by measuring the rate of formation of inositol tris- and bisphosphate. Basal phospholipase C activity was stimulated up to 3-fold by GTP or GTP-γ-S. Of the two mitogens, α-thrombin and EGF, known to stimulate DNA synthesis in Chinese hamster fibroblasts, only α-thrombin is a potent activator of PIP_2 breakdown in intact cells. Consistent with this observation, α-thrombin but not EGF potentiated GTP-γ-S-dependent phospholipase C activity in membrane preparations. These results strongly support the hypothesis that a GTP-binding protein couples α-thrombin receptor to PIP_2 hydrolysis. Because both methods used to assay phospholipase C gave identical results, we conclude that the coupling is at the level of PIP_2-phosphodiesterase activity.

GTP-binding protein; Inositol trisphosphate; Cell-free system; Phospholipase C; α-Thrombin; (CHL fibroblast)

1. INTRODUCTION

Addition of α-thrombin to resting Chinese hamster lung (CHL) fibroblasts leads to rapid activation of PIP_2-phospholipase C [1]. The two products IP_3 and diacylglycerol generated by cleavage of phosphatidylinositol 4,5-P_2 serve as intracellular second messengers. They respectively mobilize calcium from intracellular stores and activate protein kinase C [2–4]. Utilizing either permeabilized cells [5,6] or freshly isolated membranes [7–11] several groups have demonstrated that phosphatidylinositol breakdown occurs via a guanine nucleotide-modulated mechanism. We have recently shown that pretreatment of intact CHL cells with pertussis toxin inhibited thrombin-induced PIP_2 hydrolysis as well as early mitogenic events [12]. This observation, similar to those made with mast cells [13] and neutrophils [14,15], provided the first indication that a GTP-binding protein (Gp) might be associated with the activation of PIP_2 breakdown in response to thrombin. Additional evidence was obtained from studies carried out with fluoroaluminate (AlF_4^-), a phosphate analog postulated to activate directly G-proteins [16,17]. Paris and Pouysségur [18] reported that AlF_4^- activated PIP_2-phospholipase C in CHL fibroblasts in a pertussis toxin-dependent manner. The present study demonstrates directly that the thrombin stimulation of PIP_2-phospholipase C is GTP-dependent.

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2. MATERIALS AND METHODS

2.1. *Materials*

Highly purified α-thrombin (2660 NIH units/mg) was generously provided by Dr J.W. Fenton ii (New York State Department of Health, Albany, NY). Mouse EGF was purified as described by Savage and Cohen [19]. *myo*-[2-^{3}H(n)]Inositol (10–20 Ci/mmol) and [^{3}H]PIP_2 (2 Ci/mmol) were from NEN. Phosphatidylethanolamine was obtained from Avanti Polar Lipids, and GTP-γ-S, GTP and PIP_2 from Sigma.

2.2. *Cell culture*

The cell line CCL39 from CHL fibroblasts was cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2/95% air. All experiments were performed on confluent cells grown in 100 mm dishes and arrested in G_0 by serum deprivation. Inositol lipids were labelled with [^{3}H]inositol (2 μCi/ml) during the 24 h of serum starvation in inositol-free DMEM.

2.3. *Membrane preparation*

Serum-starved CCL39 cells were washed twice with Ca^{2+}- and Mg^{2+}-free PBS and rinsed once with buffer A [20 mM Tris-maleate (pH 7.4) containing 0.5 mM EGTA and 0.5 mM EDTA]. Cells were harvested by scraping with a rubber policeman into buffer A and disrupted by Dounce homogenization (40 strokes). Efficiency of disruption was monitored by phase-contrast microscopy. The broken cell preparation was centrifuged at 500 rpm for 5 min in a Sorvall RC-5B at 4°C to remove nuclei and any unbroken cells. The supernatant was centrifuged at 12000 rpm for 15 min to obtain a crude pellet enriched in plasma membranes. This pellet was resuspended in buffer A containing 20 mM LiCl at a protein concentration of 2–3 mg/ml and used immediately for assays.

2.4. *Determination of polyphosphoinositide hydrolysis with membranes prelabelled in vivo*

Assay was initiated by adding 100 μg membrane protein to a reaction mixture containing Ca^{2+} and EGTA to adjust the free Ca^{2+} concentration (final volume 200 μl). This was carried out for the time indicated at 32°C in the presence or absence of GTP-γ-S and α-thrombin. The reaction was terminated by adding 250 μl of 20% perchloric acid. Reaction mixtures were kept at 4°C for 30 min and then centrifuged for 5 min in an Eppendorf microfuge. Acid-extracted [^{3}H]inositol phosphates were analyzed on AG1-X8 columns as described by Bone et al. [20].

2.5. *Preparation of [^{3}H]PIP_2-phosphatidylethanolamine (PE) vesicles*

Vesicles were prepared essentially as described by Irvine et al. [21]. Briefly, 40 nmol PIP_2, 400 nmol PE and 2.5×10^5 cpm [^{3}H]PIP_2, per ml of stock substrate required, were mixed and carefully evaporated. They were then resuspended in 20 mM Tris-maleate, pH 7.4, to yield 1 nmol [^{3}H]PIP_2 (30000 cpm) with 10 nmol PE per 40 μl aliquot, and sonicated.

2.6. *Assay for PIP_2-phosphodiesterase activity with exogenous substrate*

Membrane-associated PIP_2-phosphodiesterase activity was determined by measuring the amount of IP_3 produced from [^{3}H]PIP_2 added as exogenous substrate. Unless otherwise indicated each standard reaction tube contained membrane protein (40–50 μg), 20 mM Tris-maleate, pH 7.4, and 100 nM free Ca^{2+} (adjusted with Ca^{2+}/EGTA). Assays were run in the presence or absence of 100 μM GTP, 10 μM GTP-γ-S, 1 U/ml α-thrombin, or 10 ng/ml EGF as indicated. The reaction was initiated by the addition of 40 μl of the stock [^{3}H]PIP_2:PE (1:10) vesicle preparation in a final volume of 100 μl. The mixtures were incubated at 37°C for various times and the reaction terminated with 750 μl ice-cold $CHCl_3$:CH_3OH (1:2, v/v). [^{3}H]Inositol phosphates were extracted and analyzed on Dowex AG1-X8 columns (formate form). Essentially all of the radioactivity was recovered in the fraction containing IP_3.

2.7. *Protein determination*

Protein concentration was estimated by the procedure of Lowry et al. [22] using bovine serum albumin as the standard.

3. RESULTS

Fig.1 shows the formation of inositol tris- and bisphosphate by labelled membranes incubated at low Ca^{2+} concentration (150 nM). The basal rate of IP_3 and IP_2 production measured over 10 min is low. Addition of 10 μM GTP-γ-S stimulates IP_3 release up to 3-fold. This stimulation occurs rapidly since a 2-fold increase is already observed after 1 min. Addition of α-thrombin alone has little effect (not shown). However, it potentiates GTP-γ-S-induced IP_3 formation, an effect which is more pronounced at 1 min than at 10 min. Under the same conditions GTP-γ-S also stimulates, although with some lag, the release of IP_2 (fig.1). In contrast, no significant formation of IP_1 or of glycerophosphoinositol is detected upon stimulation with GTP-γ-S and α-thrombin. We have also investigated the effect of Mg^{2+} on the phosphodiesterase activity. This bivalent cation has been shown to be a potent activator of PIP_2 hydrolysis in some cell systems [8–11] whereas it appears to be an inhibitor in others [23]. In the presence of Mg^{2+} only IP_2 production increased in response to GTP-γ-S, suggesting that an Mg^{2+}-dependent IP_3 phosphatase was activated (not shown).

To characterize further α-thrombin stimulation of IP_3 production we examined the effect of increasing Ca^{2+} concentrations on the phosphodiesterase activity using exogenous [^{3}H]PIP_2 as a substrate. Fig.2 illustrates the Ca^{2+}-dependent rise in IP_3 level in response to GTP-γ-S. Maximal stimulation is observed with 100 nM free Ca^{2+}, a result similar to that obtained with labelled membranes (not shown). The potentiating effect of α-thrombin also occurs in the same range of low Ca^{2+} concentration (50–200 nM). At higher Ca^{2+} levels, little if any stimulation with α-thrombin and guanine nucleotide is observed, presumably because PIP_2 hydrolytic activity was already fully stimulated. A similar low Ca^{2+} requirement for stimulation of IP_3 formation has been described in other cell-free systems [8,10,11,24,25]. Thus we decided to analyze growth factor stimulation of PIP_2-phosphodiesterase activity, in a buffer containing 100–150 nM free Ca^{2+}.

Fig.3 illustrates the time course of hydrolysis of exogenous [^{3}H]PIP_2. As previously noted with prelabelled membranes, stimulation of IP_3 formation in response to both GTP-γ-S and α-thrombin is very rapid, and α-thrombin alone is totally inef-

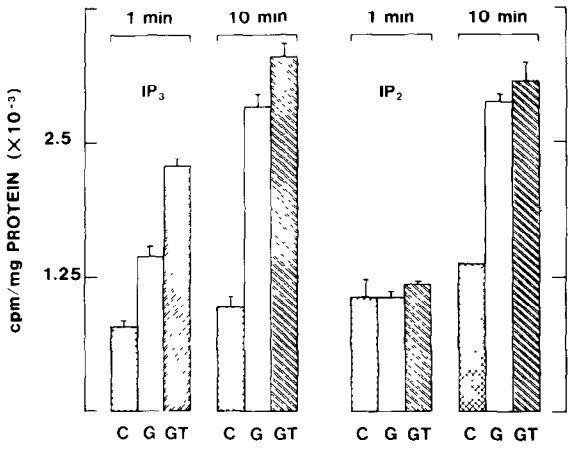

Fig.1. Inositol phosphate release in response to GTP-γ-S and α-thrombin in prelabelled membranes from G_0-arrested CCL39 cells. [^{3}H]Inositol-labelled membranes were incubated for the indicated times under control conditions (C), with 10 μM GTP-γ-S (G) or 10 μM GTP-γ-S and α-thrombin (1 U/ml) (GT). Calcium free concentration was adjusted to 150 nM using a Ca^{2+}/EGTA buffer. Results are expressed as cpm [^{3}H]IP_3 and [^{3}H]IP_2 per mg protein.

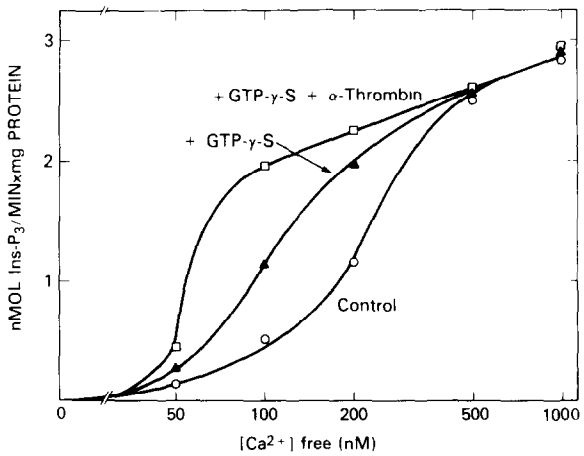

Fig.2. Effect of calcium concentration on [^{3}H]PIP_2 hydrolysis. Membranes isolated from growth-arrested CCL39 cells were incubated for 2 min at 37°C under control conditions (○—○), in the presence of 10 μM GTP-γ-S (▲—▲), or GTP-γ-S plus α-thrombin (□—□) (10 μM and 1 U/ml, respectively). The various free Ca^{2+} concentrations were adjusted as described in section 2.

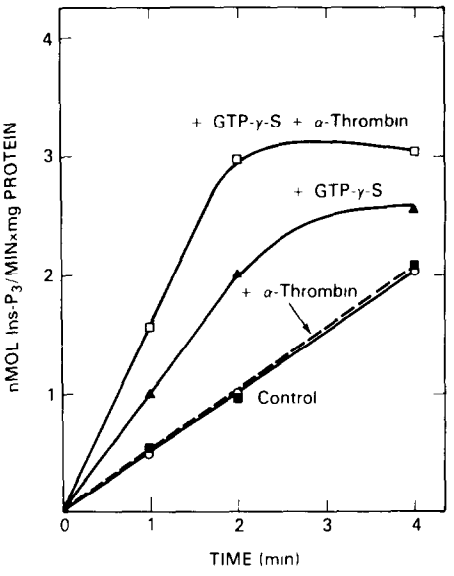

Fig.3. Time course of [^{3}H]PIP_2 hydrolysis. Membranes from growth-arrested CCL39 cells were incubated in a 100 nM Ca^{2+} free buffer under control conditions (○—○), in the presence of α-thrombin (■---■) (1 U/ml), 10 μM GTP-γ-S (▲—▲) or GTP-γ-S plus α-thrombin (□—□) (10 μM and 1 U/ml, respectively) for the times indicated.

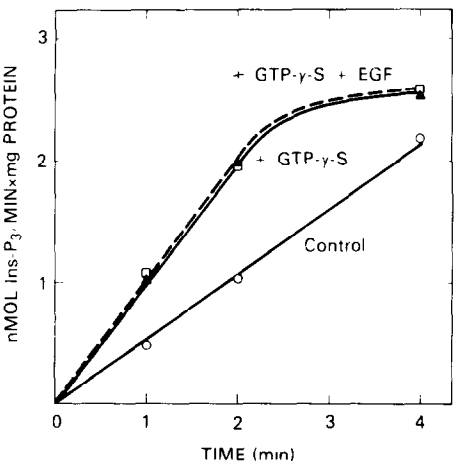

Fig.4. Time course of PIP_2-phosphodiesterase activity under basal conditions (○—○) or in the presence of 10 μM GTP-γ-S (▲—▲) and GTP-γ-S plus EGF (□—□) (10 μM and 10 ng/ml). PIP_2-phosphodiesterase activity was measured as described in the legend to fig.3.

fective. The use of high concentrations of GTP-γ-S results in a maximal activation of phospholipase C which prevents any further potentiation by α-thrombin. GTP, although less efficient, was also capable of stimulating IP_3 formation at 100 μM. When [^{3}H]PI was used instead of [^{3}H]PIP_2 as an exogenous substrate, no hydrolysis was observed. This suggests that α-thrombin directly stimulates PIP_2 and not PI-phosphodiesterase activity. PI hydrolysis could only be detected only at a much lower pH (5.5) suggesting the presence of a different phosphodiesterase activity in membrane preparations.

In previous studies with intact cells, we reported that EGF, a potent mitogen for CHL fibroblasts, failed to induce phosphoinositide breakdown [26]. In agreement with this finding, we show here that EGF does not potentiate GTP-γ-S-induced IP_3 formation in membranes (fig.4). Therefore, this membrane assay system is specific for ligand receptors coupled to PIP_2-phospholipase C.

4. DISCUSSION

Here, we have characterized a specific Ca^{2+}-sensitive PIP_2-phospholipase C activity in membranes of CHL fibroblasts. A major point of interest is that α-thrombin, a potent agonist of PIP_2 breakdown in intact CHL cells, had virtually no effect on IP_3 or IP_2 release when added alone to crude membrane preparations. Its stimulatory action could only be detected in the presence of GTP or GTP-γ-S. In contrast, EGF did not enhance GTP-dependent phospholipase C activity, a result consistent with the notion that it exerts its action through a different signal transduction pathway [26]. Therefore, it is clear that guanine nucleotides play a direct role in mediating the action of thrombin. This finding, together with the inhibitory effect of pertussis toxin on thrombin-induced PIP_2 breakdown in CHL cells [12], suggests the existence of a GTP-binding protein (Gp) coupling thrombin receptor to phospholipase C. A similar mechanism has been proposed for the action of various effectors on PIP_2-phospholipase C [5–11].

This Gp transducer protein which appears to be a target for pertussis toxin in some cells [27–30] but not in others [24,25,31] has not yet been identified. Recent studies have shown that the two

GTP-binding proteins G_o or G_i added to membranes from pertussis toxin-treated cells restore hormonal activation of phospholipase C [32]. However, this result does not prove that G_o or G_i is the G-protein involved in receptor/phospholipase C coupling. Nonetheless it validates the use of cell-free systems, similar to that presented here, to assess directly which protein of the large family of GTP-binding proteins, including those of the *ras* family, has the capacity to activate PIP_2-phospholipase C. For instance, it has been suggested that N-ras increases the efficiency of coupling between growth factor receptor and phospholipase C [33]. More direct evidence is now required to validate this interesting hypothesis.

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