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Direct evidence for two distinct G proteins coupling with thrombin receptors in human neuroblastoma SH-EP cells

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Abstract

Thrombin receptor-G protein coupling was investigated in the human epithelial neuroblastoma cell line, SH-EP. In these cells, both α-thrombin and thrombin receptor peptides, SFLLRNP (one-letter amino-acid code), which are newly exposed following cleavage by α-thrombin, stimulated GTPase activity about 2-fold over basal activity. Pertussis toxin treatment only partially attenuated α-thrombin- and SFLLRNP-stimulated GTPase activity by 50%, whereas antibody raised against synthetic heptapeptide SFLLRNP blocked α-thrombin-stimulated phosphoinositide hydrolysis more than 80%. Immunoprecipitation studies using this antibody showed that both G_i2, a subtype of guanine nucleotide-binding regulatory proteins (G proteins) mediating inhibition of adenylyl cyclase, and G_q/G_{11}, a G protein mediating stimulation of phospholipase C, were activated by α-thrombin. These data suggest that in these cells the thrombin receptor activates pertussis toxin-sensitive and pertussis toxin-insensitive G proteins simultaneously and directly couples to G_i2 and G_q/G_{11}, which mediate different signaling pathways.

Keywords: Thrombin receptor; Phosphoinositide hydrolysis; G-protein; GTPase

1. Introduction

Thrombin receptors belong to a family of seven transmembrane spanning receptors, which couple to guanine nucleotide-binding regulatory proteins (G proteins) (Vu et al., 1991). Thrombin binding leads to cleavage of its receptor between Arg^{41} and Ser^{42}. The newly formed N-terminal moiety starting from SFLLRNP acts as a tethered ligand to activate the receptor. Thrombin and thrombin receptor agonist peptides inhibit cyclic AMP production (Vassallo et al., 1992), and stimulate phosphoinositide hydrolysis, intracellular Ca^{2+} mobilization (Huang et al., 1991), mitogen-activated protein kinase, S6 kinase (Kahan et al., 1992), tyrosine kinase (Gordon and Brugge, 1989) and DNA synthesis (Ogino and Costa, 1992b; Chambard et al., 1987). Recently the G_q and G_{12} family was shown to be activated via thrombin as well as thromboxane A_2 receptors, whereas G_i was not activated in human platelet membranes (Offermanns et al., 1994).

The human epithelial neuroblastoma cell line, SH-EP, expresses thrombin receptors. These receptors mediate phosphoinositide hydrolysis activity (Ogino and Costa, 1992b), and, unlike platelets, they coupled to G_i which was ADP-ribosylated by cholera toxin in the presence of thrombin and SFLLRNP (data not shown).

This cell line, therefore, provides the opportunity of addressing the question of whether thrombin receptors in SH-EP cells can directly couple to two functionally distinct G proteins. In this paper, we have adressed this question of measurement of GTPase activity and immunoprecipitation of the two G proteins.

2. Materials and methods

2.1. Cell culture, treatment, harvesting and membrane preparation

The cell line SH-EP was obtained from Dr. June Biedler (Sloan-Kettering Institute, NY, USA) at passages 48. Cells were cultured as described before (Ogino and Costa, 1992a). Upon reaching confluency, medium was replaced

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with new growth medium containing 2% fetal calf serum for 2 days. At the final 12 h, cells were treated with or without pertussis toxin (Funakoshi, Tokyo, Japan) at a final concentration of 20 ng/ml, which was sufficient to inactivate G_i α completely in these cells (Ogino and Costa, 1992a). At the end of treatment, cells were cooled on ice and removed by scraping. After centrifugation, cell pellets were stored at −70°C.

Membranes were prepared as described (Vachon et al., 1987). The final pellets were resuspended in sucrose-free buffer to obtain 1–2 mg of protein/ml for assay of GTPase and 3–5 mg of protein/ml for immunoprecipitation. These samples were stored at −70°C.

2.2. GTPase assay

GTPase activity was assayed according to Cassel and Selinger (1976) with modification (Vachon et al., 1987). The reaction mixture contained 50 mM Hepes/Tris (pH 7.5), 0.2 mM EGTA, 0.2 mM dithiothreitol, 10 mM $MgCl_2$, 0.5 mM ATP, 1 mM AppNHp, 5 mM phosphocreatinine, 50 units/ml creatine phosphokinase, 200 nM GTP which included 10^6 cpm of [γ-^{32}P]GTP (Daiichi Radioisotope, Tokyo, Japan) and a vehicle or stimulants in a final volume of 80 μl. After equilibration at 37°C for 2 min, the reaction was started by adding the membranes in a volume of 20 μl and then incubated at 37°C for 10 min. The reaction was stopped by adding 100 μl of ice-cold 40 mM H_3PO_4 and the tubes were placed on ice. 750 μl of an ice-cold suspension of 5% activated charcoal in 20 mM H_3PO_4 was added and the tubes were then centrifuged at 4°C for 10 min. A 650 μl aliquot of supernatant fraction was assayed for radioactivity in a liquid scintillation counter. The low K_m GTPase activity was calculated by subtraction of the amount of hydrolysis observed with 50 μM GTP from that measured with 200 nM GTP.

2.3. Antibody

SFLLRNP was synthesized by solid phase synthesis using t-Boc-amino acids and conjugated to keyhole limpet hemocyanin with glutaraldehyde, and injected into rabbits. Antisera were affinity-purified on an Affi-Gel 10 column (Bio-Rad Japan, Tokyo, Japan) containing immobilized peptide.

2.4. Stimulation of phosphoinositide hydrolysis

The extent of phosphoinositide hydrolysis was determined by incubating cells for 48 h with [^{3}H]*myo*-inositol (Amersham Japan, Tokyo, Japan) to label intracellular phosphoinositides (Ogino and Costa, 1992a). The cells were washed two times in PBS (phosphate-buffered saline) at 37°C for 10 min. Cells were then incubated either with 50 μg/ml of normal rabbit antibody or an affinity purified anti-SFLLRNP antibody in the reaction buffer which contained 10 mM LiCl for 15 min. Reaction was started by adding a vehicle or 100 pM α-thrombin (final concentration) (kind gift from Dr. J.W. Fenton, II), a concentration that results in about 50% maximal hydrolysis of phosphoinositides in these cells (Ogino and Costa, 1992b), and was incubated at 37°C for 15 min. The extent of stimulation was expressed as radioactivity of total count of radiolabeled inositol mono- and bisphosphate that was eluted from the column in single fractions as described previously (Ogino and Costa, 1992b).

2.5. Immunoprecipitation and immunoblotting

Immunoprecipitation was performed according to Mateisic et al. (1989). 600 μg of membrane suspension was placed on ice, and 1 M $MgCl_2$ was added to obtain a final concentration of 3 mM. A vehicle or 10 times concentrated α-thrombin were added to obtain a final concentration of 10 nM and samples were incubated on ice for 30 min. Membranes were collected by centrifugation and resuspended in the original volume of 10 mM Tris (pH 7.5), 1 mM EDTA, 3 mM $MgCl_2$, 0.1% digitonin (Wako, Osaka, Japan), 0.02% cholate (Dojindo, Kumamoto, Japan) (TEDC buffer). Membranes were centrifuged at 27 000 × *g* for 15 min, and the pellets were resuspended in the original volume of TEDC buffer supplemented with 1% digitonin and 0.2% cholate. After a 30-min incubation on ice, the soluble fraction was obtained after a 40 000 × *g* centrifugation. Antisera (5 μl) and 1 M of NaCl were added to the soluble extract to give a final concentration of 50 μg/ml and 150 mM, respectively and incubation was conducted for 3 h at 4°C. After this period, 50 μl of protein A-agarose (25 μl of packed gel, Zymed, San Francisco, CA, USA) were added and the incubation was continued for a further 2 h. The immunocomplexes were collected as Protein A-agarose pellets by centrifugation at 1000 × *g* for 5 min at 4°C and the pellets were washed three times in Ca^{2+}/Ma^{2+}-free PBS. The final pellets were resuspended in Laemmli sample buffer and placed in a boiling water bath for 3 min. Sodium dodecyl sulfate-polyacrylamide electrophoresis was performed using resolving gels containing a linear gradient from 8–12% acrylamide (Ogino et al., 1992). Proteins were electrotransferred to Immobilon membranes (Millipore, Bedford, MA, USA). Color development procedure was used to detect immunoprecipitated G proteins. One microgram per milliliter of anti-G protein antibodies (RM, AS and QL which recognize α subunit of G_s, G_i1/G_i2 and G_{11}/G_q, respectively) was used as a first antibody and alkaline phosphatase-conjugated anti-rabbit antibody (Promega, Madison, WI, USA) as a second antibody. All anti-protein antibodies were a kind gift from Dr Andrew Shenker and their immunoblot characterics are described elsewhere (Simonds et al., 1989; Shenker et al., 1991).

Statistical significance was evaluated with unpaired Student's *t*-test.

3. Results

3.1. Stimulation of GTPase activity by α-thrombin and SFLLRNP

Fig. 1 shows that both 10 nM α-thrombin and 100 μM SFLLRNP were potent simulators of GTPase activity in SH-EP cell membranes. The extent of the stimulating activities of these agonists was similar and there was no statistical significant difference between these agonists. Absolute magnitude of GTPase activity induced by either α-thrombin and SFLLRNP was 1.9–2.4-fold over basal activity ($n = 4$). Although we have shown the presence of many mitogenic receptors in these cells (Ogino and Costa, 1992a), mitogens such as bradykinin, endothelin and angiotensin II could not stimulate GTPase activity (Ogino and Costa, unpublished observation).

Effects of anti G protein antibodies on basal and agonist-stimulated GTPase activities were performed by the addition of 10 μg/ml of AS or QL antibodies to the assay system. Neither AS nor QL antibodies reduced GTPase activity in the membranes whether these were treated with pertussis toxin or not (data not shown).

We have previously shown that an overnight incubation with 10 ng/ml of pertussis toxin is sufficient to ADP-ribosylate almost 100% of the G_i2 in SH-EP cells (Ogino and Costa, 1992a). This was reconfirmed by scanning with densitometer (data not shown). Pertussis toxin treatment reduced the GTPase activity of not only the basal activity but also the agonist-stimulated activity. Agonist-stimulated GTPase activities in pertussis toxin-treated membranes

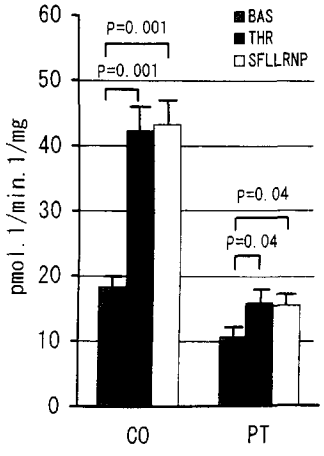

Fig. 1. Effect of pertussis toxin treatment of intact cells on thrombin- and SFLLRNP-mediated stimulation of GTPase activity. Cells were treated with (PT) or without (CO) pertussis toxin as detailed in Materials and methods. GTPase activity in the membrane was measured in the absence (BAS) or presence of 10 nM α-thrombin (THR) and 100 μM SFLLRNP (SFLLRNP). In untreated membrane, α-thrombin and SFLLRNP stimulated GTPase activity 2.3-fold and 2.4-fold over basal activity, respectively ($P = 0.001$). In pertussis toxin-treated membrane, α-thrombin and SFLLRNP stimulated GTPase activity 1.5-fold and 1.4-fold over basal activity, respectively ($P = 0.04$). There was no statistical significance between α-thrombin and SFLLRNP. Data are mean ± S.E. values of triplicate determinations.

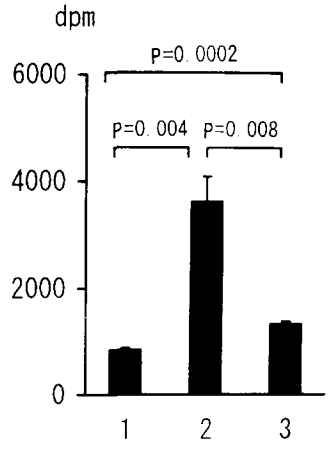

Fig. 2. Effect of anti-SFLLRNP antibody on thrombin-stimulated phosphoinositide hydrolysis. Treatment of cells and antibody concentration were described in Materials and methods. Phosphoinositide hydrolysis was measured in the presence of preimmunized rabbit antibody (1), preimmunized rabbit antibody and 100 pM α-thrombin (2) and SFLLRNP antibody and 100 pM α-thrombin (3). 100 pM α-thrombin stimulated phosphoinositide hydrolysis 4.2-fold ($P = 0.004$). This activity was attenuated by 82% in the presence of SFLLRNP antibody ($P = 0.008$). Data are mean ± S.E. values of triplicate determinations.

were 1.27–1.50-fold over basal activity ($n = 3$), suggesting that the pertussis toxin insensitive G protein(s) may be involved in thrombin receptor-mediated GTPase stimulation.

3.2. Phosphoinositide hydrolysis in the presence of anti-SFLLRNP antibodies

Phosphoinositide hydrolysis was used to characterize affinity-purified anti-SFLLRNP antibodies. As shown in Fig. 2, 82% reduction of 100 pM α-thrombin-stimulated phosphoinositide hydrolysis in the presence of 50 μg/ml anti-SFLLRNP antibody occurred when compared with that in the presence of the same concentration of affinity purified preimmunized-antibody ($n = 2$; $P = < 0.01$). The removal of antibodies before the addition of α-thrombin showed only a 34% reduction of phosphoinositide hydrolysis activity, suggesting that the antibody is specific for thrombin receptors (data not shown).

3.3. Immunoprecipitation study

We have characterized immunoreactive G proteins in SH-EP cells. The antibody, RM, recognized the 46 kDa and 44 kDa immunoreactive Gs α bands, the antibody QL recognized the 42 kDa G_q/G_{11} α band and the antibody AS recognized 40 kDa G_i2 α band in these cells (data not shown). Fig. 3 shows the extent of association of G_q/G_{11} α and G_i2 α with thrombin receptors in the absence and presence of agonists. Thrombin stimulation induced the association of both 42 kDa G_q/G_{11} α (panel A) and 40 kDa G_i2 α (panel B) with the receptor (indicated as right arrows). Because receptor cleavage by thrombin forms

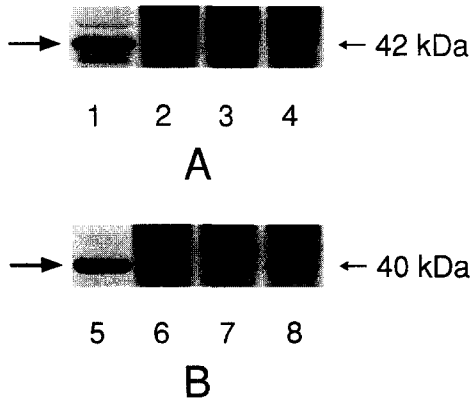

Fig. 3. Immunological identification of G α subunits in thrombin receptor-G protein complexes. Sixty μg of cell membranes are used to show native G proteins (lane 1, Lane 5). Others show immunoprecipitated samples as described in Materials and methods. Preimmunized rabbit antibody was used in lane 2 and lane 6. SFLLRNP antibody was used to precipitate receptor-G protein complexes in the absence (lane 3, lane 7) or in the presence of 10 nM α-thrombin (lane 4, and lane 8). *Top*: QL antibody (A) was used to detect G_q/G_{11}. Left arrow points native G_q/G_{11} molecules. A 42 kDa G_q/G_{11} band newly appears only in the presence of α-thrombin. *Bottom*: AS antibody (B) was used to detect G_i2. Left arrow points native G_i2. A 40 kDa G_i2 band that newly appears only in the presence of α-thrombin.

tethered receptors, retention of the cleaved fragment may be sufficient to maintain receptor stimulation and G protein coupling even after thrombin has been removed by washing. An alternative possibility of this sustained receptor-G protein coupling is an inter-receptor stimulation (Chen et al., 1994).

There was no enhancement of G_s α immunoreactivity, although the 46 kDa band was slightly hidden by immunegroblin G heavy chain (data not shown).

4. Discussion

Thrombin is a potent stimulator of phosphoinositide hydrolysis and DNA synthesis in SH-EP cells (Ogino and Costa, 1992b) and in CCL39 cells (Chambard et al., 1987). But α-thrombin-stimulated phosphoinositide hydrolysis was not affected by pertussis toxin treatment in SH-EP cells. In addition, cholera toxin catalyzed ADP ribosylation of G_i α in the presence of thrombin and SFLLRNP in these cells (Ogino and Costa, data not shwon). These suggest that the roles of β γ subunit to activate phospholipase C and receptor-G protein coupling are different from those of platelet in these cells (Birnbaumer, 1992; Bristol and Rhee, 1994; Offermanns et al., 1994). We have designed two independent experiments to clarify that these G_q/G_{11} α- and G_i α-mediated events occur independently through thrombin receptor-G protein coupling after agonist stimulation.

First, these agonists stimulated pertussis toxin-sensitive and -insensitive GTPase activities. The pertussis toxin concentration used here is sufficient to ADP-ribosylate almost 100% of G_i, suggesting that this small but significant increase originates from pertussis toxin-insensitive G protein(s). EC_{50} of SFLLRNP to activate GTPase is about 2 μM in both pertussis toxin-treated and non-treated membranes (paper in preparation) and this EC_{50} is almost the same as that of phosphoinositide hydrolysis and thymidine incorporation by SFLLRNP in intact cells (Nose et al., 1993). Although α_2-adrenoceptors showed a biphasic activation of G_i and G_s with different EC_{50} values and was supposed to have secondary pathway for G_s activation (Eason et al., 1992), the lack of dissociation between EC_{50} of pertussis toxin-sensitive and -insensitive GTPase activity suggests a direct receptor-G protein coupling in SH-EP cells.

There is growing evidence that antibodies against the C-terminal regions of G α subunits can effectively block these G protein-mediated effector functions (Eason et al., 1992; Simonds et al., 1989; Shenker et al., 1991; Gutowski et al., 1991). We were unable to show a reduction of GTPase activity in the presence of AS and QL antibodies. However, it is difficult to conclude that the lack of effect of the AS antibodies on GTPase activity implies the presence of new G proteins which are the substrate of pertussis toxin. One possible explanation for this pertussis toxin-insensitive element is that α_{14} and α_{16} are involved in agonist-stimulated GTPase activity, because pertussis-toxin-insensitive α_q, α_{11}, α_{14} and α_{16} directly activate phospholipase C-β_1 and stimulate phosphoinositide hydrolysis (Birnbaumer, 1992).

Second, immunoprecipitation studies demonstrated that thrombin receptors directly activated both G_i2 and G_q/G_{11} but not G_s. This result coincides with thrombin-stimulated cellular responses. It also suggests that the G proteins responsible for GTPase activation may be G_i2 and G_q/G_{11}.

Receptor-G_i coupling has been studied through immunoprecipitation analysis and [^{32}P]γ-azidoanilido GTP binding to receptors. Human α_2-adrenoceptors transfect into Rat 1 fibroblast coupled to G_i2 and G_i3 by using cholera toxin-catalyzed ADP-ribosylation and immunoprecipitation (Milligan et al., 1991). Muscarinic acetylcholine receptors associated with G_i1, G_i2 and G_o with different affinities in different tissues (Mateisic et al., 1991). After the purification (Taylor et al., 1990), cloning (Strathmann and Simon, 1990) and characterization of G_q (Smrcka et al., 1991), receptor-G_q coupling has also been demonstrated. Vasopressin receptor stimulation enhanced [^{32}P]γ-azidoanilido GTP labeling of G_q in rat plasma membrane (Wange et al., 1991). Thrombin and thromboxane A_2 receptors stimulation accelerated [^{32}P]γ-azidoanilido GTP coupling to the G_q and G_{12} family (G_{12}, G_{13}) in platelet membranes (Offermanns et al., 1994). In the last paper, thrombin receptors did not couple G_i in spite of its presence in membranes. Analogous to the situation with muscarinic acetylcholine receptors in brain and heart (Mateisic et al., 1991), the diversity of thrombin

receptor-G protein coupling may depend on the cell type used.

Taken together the data presented here, we suggest that the thrombin receptor directly couples to two distinct G proteins which link to different signaling pathways.

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