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Treatment of human platelets with trypsin, thrombin, or collagen inhibits the pertussis toxin-induced ADP-ribosylation of a 41-kDa protein

(GTP-binding proteins/phospholipase C/inositol phospholipids/protein kinase C/platelet activation)

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ABSTRACT **Permeabilization of human platelets with saponin (15–25 μg/ml) allows the determination of the ADP-ribosylation of a 41-kDa protein by pertussis toxin. The ADP-ribosylated protein is present in the particulate fraction. ADP-ribosylation of the 41-kDa protein increases for 20 min; it is not affected by indomethacin, prostacyclin, and 1,2-diacylglycerols but is inhibited by 1 mM Ca^{2+} and phorbol esters. Treatment of platelets with trypsin, thrombin, or collagen before saponin addition precludes subsequent pertussis toxin-induced ADP-ribosylation of the 41-kDa protein. The effect of trypsin or thrombin is blocked by soybean trypsin inhibitor and leupeptin. Trypsin proteolytically cleaves the ADP-ribosylated 41-kDa protein to an ADP-ribosylated fragment slightly smaller than 20 kDa. The results suggest that a modification of a guanine nucleotide-binding regulatory protein is associated with the actions of trypsin, thrombin, and collagen on platelet activation.**

Two guanine nucleotide-binding regulatory proteins, G_s (also called N_s) and G_i (N_i), modulate the stimulation and inhibition of adenylate cyclase, respectively (1–13). These proteins contain α, β, and γ subunits. Cholera toxin catalyzes ADP-ribosylations of the α subunit (α_s, 45 kDa) of G_s (1–10), whereas pertussis toxin ADP-ribosylates the α subunit (α_i, 41 kDa) of G_i (11–15). Pertussis toxin-catalyzed ADP-ribosylation inhibits the ability of G_i to mediate receptor-coupled inhibition of adenylate cyclase (10). Treatment of various cell types with pertussis toxin causes inhibition of agonist-mediated responses such as chemotaxis; secretion; increases in intracellular free Ca^{2+}, superoxide production, cGMP phosphodiesterase, and cAMP phosphodiesterase; liberation of arachidonic acid from phospholipids; and degradation of the inositol phospholipids (16–25).

Platelets lack cell surface receptors for pertussis toxin (10), which precludes the study of its effect on platelet activation. However in platelet membranes, ADP-ribosylation of α_i has been demonstrated (13). Thrombin activates G_i in these membranes, with subsequent inhibition of adenylate cyclase (26). Platelets permeabilized with saponin have been used to study the effect of molecules that normally do not penetrate the cell, such as inositol 1,4,5-trisphosphate (27–29). Platelet permeabilization with saponin allows pertussis toxin penetration, which permits ADP-ribosylation of α_i. We have observed that stimulation of platelets with trypsin, thrombin, or collagen before addition of saponin inhibits the effect of pertussis toxin on the ADP-ribosylation of α_i.

EXPERIMENTAL PROCEDURES

Materials. Pertussis toxin (islet-activating protein) and cholera toxin were from List Biological Labs, Campbell, CA. [*adenylate*-^{32}P]NAD was from New England Nuclear. Thrombin, calcium ionophore A23187 (calimycin), phorbol 12,13-dibutyrate, indomethacin, NAD, soybean trypsin inhibitor, and leupeptin were from Sigma. Trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) was from Worthington. Collagen (1 mg of suspended equine collagen fibrils per ml of isotonic glucose solution) was from Hormon-Chemie, Munchen, F.R.G. Prostacyclin was supplied by Wellcome. 1,2-Didecanoylglycerol and 1,2-dioctanoylglycerol were from Nova Biochem, Läufelfingen, Switzerland, and saponin was from Fisher. Guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) was from Boehringer Mannheim.

Isolation of Human Platelets. Blood (100 ml) was obtained from healthy human volunteers (age 20–45 years) who had not been medicated in the previous 3 weeks. Blood was mixed with 0.20 volume of ACD buffer (85 mM trisodium citrate/111 mM dextrose/71 mM citric acid, pH 5.5) to prevent coagulation. Platelet-rich plasma was obtained by centrifugation at 200 × *g* for 20 min; it was further centrifuged at 250 × *g* for 15 min. The platelet pellet obtained was washed once in 30 ml of a modified Hepes-buffered Tyrode's solution (134 mM NaCl/12 mM $NaHCO_3$/2.9 mM KCl/0.36 mM NaH_2PO_4/1 mM $MgCl_2$/10 mM glucose/10 mM Hepes, pH 7.4) containing 5 ng of prostacyclin per ml, and platelets were resuspended in the same buffer without prostacyclin, at a concentration of 9.0×10^8 per ml, as determined with a Coulter counter 2F.

[^{32}P]ADP-Ribosylation with Pertussis Toxin in Platelets Permeabilized with Saponin. ADP-ribosylation by pertussis toxin was determined from the incorporation of ^{32}P from [*adenylate*-^{32}P]NAD into membrane proteins of platelets permeabilized with saponin at 20 μg/ml. The standard ADP-ribosylation reaction was carried out in 0.1 ml that contained 0.085 ml of the platelet suspension. Final concentrations of the reaction-mixture constituents were as follows: saponin, 20 μg/ml; pertussis toxin, 5 μg/ml; NAD, 20 μM; [*adenylate*-^{32}P]NAD, 10 μCi/ml (specific activity ≈ 707 Ci/mmol; 1 Ci = 37 GBq); ATP, 1 mM; $MgCl_2$, 1.5 mM; EDTA, 1 mM; dithiothreitol, 1 mM; thymidine, 10 mM; platelets, 7.7×10^8 per ml. Incubations were carried out at 37°C in a shaking incubator bath for 1–60 min. Ten- or twenty-minute experiments were most common. In some experiments, platelets were incubated with trypsin, thrombin, collagen, chymotrypsin, 1,2-didecanoylglycerol, phorbol 12,13-dibutyrate, or GTP[γ-S] for up to 5 min before ADP-ribosylation in the presence of saponin. When trypsin was used, a trypsin inhibitor (soybean trypsin inhibitor or leupeptin) was added before addition of saponin. Reactions were stopped by centrifugation for 90 sec in a Beckman Microfuge B, the supernatant was discarded, the

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Abbreviations: G_i and G_s, guanine nucleotide-binding regulatory proteins that respectively inhibit and stimulate adenylate cyclase; α_i and α_s, α subunits of G_i and G_s; GTP[γ-S], guanosine 5′-[γ-thio]-triphosphate.

pellet was resuspended in 0.1 ml of Laemmli sample buffer (30), and the proteins were separated in a sodium dodecyl sulfate/11% polyacrylamide gel (27, 28). In some experiments, reactions were stopped by adding trichloroacetic acid to a final concentration of 20% (wt/vol), followed by centrifugation. Gels were stained with Coomassie brilliant blue and dried; radioactivity was determined by autoradiography.

G_i was isolated from rat brain membranes according to the method of Bokoch et al. (11, 12). The identity of G_i was confirmed by ADP-ribosylation induced by pertussis toxin, GTP[γ-^{35}S] binding activity, and the apparent size of the protein in $NaDodSO_4$/polyacrylamide gel electrophoresis (see Fig. 2).

RESULTS

Pertussis Toxin Induces ADP-Ribosylation of a 41-kDa Protein in Human Platelets Permeabilized with Saponin. Saponin at 15 μg/ml permits permeabilization of human platelets to pertussis toxin (5 μg/ml), as determined by ADP-ribosylation of a 41-kDa protein (Fig. 1). ADP-ribosylation is maximized with 20–25 μg of saponin per ml (Fig. 1). At 20 μg of saponin per ml, pertussis toxin induces maximal ADP-ribosylation at concentrations of 5–50 μg/ml (data not shown). ADP-ribosylation is not observed without saponin. Under the same conditions, thrombin (2–10 units/ml) does not catalyze ADP-ribosylation of platelet proteins (Fig. 1).

Fig. 2 shows that the ADP-ribosylated protein from human platelets treated with pertussis toxin has the same apparent molecular mass as the 41-kDa α_i subunit of G_i isolated from brain membranes (11, 12).

Another GTP-binding protein, G_o, has recently been isolated from brain tissue (31). Its α subunit, α_o, has a molecular mass of 39 kDa and is also ADP-ribosylated by pertussis toxin. Discrimination of the α_i or α_o subunits is difficult; the ADP-ribosylated protein in platelets could be either α_i or α_o. Collagen (100 μg/ml) or thrombin (1 unit/ml) does not change the pertussis toxin-induced ADP-ribosylation of the α_i or α_o subunits (data not shown) isolated from brain membranes (11, 12).

The ADP-ribosylated platelet protein is different from the phosphorylated protein that appears in stimulated platelets as a consequence of protein kinase C activation (27, 28). It has commonly been recognized as a 40-kDa protein (27, 28), but we find that its molecular mass is higher than that of α_i. The molecular mass of the protein phosphorylated by protein kinase C in stimulated platelets is indicated as 47 kDa on Fig. 2. The figure also shows that the α_s subunit ribosylated by cholera toxin has an apparent molecular mass slightly higher than that of the α_i subunit.

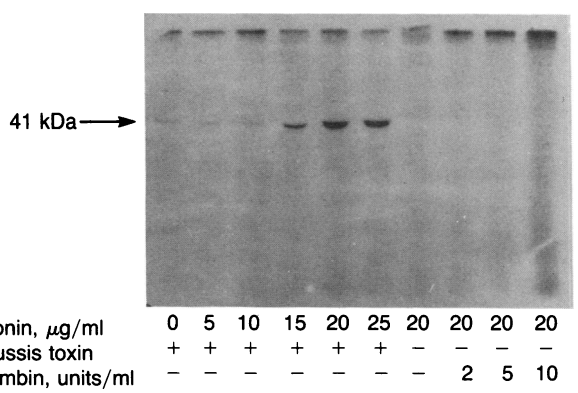

FIG. 1. Pertussis toxin-induced ADP-ribosylation of a 41-kDa protein in human platelets. Platelets were incubated for 20 min in the presence of various concentrations of saponin with pertussis toxin (5 μg/ml) or thrombin.

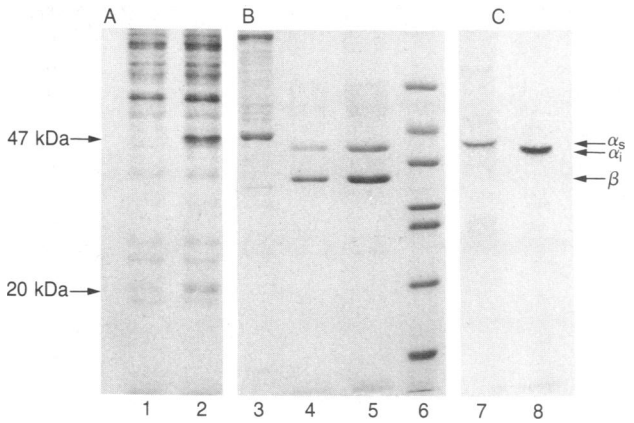

FIG. 2. Electrophoretic mobility of the ADP-ribosylated protein from pertussis toxin-treated platelets. (*A*) Autoradiograph. Platelets were prelabeled with [^{32}P]phosphate and then were incubated without thrombin (lane 1) or with thrombin (lane 2). Among the major labeled proteins in thrombin-stimulated platelets are a 47-kDa species (often identified as a 40-kDa protein), phosphorylated by protein kinase C, and a 20-kDa species that is the substrate for myosin light chain kinase. (*B*) Coomassie blue-stained gel. Lane 3: platelet proteins; the major band is actin. Lanes 4 and 5: α_i and β subunits of G_i prepared from brain membranes. Lane 6: protein standards (from top to bottom: 66, 45, 36, 29, 24, 20.1, and 14.2 kDa); note that these standards do not apply to *A*. (*C*) Autoradiograph. Lane 7 shows the cholera toxin (10 μg/ml)-induced ADP-ribosylation of the α_s subunit in platelets permeabilized with saponin for 3 hr. Lane 8 shows the pertussis toxin-induced ADP-ribosylation of the α_i subunit in platelets permeabilized with saponin for 20 min. The [α-^{32}P]ADP-ribosylated protein (lane 8) that is formed in platelets treated with saponin and pertussis toxin has the same molecular mass (41 kDa) as the α_i subunit purified from brain membranes (lanes 4 and 5). Lanes 4 and 5 also show the β subunit (35 kDa). Cholera toxin ADP-ribosylates the α_s subunit of G_s in platelets (lane 7). Both α_i (lanes 4, 5, and 8) and α_s (lane 7) differ from the protein that is phosphorylated by protein kinase C in platelets prelabeled with ^{32}P and stimulated with thrombin (lane 2).

Pertussis toxin-induced ADP-ribosylation of the 41-kDa protein increases with time for up to 20 min and remains constant up to 60 min (data not shown). Pertussis toxin-induced ADP-ribosylation is not affected by prostacyclin (1 μg/ml) or the cyclooxygenase inhibitor indomethacin (20 μM). Addition of Ca^{2+} (1 mM) inhibits ADP-ribosylation induced by pertussin toxin (data not shown).

Stimulation of Intact Platelets with Trypsin, Thrombin, or Collagen Inhibits Subsequent Pertussis Toxin-Induced ADP-Ribosylation of the 41-kDa Protein. Pretreatment of platelets with trypsin (2–5 μg/ml) for 2 min prevents subsequent pertussis toxin-induced ADP-ribosylation of the 41-kDa protein (Fig. 3). In the experiment represented in Fig. 3, trypsin inhibitor (soybean trypsin inhibitor or leupeptin) was added for 2 min after pretreatment of platelets with trypsin and before addition of saponin and pertussis toxin. However, when platelets are treated with soybean trypsin inhibitor or leupeptin before addition of trypsin, the subsequent ADP-ribosylation induced by pertussis toxin is not decreased (Fig. 4*B*). ADP-ribosylated protein is degraded by trypsin to an ADP-ribosylated protein of slightly less than 20 kDa (Fig. 4*A*). Chymotrypsin is much less effective in inducing the same proteolytic product, and thrombin is completely ineffective (Fig. 4*A*). Stimulation of platelets with collagen (25–200 μg/ml) for 3 min decreases pertussis toxin-induced ADP-ribosylation of the 41-kDa protein; at 200 μg of collagen per ml, ADP-ribosylation is almost absent (Fig. 5*A*). Similarly, treatment of platelets with 2 units of thrombin per ml for 1 or 3 min substantially prevents subsequent protein ADP-ribosylation that is induced by pertussis toxin in the presence of saponin (Fig. 5*B*). Pretreatment of platelets with 5–20 units

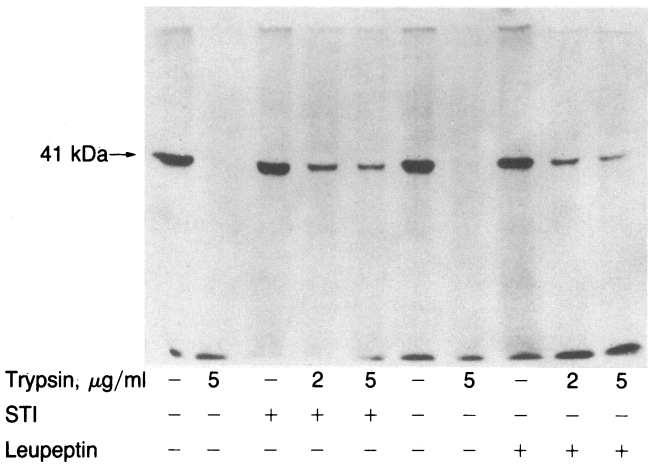

FIG. 3. Treatment of platelets with trypsin inhibits the pertussis toxin-induced ADP-ribosylation of a 41-kDa protein. Human platelets were treated with trypsin (2–5 μg/ml) for 2 min. Then soybean trypsin inhibitor (STI, 100 μg/ml) or leupeptin (200 μM) was added for another 2 min. Subsequently, saponin (20 μg/ml) and pertussis toxin were added for 15 min.

of thrombin per ml totally blocks the pertussis toxin-induced ADP-ribosylation. Preincubation of platelets with 2 μg of prostacyclin per ml does not prevent this effect of thrombin on pertussis toxin-induced ADP-ribosylation. Leupeptin (500 μg/ml) or soybean trypsin inhibitor (100 μg/ml), when added before thrombin, inhibits the decrease of ADP-ribosylation that is caused by thrombin at 1 unit/ml.

The inhibition of ADP-ribosylation by trypsin, thrombin, or collagen does not seem to be related to the activation of protein kinase C. Phorbol 12,13-dibutyrate (100 nM), 1,2-dioctanoylglycerol (10 μM), and 1,2-didecanoylglycerol (10–20 μM) maximally stimulate platelet kinase C in intact platelets (32–34). However, phorbol 12,13-dibutyrate and 1,2-diacylglycerols have an opposite effect on the ADP-ribosylation. Pretreatment (up to 2 min) with phorbol 12,13-dibutyrate inhibits subsequent ADP-ribosylation induced by pertussis toxin, whereas pretreatment with 1,2-diacylglycerols has no effect (data not shown). ADP-ribosylation is

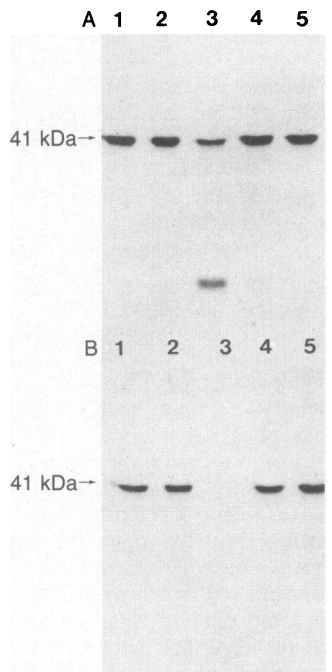

FIG. 4. (*A*) Proteolytic degradation of the ADP-ribosylated α_i subunit by trypsin. Platelets were treated with saponin and pertussis toxin for 20 min and subsequently incubated for 5 min without addition (lanes 1 and 5), with 5 units of thrombin per ml (lane 2), with 5 μg of trypsin per ml (lane 3), or with 100 μg of chymotrypsin per ml (lane 4). (*B*) Inhibition of ADP-ribosylation by trypsin. Platelets were incubated for 2 min with 100 μg of soybean trypsin inhibitor per ml (lanes 2 and 4) or 200 μg of leupeptin per ml (lane 5) and then further incubated with 5 μg of trypsin per ml for 2 min (lanes 3–5) before addition of saponin and pertussis toxin for 20 min. Lanes: 1, control; 2, soybean trypsin inhibitor; 3, trypsin; 4, soybean trypsin inhibitor plus trypsin; lane 5, leupeptin plus trypsin.

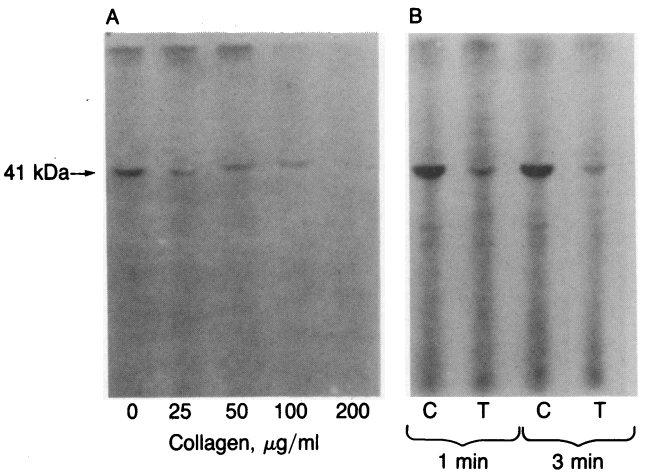

FIG. 5. Treatment of platelets with collagen or thrombin inhibits the pertussis toxin-induced ADP-ribosylation of a 41-kDa protein. Human platelets were treated with saponin (20 μg/ml) and pertussis toxin for 10 min after treatment with collagen and thrombin. Thrombin or collagen remained during the entire incubation period. (*A*) Platelets were incubated with various concentrations of collagen for 3 min. (*B*) Platelets were incubated with (lanes T) or without (lanes C, control) thrombin (2 units/ml) for 1 or 3 min.

blocked by pretreatment for 3 min with GTP[γ-S] (100–200 μM) before addition of saponin and pertussis toxin.

Pretreatment of platelets with vasopressin (50 μM), epinephrine (1 mM), or a combination of both agonists does not affect the subsequent ADP-ribosylation that is induced by pertussis toxin (data not shown).

DISCUSSION

Pretreatment of various cell types with pertussis toxin produces inhibition of agonist-induced responses (14–23). In all cases, an association of the G_i protein with those cell responses has been indicated. The mechanism by which these receptors interact with G_i protein to effect those specific responses is not understood.

Pertussis toxin modifies and inactivates G_i, the guanine nucleotide-binding regulatory protein that is involved in the receptor-coupled inhibition of adenylate cyclase activity (10–15). Pertussis toxin catalyzes the ADP-ribosylation of the α_i subunit of G_i (10–15). This ADP-ribosylation is inhibited when α_i is dissociated from the β subunit of G_i (10). The ability of GTP[γ-S] to inhibit pertussis toxin-induced ADP-ribosylation of G_i is consistent with this hypothesis. A similar G protein (G_o), which has recently been identified in brain membranes, can also be ADP-ribosylated by pertussis toxin (31). The α_o subunit is only slightly smaller (39 kDa) than the α_i subunit (41 kDa). Therefore, it is difficult to separate α_i from α_o by $NaDodSO_4$/polyacrylamide gel electrophoresis (31), and whether or not the ADP-ribosylated protein in platelets is α_o or α_i or a mixture of both remains to be determined. The present study demonstrates that the substrate phosphorylated by protein kinase C, which is often referred to as a 40-kDa protein, is not a subunit of the G protein. In fact, the protein that is the substrate for protein kinase C is larger than the α_i subunit, with a molecular mass of about 47 kDa (Fig. 2).

The exposure of intact platelets to collagen, thrombin, or trypsin prevents the subsequent ADP-ribosylation of G_i by pertussis toxin. However, the ADP-ribosylation induced by pertussis toxin is not affected by cyclooxygenase inhibitors, prostacyclin, or 1,2-diacylglycerols, but it is decreased by phorbol 12,13-dibutyrate. These results suggest that the impairment of ADP-ribosylation by collagen, thrombin, or

trypsin is not the consequence of activation of phospholipase A_2, phospholipase C, or protein kinase C and might reflect an alteration of the α and β subunits of G_i. This alteration might not be a simple dissociation of the α and β subunits of G_i (or G_o), because vasopressin and epinephrine; agonists that inhibit adenylate cyclase by interacting with receptors and G_i, do not have the same inhibitory action on pertussis toxin-induced ADP-ribosylation. Alternatively, the lack of an effect by vasopressin and epinephrine might reflect that only a small fraction of the G_i molecules are coupled to their respective receptors, and this alteration cannot be detected by the present techniques. Recent data suggest that the maximal ratio of epinephrine α_2-receptor to G_i protein is 1:20 (35). Since thrombin is able to completely inhibit ADP-ribosylation, this suggests that thrombin can fully activate G_i. This may represent a mechanism for thrombin action that has not been described. However, it might be related to the effect that proteases activated by thrombin could have on G_i. Leupeptin inhibits thrombin-induced responses such as secretion and aggregation, without affecting the action on clot formation (36, 37), which indicates that the action of thrombin on platelet proteases is essential for platelet activation. This hypothesis is also consistent with the irreversible effect of thrombin on platelet responses and the fact that proteolytic inhibitors can prevent the effect of trypsin and thrombin on ADP-ribosylation induced by pertussis toxin.

Addition of GTP[γ-S] to platelets permeabilized by electrical stimulation (38) or saponin (unpublished results) provokes activation of phospholipase C, as determined by the formation of 1,2-diacylglycerol (38) and inositol trisphosphate and the degradation of inositol phospholipids (unpublished results). Similarly, GTP[γ-S] activates phospholipase C in plasma membranes obtained from human neutrophils, blowfly salivary gland, and liver membranes (39–41).

The exposure of intact platelets to trypsin induces stimulation of phospholipase C, which is illustrated by the formation of inositol phosphates (36). Our present information indicates that trypsin proteolytically alters the α_i subunit and precludes subsequent ADP-ribosylation. It is possible that activation of phospholipase C is related to this alteration of α_i. Similarly, thrombin and collagen effects might be associated with activation of proteases that cause a proteolytic modification of G_i that leads to phospholipase C activation.

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