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Agonist-induced tyrosine phosphorylation of $G_q/G_{11}\alpha$ requires the intact structure of membrane domains☆

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Abstract

Stimulation of receptors coupled to G_q/G_{11} protein may induce phosphorylation on a tyrosine residue of the α subunit of this G protein, which is an essential event for G_q/G_{11} activation. Here we observed that in HEK293 cells stably expressing high levels of thyrotropin-releasing hormone (TRH) receptors and $G_{11}\alpha$ protein the maximal tyrosine phosphorylation of $G_q/G_{11}\alpha$ was reached within 10 min of TRH stimulation and then it faded away at longer time periods of agonist exposure. The $G_q/G_{11}\alpha$ protein levels did not change during this treatment. Incubation of intact cells with β-cyclodextrin (βCD) for 40 min prior to hormone exposure significantly decreased the rapid transient tyrosine phosphorylation. Subsequent replenishment of cholesterol levels reversed the former negative effect of βCD. Isolation of caveolin-enriched, detergent-resistant membrane domains indicated destruction of these structures in βCD-treated cells. These data indicate that the preserved integrity of plasma membrane domains/caveolae is required for complete agonist-induced phosphorylation of $G_q/G_{11}\alpha$.

Keywords: Trimeric G proteins; $G_q/G_{11}\alpha$; Tyrosine phosphorylation; Membrane domains

Trimeric GTP-binding regulatory proteins (G proteins), which consist of α, β, and γ subunits, transmit signals from G protein-coupled receptors (GPCRs) located on the cell surface to various intracellular effectors. When G protein is activated, GDP on the α subunit is exchanged for GTP, the activated α subunit dissociates from the βγ complex and both these subunits are then capable to regulate activity of numerous enzymes responsible for the formation of secondary messengers [1,2]. The cellular localisation of GPCRs and their cognate G proteins has been studied for a number of years. More recently, a great attention has been paid to studies of "lipid rafts" or "membrane domains" in signal transduction initiated by GPCRs. Lipid rafts are conceived as specific plasma membrane regions (about 100 nm in diameter) with characteristic lipid and protein composition, which are resistant to treatment with non-ionic detergents such as Triton X-100 [3,4]. Hence, they are called "detergent-resistant membrane domains" (DRMs) or "detergent-insoluble membranes" (DIMs).

☆ *Abbreviations:* DRMs, detergent-resistant membrane domains; DMEM, Dulbecco's modified Eagle's medium; G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; $G_s\alpha$, G protein stimulating adenylyl cyclase activity; $G_i\alpha1/G_i\alpha2$, G proteins inhibiting adenylyl cyclase activity in pertussis-toxin sensitive manner; $G_q\alpha/G_{11}\alpha$, G proteins stimulating phosholipase C in pertussis-toxin independent manner; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; GPI, glycosylphosphatidylinositol; PBS, phosphate-buffered saline; PM, plasma membranes; PMSF, phenylmethylsulphonyl fluoride; SLB, solubilisation lysis buffer; TBS, Tris-buffered saline; TRH, thyrotropin-releasing hormone; TRH-R, thyrotropin-releasing hormone receptor.

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They are enriched in some molecules engaged in signal transduction such as caveolins, Src and Fyn kinases, GPI-bound proteins, and NO synthase [5–7]. There are some indications that DRMs might play a role in G protein-mediated transmembrane signalling but it is not quite clear how many GPCRs and G proteins are actually present in DRMs. Whereas muscarinic acetylcholine receptors and IP prostanoid receptors were found in a bulk plasma membrane fraction, β-adrenergic, bradykinin, and endothelin receptors occur mostly in membrane domains [8–13]. Relatively large quantities of various G protein α subunits were also identified in membrane domains [14–16].

We have previously reported that subcellular distribution of G proteins might be affected by prolonged stimulation of GPCRs. Sustained hormonal or neurotransmitter stimulation resulted in internalisation, solubilisation, and overall decrease of G protein levels in the cell [17–19]. Long-term agonist treatment was also accompanied by depletion of G proteins from DRMs [13,20,21]. On the other hand, short-term (minutes) agonist stimulation did not alter the level of $G_q/G_{11}\alpha$ in DRMs as well as other subcellular membrane fractions. Previous studies of agonist-induced subcellular redistribution of G proteins, however, were not accompanied by any analysis of the functional state of these regulatory proteins in control and hormone-exposed cells.

Besides stimulation of the GDP/GTP exchange reaction, several G_q/G_{11}-coupled receptors were reported to induce tyrosine phosphorylation of cellular proteins [22]. In rat fibroblasts, tyrosine phosphorylation and activity of $G_q/G_{11}\alpha$ proteins were enhanced by Src-oncogene [23]. More specifically, activation of metabotropic glutamate receptors (mGluR1α) led to increased phosphorylation of $G_q/G_{11}\alpha$ at tyrosine residue Tyr^{356} and this tyrosine phosphorylation was suggested to be essential for full activation of $G_q/G_{11}\alpha$ as well as for agonist-stimulated inositol-1,4,5-trisphosphate production [24]. Tyrosine phosphatases (PTPs) also participated in these regulatory loops because the agonist-induced formation of IP_3 was blocked by both PTK and PTP inhibitors [25]. More recently, tyrosine phosphorylation of $G_q\alpha$ was found to potentiate signalling through $G_q\alpha$-coupled bombesin receptor [26].

In the present study, we aimed to evaluate the role of intact membrane domains in the thyrotropin-releasing hormone (TRH)-induced phosphorylation of $G_q/G_{11}\alpha$ protein and we used the stable clonal cell line derived from epithelial human embryonic kidney cells (clone E2M11 of HEK293 cells) expressing high levels of rat TRH receptor and murine $G_{11}\alpha$ protein for these investigations. TRH is an important hypothalamic peptide that controls production of thyrotropin and prolactin [27]. This hormone binds to TRH receptor, which is coupled to G_q/G_{11}, and the result of this interaction is activation of phospholipase Cβ enzyme activity and formation of IP_3 [27,28]. Here we have demonstrated that stimulation of E2M11 cells with TRH may lead to rapid tyrosine phosphorylation of $G_q/G_{11}\alpha$ protein and that breakage of membrane domains caused by cholesterol depletion abolishes this $G_q/G_{11}\alpha$ phosphorylation.

Materials and methods

Chemicals. Tissue culture reagents and media were supplied by Gibco-BRL (Renfrewshire, UK) or Sevac (Prague, Czech Republic). All other chemicals were purchased from Sigma (St. Louis, MO, USA). Monoclonal anti-caveolin 2 antibody was from BD Biosciences (San Jose, CA, USA) and anti-phosphotyrosine antibody was obtained form the Institute of Molecular Genetics, AS CR (Prague, Czech Republic). Rabbit polyclonal CQ antiserum against the C-terminal decapeptide of $G_q/G_{11}\alpha$ was characterised previously [29].

Cell culture. HEK293 cells stably expressing TRH receptor and murine $G_{11}\alpha$ (clone E2M11) were cultivated in Dulbecco's modified Eagle's medium supplemented with selection markers (800 μg/ml geneticin and 200 μg/ml hygromycin B) at 37 °C under 5% CO_2 atmosphere [30]. When treated with βCD, E2M11 cells were incubated for 2 h in serum-free medium and βCD (10 mM final concentration) was added for 40 min. If needed, the negative effect of βCD could be reversed by subsequent incubation of cells in 2.5 mM βCD and 0.25 mM cholesterol at 37 °C for 2 h [31,32].

Cell membrane preparation. HEK293 cells were harvested from 7 culture flasks (80 cm^2 each) by centrifugation at 1800 rpm for 10 min. Homogenisation was performed in STME medium (0.25 M sucrose, 50 mM Tris–HCl, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4) containing 1 mM sodium orthovanadate, freshly added 1 mM PMSF, and Complete protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). The tightly fitting Teflon-glass homogeniser was applied for 5 min at 1700 rpm and the resulting homogenate was centrifuged for 5 min at 1000*g*. The total membrane preparation was sedimented from thusly obtained post-nuclear supernatant by centrifugation for 2 h at 250,000*g*. The high-speed membrane pellet was re-homogenised mildly by hand in STME medium (10–15 mg protein/ml), snap-frozen in liquid nitrogen, and stored at −80 °C until use.

Isolation of detergent-resistant membrane domains. Membrane suspension (1 ml) was mixed with 1 ml of 2% v/v Triton X-100, 20 mM Tris–HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, and 1 mM PMSF. After 60 min on ice, the resulting detergent lysate (2 ml) was mixed with 2 ml of ice-cold 80% sucrose and transferred into a Beckman SW41 centrifuge tube. This sample was then overlaid with 35%, 30%, 25%, 20%, 15%, 10% (1 ml each), and 5% (w/v) sucrose (1.5 ml) in 20 mM Tris–HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM EDTA, and 0.15 M NaCl. The density gradients were centrifuged at 200,000*g* for 24 h at 0–4 °C and fractions were collected manually from the meniscus by 0.5 ml (the first fraction) and 1 ml (all the remaining fractions). Constant volume aliquots (20 μl) were analysed by SDS–PAGE and immunoblotting.

Immunoblot analysis. The samples were solubilised in Laemmli buffer (50 mM Tris–HCl, 6% dithiothreitol, 5% SDS, and 0.005% bromphenol blue, pH 8.0) and proteins were resolved by standard SDS–PAGE in 10% gel (200 V for 1 h, Bio-Rad Mini Protean II gel kit). After SDS–PAGE, proteins were transferred to nitrocellulose (NC) membrane by semi-dry blotting and the NC membrane was blocked for 1 h in 5% fat-free milk in PBS–T buffer (PBS containing 0.05% v/v Tween 20), washed once, and then incubated for 2 h at room temperature with appropriate primary antibody diluted in 1% fat-free milk/PBS–Tween. After removal of primary antibody, the NC membrane was washed three times for 10 min in PBS–T buffer and incubated with secondary antibody in 1% fat-free milk/PBS–T for 1 h.