

Original Article

Identification and subcellular localization of molecular complexes of $G_{q/11}\alpha$ protein in HEK293 cells

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Heterotrimeric G-proteins localized in the plasma membrane convey the signals from G-protein-coupled receptors (GPCRs) to different effectors. At least some types of G-protein α subunits have been shown to be partly released from plasma membranes and to move into the cytosol after receptor activation by the agonists. However, the mechanism underlying subcellular redistribution of trimeric G-proteins is not well understood and no definitive conclusions have been reached regarding the translocation of $G\alpha$ subunits between membranes and cytosol. Here we used subcellular fractionation and clear-native polyacrylamide gel electrophoresis to identify molecular complexes of $G_{q/11}\alpha$ protein and to determine their localization in isolated fractions and stability in naïve and thyrotropin-releasing hormone (TRH)-treated HEK293 cells expressing high levels of TRH receptor and $G_{11}\alpha$ protein. We identified two high-molecular-weight complexes of 300 and 140 kDa in size comprising the $G_{q/11}$ protein, which were found to be membrane-bound. Both of these complexes dissociated after prolonged treatment with TRH. Still other $G_{q/11}\alpha$ protein complexes of lower molecular weight were determined in the cytosol. These 70 kDa protein complexes were barely detectable under control conditions but their levels markedly increased after prolonged (4–16 h) hormone treatment. These results support the notion that a portion of $G_{q/11}\alpha$ can undergo translocation from the membrane fraction into soluble fraction after a long-term activation of TRH receptor. At the same time, these findings indicate that the redistribution of $G_{q/11}\alpha$ is brought about by the dissociation of high-molecular-weight complexes and concomitant formation of low-molecular-weight complexes containing the $G_{q/11}\alpha$ protein.

Keywords trimeric $G_{q/11}$ protein; clear native electrophoresis; protein complex; subcellular localization

Introduction

Heterotrimeric G-proteins, which are composed of α , β , and γ subunits, constitute an important regulatory part of the signaling pathways initiated by G-protein-coupled receptors (GPCRs). These membrane-bound receptors are activated by ligand binding, which causes a conformational change in their seven transmembrane helices. A subsequent change in the steric configuration of their cognate G-proteins allows the release of GDP and the binding of GTP on the α subunit [1,2]. Next, the signal is transduced to different effector molecules (e.g. phospholipases, adenylyl cyclases, and ion channels) both by the α and $\beta\gamma$ subunits [3]. It is well known that the cell response may depend on different factors, such as the character of the signal, ligand concentration and treatment interval, receptor–ligand affinity, and receptor oligomerization [4–6]. It has been frequently observed that treatment with ligands of different GPCRs leads to a desensitization of the corresponding signaling pathway, which includes such phenomena as phosphorylation, sequestration, and internalization of the receptors [7,8]. During this process, part of the activated and subsequently desensitized receptors may undergo intracellular trafficking from the cell surface into the cell interior and subsequent degradation or recycling to the plasma membrane (PM) [9,10].

Cellular trafficking after ligand binding was also demonstrated for some trimeric G-proteins, mainly in the case of $G_s\alpha$. Using cell fractionation, immunofluorescence microscopy, and live cell imaging, it has been shown that redistribution of $G\alpha$ subunits is a very rapid process, occurring mostly within 1–20 min after the addition of the agonist [11]. Translocation of $G_s\alpha$ from the PM into the cytosol triggered by cholera toxin-mediated activation, receptor-mediated activation, or by constitutively active mutant receptors was described in HEK293, MCF7, C6, or S49 cell lines [12–17]. The observed subcellular redistribution

Received: February 20, 2012 Accepted: April 25, 2012

of G_sα was found to be associated with the activation-dependent depalmitoylation of the α subunit [18]. However, in other cases, the release from the PM of activated G_sα was not detected [19,20].

In the case of the G_{q/11} protein family, the subcellular redistribution of G_qα was reported after the activation of rhodopsin in *Drosophila* eyes or after the activation of the angiotensin II receptor or thyrotropin releasing-hormone receptor-1 (TRH-R1) in HEK293 cells [11]. Although the soluble form of G_qα was observed in rhodopsin and angiotensin II receptor studies as early as within 5–20 min after stimulation by agonists [21–23], translocation of G_{q/11}α to the cytosol was not detectable earlier than 2 h after TRH-R1 stimulation [24]. The mechanism of trafficking G_q subunits from the PM to the cytosol is not entirely understood. It is supposed that this phenomenon is related to receptor activation and depalmitoylation of G_q [11,12].

Recently, we have successfully identified a pre-associated molecular complex of TRH-R1 and G_{q/11} protein after detergent solubilization and separation by colorless native electrophoresis (CN-PAGE) [25]. Two distinct membrane-bound complexes observed in the 140 kDa region were detected mainly after solubilization with lauryl maltoside (LM) and they are likely to represent pre-associated complexes of the heterotrimeric G_{q/11} proteins with particular GPCRs. In the present study, we were also able to detect other molecular complexes containing G_{q/11}α proteins. Besides the 140 kDa pre-associated TRH-R1/G_{q/11} complex, we succeeded in determining a high-molecular-weight complex of G_{q/11}α with a molecular size of ~300 kDa localized in the PM-enriched fraction and other 70 kDa complexes in the cytosol. Prolonged treatment (10 min to 16 h) of HEK293 cells with TRH resulted in a gradual release of G_{q/11}α from the PM-enriched fraction into the cytosol and these proteins were successively translocated from both membrane-associated complexes (300 and 140 kDa) to the cytosolic 70 kDa protein complex.

Materials and Methods

Materials

All materials for tissue culture were supplied by NUNC (Rochester, USA), Gibco (Carlsbad, USA), and PAA (Pasching, Austria). Complete Protease Inhibitor Cocktail was from Roche (Basel, Switzerland). Nitrocellulose membrane was purchased from Schleicher–Schuell (Erdmannhausen, Germany), polyvinylidene difluoride membrane from Bio-Rad (Hercules, USA), Whatman GF/C filters from Whatman Ltd (Oxford, UK), acrylamide and bis-acrylamide from Serva (Heidelberg, Germany), and secondary anti-rabbit antibody labeled with horseradish peroxidase from GE Healthcare (Chalfont St Giles, UK). Preparation and characterization of rabbit polyclonal serum against G_{q/11}α

protein was described previously [24]. All other primary antibodies and secondary anti-goat antibody labeled with horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, USA).

Cell culture

HEK293 cells [clone E2M11 stably expressing the long isoform of the rat TRH receptor (TRH-R1) and the mouse G₁₁α protein] prepared in Dr G. Milligan's laboratory (University of Glasgow, Glasgow, UK) were obtained by courtesy of Dr P. Svoboda (Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic). HEK293 cells were grown in DMEM supplemented with 10% heat-inactivated newborn calf serum, geneticin (0.8 mg/ml) and hygromycin B (0.2 mg/ml) at 37°C in 5% CO₂ humidified atmosphere [26].

Preparation of the postnuclear supernatant, PM-enriched fraction, and cytosol

Nearly confluent cell cultures grown in 80-cm² tissue culture flasks (15 flasks per sample) were treated with or without 0.001–10 μM TRH for 10, 30 min, 1, 2, 4, 8, or 16 h and subsequently harvested. After collection by centrifugation at 500 g for 10 min in Hettich Universal 30RF Centrifuge (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany), the cell samples were homogenized in 2 ml of chilled homogenization buffer H (750 mM aminocaproic acid, 50 mM Bis–Tris, 0.5 mM EDTA; pH 7.0) supplemented with Complete Protease Inhibitor Cocktail in a Teflon-glass homogenizer at 1500 rpm for 5 min on ice. The homogenate was centrifuged for 3 min at 350 g at 4°C in a Hettich Centrifuge and samples of the postnuclear supernatant (PNS) were used for fractionation of Percoll density gradient or frozen in liquid nitrogen and stored at –80°C. The resulting PNS was applied on the top of 25% (w/v) Percoll[®] in TMEN buffer (20 mM Tris–HCl, 3 mM MgCl₂, 1 mM EDTA, 150 mM NaCl; pH 7.4). After centrifugation in a Beckman Ti50.2 rotor at 50,000 g for 15 min at 4°C, two distinct layers of protein-rich opalescent materials were separated. The upper fraction (with lower buoyant density) enriched with PMs was diluted in TMEN buffer and centrifuged in a Beckman Ti50.2 rotor at 150,000 g for 1 h at 4°C. Membrane pellet was re-suspended in a small volume of TMEN buffer. The cytosol was prepared by high-speed centrifugation (300,000 g, 1 h) of the fraction collected from the top of the gradient. Samples of the membrane and cytosolic fractions were then frozen in liquid nitrogen and stored at –80°C.

Colorless native electrophoresis

Samples (50 μg protein) of the PNS or the PM-enriched fraction were solubilized in sample buffer (75 mM Bis–Tris, 750 mM ε-aminocaproic acid, 0.5% Coomassie

G-250) containing various concentrations of different detergents (digitonin, lauryl maltoside, Brij 56, Triton X-100, and CHAPS) for 30 min on ice. The insoluble material was removed by centrifugation at 7,500 g for 10 min at 4°C and subsequent separation of protein complexes was performed on 6%–15% or 12%–15% linear gradient polyacrylamide slab gels cooled to 2°C using the Mighty Small SE 260 apparatus (GE Healthcare). A discontinuous buffer system using different anode (50 mM Bis-Tris, pH 7.0) and cathode (50 mM tricine and 15 mM Bis-Tris, pH 7.0) buffers was employed in the current CN-PAGE. Electrophoresis was carried out at a constant voltage of 50 V for 30 min and then at a constant current 15 mA for 3 h until the Coomassie G-250 dye reached the end of the gel. After electrophoresis, proteins were transferred onto the polyvinylidene difluoride (PVDF) membrane at a constant voltage of 100 V for 2 h. After blocking with 5% fat-free milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20; pH 8.0), PVDF membranes were incubated with appropriate primary antibodies overnight and subsequently with horseradish peroxidase-conjugated secondary antibodies for 1 h. The blots were developed with the SuperSignal chemiluminescent substrate (Pierce Biotechnology, Rockford, USA) and exposed to Kodak MXB film (Eastman Kodak Company, Rochester, USA). Western blots for each detergent and antibody were replicated at least three times and one representative blot was chosen for display. The density of some selected spots was quantified by ImageQuant™ TL software (GE Healthcare).

CN/SDS-PAGE

After the first dimension CN-PAGE, the gel strips were cut and incubated with 1% dithiothreitol (DTT) in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 0.1 mM EDTA, 2% SDS, 30% glycerol, 0.01% bromophenol blue) for another 15 min and subsequently with 2.5% iodoacetamide (IAA) in equilibration buffer for 15 min. The strips were then placed onto denaturing gels (4% stacking and 10% separating gel). The electrophoresis was performed in Laemmli running buffer (25 mM Tris, 192 mM glycine, 1% (w/v) SDS) at a constant voltage of 200 V at room temperature. The separated proteins were transferred onto the nitrocellulose membrane and probed with specific primary antibodies against G_{q/11}α proteins. After the incubation of membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 60°C for 30 min, membranes were re-probed with primary antibody against the Gβ subunit. Western blots for each detergent and antibody were replicated at least three times and one representative blot was chosen for display. The density of some selected spots was quantified by ImageQuant™ TL software (GE Healthcare).

Results

Determination of G_{q/11}α and Gβ proteins by SDS-PAGE and characterization of fractions from Percoll density gradient

Before using specific antibodies against G_{q/11}α and Gβ proteins for the identification of molecular complexes, we checked their specificity by analysing proteins in the PNS separated by SDS-PAGE. Two discrete bands were detected using an antibody against G_{q/11}α [Fig. 1(A)]. The band with a molecular size of 43 kDa corresponds to G₁₁α because its intensity was much stronger than the intensity of the band with a size of 40 kDa, which corresponds to G_qα. These observations are in line with previous findings that G_qα exhibits higher mobility in polyacrylamide gels than G₁₁α [27,28]. Moreover, we have demonstrated in our preceding study that a peptide derived from G₁₁α was able to selectively block the signal corresponding to G₁₁α on immunoblots [25]. The Gβ subunit was detected as a single strong band with a molecular size of 35 kDa [Fig. 1(B)].

For subsequent experiments dealing with the determination of the presumed G_{q/11} protein complexes, the PNS obtained from HEK293 cells was separated on a Percoll density gradient and the resulting cytosolic and

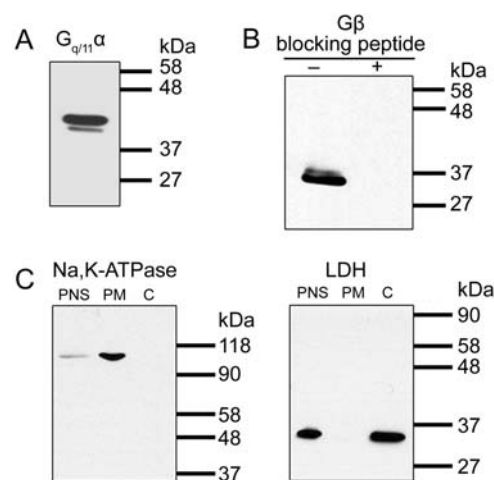


Figure 1 Determination of G_{q/11}α and Gβ proteins by SDS-PAGE and characterization of the fractions from Percoll density gradient Twenty micrograms of the postnuclear supernatant (PNS) from control HEK293 cells were resolved on 10% SDS-polyacrylamide gels and proteins were electroblotted onto the nitrocellulose membrane. Using specific antibodies against G_{q/11}α and Gβ, two individual bands corresponding to G₁₁α (43 kDa) or G_qα (40 kDa) (A) and one band corresponding to Gβ (B) were observed. The specificity of the Gβ antibody was confirmed using the corresponding blocking peptide at 0.5 μg/ml in the incubation mixture of primary antibody (B). In order to determine the quality of fractions purified by the Percoll density gradient, 20 μg of the PNS, the PM-enriched fraction or cytosol were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Na,K-ATPase and lactate dehydrogenase (LDH) were used as markers of the PM-enriched fraction and cytosol, respectively (C).

PM-enriched fractions were collected. Twenty micrograms of the PNS, PM-enriched, or cytosolic fraction was separated by SDS-PAGE and specific antibodies against Na,K-ATPase and lactate dehydrogenase (LDH) were used as markers of the PM and cytosol, respectively. As shown in **Fig. 1(C)**, the signal of Na,K-ATPase was markedly increased in the PM-enriched fraction as compared with the PNS but no signal was observed in the cytosol. On the contrary, an increased amount of LDH was present in the cytosol as compared with the PNS and no immunoblot signal was detected in the PM-enriched fraction. Hence, these fractions were found to be suitable for the next experiments determining the localization of the presumed $G_{q/11}$ protein complexes.

Identification and subcellular localization of $G_{q/11}\alpha$ protein complexes by detergent solubilization and CN-PAGE

The PNS from control (naïve) HEK293 cells was incubated with the non-ionic detergent Brij 56 at different concentrations (0.1%–4%) and the resulting mixtures of solubilized proteins were resolved by CN-PAGE. In this way, a protein complex of $G_{q/11}\alpha$ with molecular mass ~ 300 kDa was identified using a specific $G_{q/11}\alpha$ protein antibody [**Fig. 2(A)**]. Interestingly, this complex was not detectable after solubilization with the other applied detergents, that is

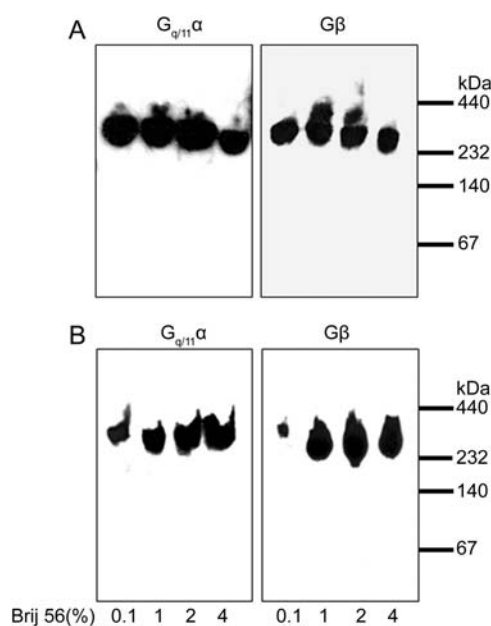


Figure 2 Detergent solubilization and detection of protein complexes containing $G_{q/11}\alpha$. Protein complexes in samples of PNS or PM from the control HEK293 cells were solubilized by different concentrations of Brij 56 and separated on 6%–15% linear gradient polyacrylamide gels under native conditions. After electrotransfer onto PVDF membranes, the separated complexes were detected with specific antibodies against $G_{q/11}\alpha$ or $G\beta$. Protein complexes with a size of 300 kDa were observed in the PNS (A) and PM-enriched fraction (B) after solubilization with Brij 56.

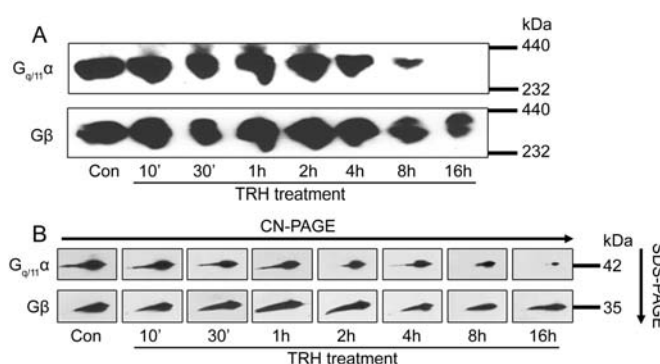


Figure 3 Effect of TRH treatment on the stability of the 300 kDa protein complex separated by CN-PAGE and CN/SDS-PAGE. Samples of the PM-enriched fraction from the control and TRH-treated (10 μ M; 10 min to 16 h) HEK293 cells were solubilized with 1% Brij 56 and separated on 6%–15% linear gradient non-denaturing polyacrylamide gels followed by immunoblotting with antibodies against $G_{q/11}\alpha$ and $G\beta$ proteins (A). In the case of CN/SDS-PAGE, the excised gel strips after the first dimension were preincubated with 1% DTT and then with 2.5% IAA and applied on standard 10% SDS-polyacrylamide gels and the dissociated components of protein complexes were detected by immunoblotting with specific antibodies (B).

Triton X-100, digitonin, and CHAPS. In the following experiments, high-molecular-weight complexes of $G\beta$ protein were observed under the same conditions. The immunoblot signals were detected in the region of molecular size of ~ 300 kDa after solubilization with Brij 56 [**Fig. 2(A)**]. These results strongly suggest that this complex contains the $G_{q/11}$ protein.

In order to determine the cellular localization of this complex, the PNS of control and TRH-treated cells was fractionated on a Percoll density gradient and the top portion of the gradient containing the soluble proteins was further centrifuged at high speed (300,000 g) to obtain a pure cytosolic fraction. The PM-enriched and cytosolic fractions were solubilized under the same detergent conditions as in case of the experiments with the PNS and separated by CN-PAGE. Both $G_{q/11}\alpha$ and $G\beta$ proteins were identified in all detected complexes from the PM-enriched fraction [**Fig. 2(B)**] but not in the cytosol (data not shown) suggesting that this complex is bound to membranes. Based on the results of these preliminary observations, all the next solubilization experiments were performed using 1% detergents.

In the next experiments we checked whether the 300 kDa complex may contain some GPCRs, because these receptors were previously found to constitute protein complexes with a molecular size of ~ 140 kDa [25]. However, no immunoblot signals of the TRH receptor or α_1 -adrenergic receptor were found in the 300 kDa region. These data suggest that the observed 300 kDa molecular complex does not contain GPCRs. Thus, it can be assumed

that this complex does not participate directly in the signal transduction via the GPCR-coupled pathways.

TRH-induced dissociation of high-molecular-weight G_{q/11}α protein complexes

HEK293 cells were treated with TRH for different time intervals (10, 30 min, 1, 2, 4, 8, and 16 h) and the PM-enriched fraction prepared from control (naïve) and hormone-treated cells was solubilized with Brij 56 and separated by CN-PAGE. The high-molecular-weight complex of 300 kDa was detectable on immunoblots using an anti-G_{q/11}α and anti-Gβ antibody and the signal intensity of both these proteins weakened after long-term hormone treatment (4–16 h) [Figs. 3(A) and 4(A)]. This can be attributed to a partial dissociation of this complex or perhaps to a changed protein conformation in a particular complex. Therefore, it was important to find out whether long-term treatment with TRH may induce dissociation of this G_{q/11} protein complex or only its steric reorganization. Protein complexes were separated by CN/SDS-PAGE and immunoblots were developed with specific antibodies in order to assess the relative protein quantities in the analyzed complexes [Figs. 3(B) and 4(B)]. Interestingly, a disintegration process of the 300 kDa complex started roughly after 4 h of hormone treatment, and since then the signal gradually faded away. A similar pattern of immunoblot signals was previously observed in the 140 kDa molecular complexes [25].

TRH-induced formation of low-molecular-weight G_{q/11}α protein complexes

It was reported previously that G_{q/11}α proteins are partly translocated from PMs to the cytosol after long-time treatment with TRH of HEK293 cells [24]. Our current findings of the dissociation of the two high-molecular-weight G_{q/11} complexes (300 and 140 kDa) after long-term TRH treatment may indicate that G_{q/11}α released from these complexes could be transferred to the cell cytosolic compartment. It seemed of interest, therefore, to find out whether some G_{q/11}α protein complexes could also occur in the cytosol after TRH treatment. In the course of these experiments, three distinct complexes with a size of ~70 kDa were separated and detected using CN-PAGE and specific primary anti-G_{q/11}α antibodies [Fig. 5(A)]. These complexes were solubilized and identified with most of the applied detergents (LM, Brij 56, Triton X-100, and CHAPS). They were not detectable in our preliminary experiments when an optimal concentration of detergents was tested apparently because of the low amounts of these complexes in the samples of the PNS obtained from naïve cells. Intriguingly, they became clearly visible only in the experimental conditions used for investigating the presumed effect of TRH on the stability of the high-molecular-weight

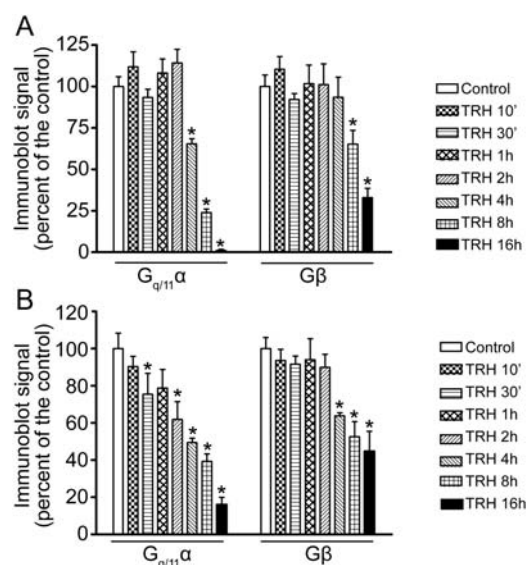


Figure 4 Quantitation of the effect of TRH on the levels of G_{q/11}α and Gβ in the 300 kDa G_{q/11} protein complex. The optical density of the immunoblot bands in the 300 kDa region corresponding to G_{q/11}α and Gβ resolved by CN-PAGE (A) or CN/SDS-PAGE (B) was quantified by ImageQuant™ TL software (GE Healthcare). Data are expressed as a percentage of the immunoblot signals determined in the corresponding controls (100%) and represent the mean ± SEM of three separate experiments; *P < 0.05 compared with the controls.

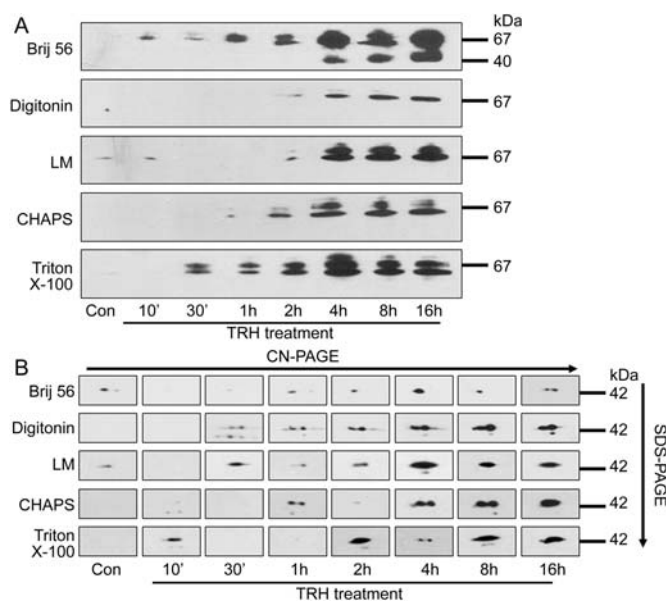


Figure 5 Detection by CN-PAGE of the 70 kDa protein complexes in the cytosol and their formation after TRH treatment. Samples of the cytosol from the control and TRH-treated (10 μM; 10 min to 16 h) HEK293 cells were solubilized with 1% Brij 56, 1% digitonin, 1% lauryl maltoside, 1% CHAPS, or 1% Triton X-100 and separated on 12%–15% linear gradient non-denaturing polyacrylamide gels. The resolved proteins were electrotransferred onto PVDF membranes and probed with anti-G_{q/11}α antibody (A) or the excised gel strips incubated with 1% DTT and then with 2.5% IAA were applied on standard 10% SDS-polyacrylamide gels and the dissociated components of protein complexes were detected by immunoblotting with anti-G_{q/11}α antibody (B).

$G_{q/11}$ protein complexes. Although there were some differences in the signal intensity of individual samples after solubilization with different detergents, a marked increase in the amount of these complexes was usually detected after 2 h of TRH treatment, with the largest intensity of bands visible after long time periods (4–16 h) [Figs. 5(A) and 6(A)]. After solubilization with the detergent Brij 56, besides the 70 kDa complex another band of ~45 kDa was observed, which obviously corresponds to the individual subunits of the $G_{q/11}\alpha$ protein.

To assess the relative quantity of the 70 kDa complexes, samples were separated by CN/SDS-PAGE. As shown in Fig. 5(B), solubilization with different detergents enabled us to detect two intensive spots with a size of 43 kDa corresponding to $G_{11}\alpha$ protein and, in some cases, two fainter spots with a size of 40 kDa corresponding to $G_q\alpha$ protein. A slight increase in the immunoblot signals of $G_{q/11}\alpha$ in these complexes was observed after short-term TRH treatment (30 min to 2 h) followed by sample solubilization with some detergent, but only prolonged treatment (4–16 h) with the hormone resulted in a massive formation of these complexes, which was reflected by an enormous rise of signal intensity [Figs. 5(B) and 6(B)]. It appears that both the 70 kDa complexes are composed of $G_{11}\alpha$ as well as $G_q\alpha$ and a slight difference in the size of these complexes is not substantiated by the presence of only one type of $G_{q/11}\alpha$ protein in a particular complex. It might be speculated that these complexes can be trimeric $G_{q/11}\alpha$

proteins varying in the type of $\beta\gamma$ heterodimer. In order to determine whether these complexes include the β subunit of trimeric G-proteins, a specific primary antibody against $G\beta$ protein was used for immunodetection. Although this experiment was repeatedly conducted after sample solubilization with different detergents by CN-PAGE as well as CN/SDS-PAGE, no immunoblot signal of $G\beta$ was observed in the region of 70 kDa (data not shown). To determine whether the 70 kDa complexes or also possibly the 300 kDa complex may contain some membrane scaffolding proteins (e.g., caveolin and flotillin), specific antibodies against caveolin-1 or flotillin-2 were employed in immunochemical detection experiments after the separation of these complexes by CN-PAGE or CN/SDS-PAGE. However, no immunoblot signals were detected on the whole blots in this case (data not shown).

The effect of TRH concentration on the dissociation/association of $G_{q/11}\alpha$ complexes

In the previous set of experiments, TRH was added at a final concentration of 10 μM so that the hormone effect could be detected well. Nevertheless, the ligand concentration is one of the most significant factors determining which processes will actually take place in the cell. As 10 μM TRH is a very high concentration, which can hardly be considered physiological, it was important to check whether the same dissociation/association patterns of the observed protein complexes would also be detectable with lower concentrations of the hormone. Therefore HEK293 cells were treated for 16 h with TRH in the 10^{-5} – 10^{-9} M range. The PNS from the control and TRH-treated cells were then solubilized by LM or Brij 56, protein complexes separated by CN-PAGE and $G_{q/11}\alpha$ proteins were detected by using specific antibodies (Fig. 7).

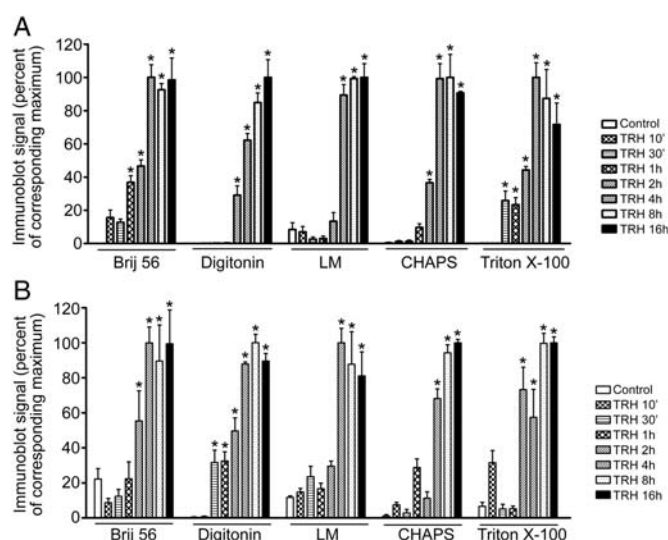


Figure 6 Quantitation of the effect of TRH on $G_{q/11}\alpha$ level in the 70 kDa $G_{q/11}$ protein complex The optical density of the immunoblot bands in the 70 kDa region corresponding to $G_{q/11}\alpha$ resolved by CN-PAGE (A) or CN/SDS-PAGE (B) was quantified by ImageQuantTM TL software (GE Healthcare). Data are expressed as a percentage of the maximal immunoblot signal (100%) in each group of samples and represent the mean \pm SEM of three separate experiments; * P < 0.05 compared with the corresponding control value.

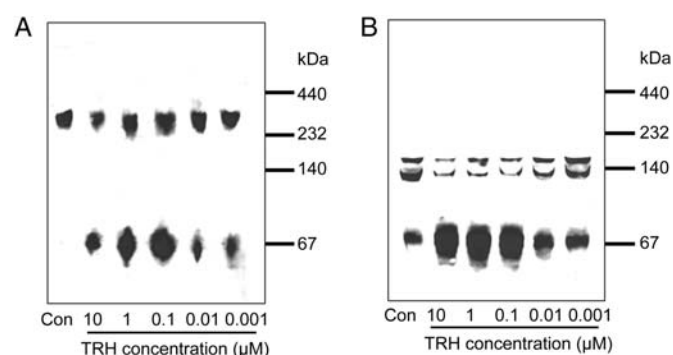


Figure 7 Effect of TRH concentration on dissociation/association of $G_{q/11}\alpha$ complexes HEK293 cells were treated with TRH at different concentrations (10, 1, 0.1, 0.01, and 0.001 μM) for 16 h. Samples of the postnuclear supernatants from the control and TRH-treated cells were solubilized with Brij 56 (A) or LM (B) and separated on 6%–15% linear gradient non-denaturing polyacrylamide gels. The resolved complexes were identified after electrotransfer onto PVDF membranes and incubation with anti- $G_{q/11}\alpha$ antibody.

Whereas dissociation of the G_{q/11} protein complex in the 140 kDa region was more pronounced in samples of cells treated with higher concentrations of TRH (10^{-5} – 10^{-7} M) and it was not observed in the case of 1×10^{-9} M TRH, the decomposition of the 300 kDa G_{q/11} protein complex was detected only in the presence of 1×10^{-5} M TRH. The formation of low-molecular-weight (70 kDa) complexes was quite prominent in samples of cells treated with TRH in the concentration range 10^{-5} – 10^{-7} M and the lower (physiological) hormone concentrations (10^{-8} – 10^{-9} M) produced similar changes but to a lesser extent. These results indicate that the observed dissociation/association of G_{q/11} protein complexes represents a general process induced by a wide range of TRH concentrations, which can apparently also occur under physiological conditions.

Determination of G₁₁α levels in the PNS, PM-enriched fraction and cytosol

To assess the presumed changes in subcellular distribution of G_{q/11}α protein and its possible down-regulation, samples of the PNS, PM-enriched, and cytosolic fractions from the control and TRH-treated cells (1×10^{-5} M; 10 min to 16 h) were resolved on 10% polyacrylamide gels by SDS-PAGE and G_{q/11}α was determined by immunoblotting. The

amount of G_{q/11}α in the PNS gradually decreased during the prolonged (4–16 h) treatment of cells with TRH, which indicates that the hormone caused the down-regulation of this protein [Fig. 8(A)]. In the same time interval of TRH treatment, the G_{q/11}α levels decreased in the PM-enriched fraction and increased in the cytosol [Fig. 8(B,C)], which reflects a partial subcellular redistribution of the protein.

Discussion

The translocation of Gα proteins (G_sα and G_{q/11}α) between PMs and the cytosol was observed in many previous studies using cell fractionation, immunofluorescence microscopy and cell live imaging [12–17,21–23]. Although the release of G-protein α subunits from the PM and their movement into the cytosol occurred within 1–20 min in most cases, the trafficking of G₁₁α protein in HEK293 caused by TRH receptor activation was detected only after 2 h of TRH treatment [24].

In the present study, we aimed to identify the presumed molecular complexes of the G_{q/11}α protein that may participate in the G-protein translocation processes after activation of the TRH receptor. We used HEK293 cell line expressing a high level of rat TRH-R1 and mouse G₁₁α protein, in which a subcellular redistribution of G₁₁α was investigated previously by immunofluorescence microscopy [24]. Using CN-PAGE and CN/SDS-PAGE, we were able to identify high-molecular-weight and membrane-bound G_{q/11} protein complexes with apparent sizes of ~300 and 140 kDa. Long-term treatment with TRH caused gradual decomposition of all these complexes and a concomitant accumulation of low-molecular-weight complexes of 70 kDa in the cytosol.

Interestingly, the 300 and 140 kDa complexes were only detected by CN-PAGE after solubilization with detergent Brij 56 and lauryl maltoside, respectively. The 140 kDa complexes were observed after solubilization with LM, digitonin, or Triton X-100 and subsequent separation by CN/SDS-PAGE. On the contrary, the 70 kDa complex was solubilized with all the applied detergents (Brij 56, LM, CHAPS, digitonin, and Triton X-100). These differences can be attributed to the specific properties of particular detergents and also to a different subcellular localization of the analyzed G_{q/11} protein complexes. It was reported previously that the solubilization efficiency depends not only on the content of membrane proteins and lipids, but also on the way of sample treatment [29–32]. Highly efficient solubilization of the 70 kDa complexes with all the used detergents can be explained by cytosolic localization of these complexes. On the contrary, the 300 and 140 kDa complexes associated with the membrane were only solubilized by some detergents. The observed variable efficiency of solubilization with different detergents of these complexes can

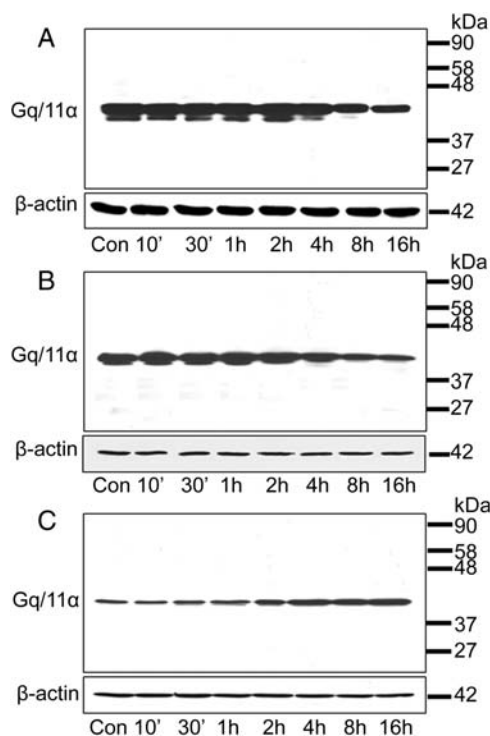


Figure 8 Assessment of TRH-induced redistribution and down-regulation of G_{q/11}α proteins. Samples of the PNS (A), PM-enriched fraction (B) and cytosol (C) from the control and TRH-treated (10 μM; 10, 30 min, 1, 2, 4, 8, and 16 h) HEK293 cells were resolved by SDS-PAGE. After electrotransfer onto nitrocellulose membranes, G_{q/11}α proteins were detected by the G_{q/11}α-specific antibody.

suggest that they are localized in distinct membrane areas which can perhaps differ in protein and lipid composition and/or structural membrane organization.

The protein complex in the 300 kDa region was shown not to contain the TRH receptor. Therefore, it seems that this complex is not directly engaged in TRH-R signaling. The observed dissociation of this complex after TRH treatment can be explained by the down-regulation of G_{q/11}α and/or its translocation from the PM to the cytosol. As the amount of a particular molecular complex obviously depends on the amounts and availability of its individual components, it seems to be quite logical that the hormone-induced decrease in the level of membrane-bound G_{q/11}α will lead to reduction of G_{q/11}α protein complexes in the PM.

Oh and Schnitzer [33] in a study dealing with the presumed interactions of caveolin-1 and -2 with different Gα proteins applied co-immunoprecipitation experiments to show that G_{q/11}α can associate with caveolin-1. It was demonstrated that the N-terminal domain of caveolin can interact with some types of Gα subunits with bound GDP and thus prevent the GDP/GTP exchange [34]. Therefore, it was of interest to examine whether the 300 or 70 kDa complexes involve caveolin or flotillin, which are both typical scaffolding proteins located in the caveolae [35]. Our experiments using CN-PAGE as well as CN/SDS-PAGE and specific antibodies against caveolin-1 and flotillin-2 failed to detect any signal on the blots. The obvious absence of caveolin-1 and flotillin-2 in our samples can be explained by a natural propensity of caveolae and lipid rafts not dissolving during solubilization by non-ionic detergents [36]. These membrane species are likely to be pelleted by centrifugation during the first step of protein fractionation after detergent solubilization and thus unavailable for further analyses.

A relatively high (10 μM) TRH concentration mostly used in the present work is not physiological and it may potentially cause cell damage or death [37]. However, we have previously found that the addition of 10 μM TRH to the HEK293 cell line stably expressing TRH-R1 and G₁₁μ protein does not lead to apoptosis, although the cells stop growing [38]. On the contrary, a prolonged incubation of these cells with TRH increased the Bcl-2/Bax ratio and elevated the level of some proteins with anti-apoptotic and protective effects. Here we investigated whether different TRH concentrations may exert similar effects on the stability of the identified protein complexes. Importantly, the treatment of HEK293 cells with a wide range of TRH concentrations (10⁻⁵–10⁻⁸ M, 16 h) caused dissociation of the G_{q/11} protein complex in the 140 kDa region and the formation of low-molecular-weight complexes comprising G_{q/11}α. As expected, the stability of these complexes was affected more significantly by higher hormone doses. Our results indicate that the higher the hormone concentration applied, the larger the extent of G_{q/11}α down-regulation and

translocation is detected. As insinuated above, the loss of G_{q/11}α from the PM could compromise the stability of the G_{q/11} complexes and dissociation of these complexes would thus ensue as a logical consequence of hormone action. The 140 kDa G_{q/11} protein complex was decomposed to a larger extent than the G_{q/11} protein complex in the 300 kDa region, which can be explained by the different character of these two membrane-bound complexes. Because the 140 kDa G_{q/11} protein complex is directly engaged in TRH receptor-coupled signaling, it could primarily dissociate in order to desensitize the hormone response.

One might envisage that the overexpression of proteins in transfected cells may significantly affect the formation of protein complexes and some artifacts can be encountered. Nevertheless, we have previously shown that the amount of TRH receptors and G_{q/11}α proteins in HEK293-E2M11 cells which were used in the present experiments is not excessively high and corresponds well to the endogenous expression levels of these proteins in rat brain cortex [25]. Hence, it might be assumed that the observed receptor and G-protein complexes may also exist in native cells and tissues.

In conclusion, our current results suggest that a portion of G_{q/11}α is translocated from the high-molecular-weight membrane-bound complexes of the sizes 300 and 140 kDa into the cytosolic fraction during TRH stimulation of HEK293 cells. This translocation is accompanied by a concomitant formation of low-molecular-weight complexes of 70 kDa. Both high-molecular-weight complexes contain the G_{q/11} protein and long-term treatment (4–16 h) with TRH induces the dissociation of these complexes, which can be caused partly by down-regulation of G_{q/11}α and partly by its translocation. A gradual reduction of the G_{q/11}α levels in the membrane-bound complexes observed during prolonged hormone stimulation can contribute to desensitization of the signal transduction via G_{q/11}-coupled receptors and, in parallel, G_{q/11}α released into the cytosol can transfer the signals to some cytosolic signaling proteins.

Acknowledgement

We are very grateful to Dr P. Svoboda (Institute of Physiology, Academy of Sciences of the Czech Republic) for allowing us to use HEK293 cell line expressing TRH receptors and G₁₁α protein.

Funding

This work was supported by grants from the Charles University Grant Agency (GAUK 6409) and the Ministry of Education, Youth and Sports of the Czech Republic (MSM0021620858).

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