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# The Time-Course of Agonist-Induced Solubilization of Trimeric $G_q\alpha/G_{11}\alpha$ Proteins Resolved by Two-Dimensional Electrophoresis

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**Summary**

Prolonged agonist stimulation results in specific transfer of activated Gα subunits of $G_q\alpha/G_{11}\alpha$ family from particulate membrane fraction to soluble (cytosol) cell fraction isolated as 250 000 x *g* supernatant. In this study, we have used 2D electrophoresis for more defined resolution of Gα subunits of $G_q\alpha/G_{11}\alpha$ family and followed the time course of solubilization effect. The small signal of soluble G proteins was already detected in control, hormone-unexposed cells. Hormone stimulation resulted in a slow but continuous increase of both intensity and number of immunoreactive signals/spots of these G proteins (10, 30, 60, 120 and 240 min). At longer times of agonist exposure (>2 hours), a marked increase of $G_q\alpha/G_{11}\alpha$ proteins was detected. The maximal level of soluble $G_q\alpha/G_{11}\alpha$ proteins was reached after 16 hours of continuous agonist exposure. At this time interval, eight individual immunoreactive signals of $G_q\alpha/G_{11}\alpha$ proteins could be resolved. The relative proportion among these spots was 15:42:10:11:7:7:2:5. Solubilization of this class of Gα proteins was thus observed after prolonged agonist stimulation only, induced by ultra high concentration of hormone and in cells expressing a large number of GPCRs. Our data therefore rather indicate tight/persisting binding of $G_q\alpha/G_{11}\alpha$ proteins to the membrane.

**Key words**

G proteins • Solubilization • $G_q\alpha/G_{11}\alpha$• Two-dimensional electrophoresis • Thyrotropin-releasing hormone • TRH receptor

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## Introduction

Number of independent studies indicated that the exposure of intact cells to hormonal stimulation results in redistribution of the cognate G-proteins from plasma membrane to the cell interior (internalization). In biochemical studies, G-protein α subunits were redistributed from plasma membranes to the light-vesicular membrane fractions distinct from plasma membrane (Haraguchi and Rodbell 1990, Svoboda *et al.* 1992, 1996, Kvapil *et al.* 1994, Svoboda and Milligan 1994). When analyzed by confocal immunofluorescence microscopy, Gα subunits were transferred from the cell surface into the intracellular sites of various origin (Drmota *et al.* 1998, 1999). Transfer of Gα subunits from the particulate membrane fraction to the soluble cell fraction (solubilization) was also demonstrated (for review see Svoboda and Novotný 2002). The agonist-induced subcellular redistribution of trimeric G-protein subunits is worth of further studies because it represents a potential mechanism for heterologous desensitization of the hormone response (Lohse 1993).

Solubilization of G-protein α subunits has been reported for the first time in S49 lymphoma cells. Stimulation of $\beta_2$-adrenergic receptors by isoprenaline resulted in redistribution of about half of the cellular $G_s\alpha$ from membranes to 250 000 x *g* supernatant (Ransnas *et al.* 1989). The agonist-induced solubilization of $G_s\alpha$ was also reported in myocardial membranes when exposed to the same agonist (Ransnas *et al.* 1991, 1992). In mouse mastocytoma cells, solubilization of $G_s\alpha$ was induced by iloprost (*via* stimulation of IP prostanoid receptors).

Thrombin-induced translocation of $G_{i2}\alpha$ from membranes to soluble fraction was also reported in these cells (Takahashi *et al.* 1991, Negishi *et al.* 1992). Agonists acting on receptors for vasoactive intestinal peptide in rat pituitary GH4C1 cells induced release of activated $G_s\alpha$ from membranes to cytosol along with its down-regulation, i.e. decrease of total cellular amount (Yajima *et al.* 1998). Besides G-proteins of $G_s\alpha$ and $G_i\alpha/G_o\alpha$ families, evidence for solubilization of $G_q\alpha/G_{11}\alpha$ proteins has also been provided. In stably transfected HEK-293 cells, prolonged stimulation by thyrotropin-releasing hormone receptors caused a dramatic shift of $G_q\alpha/G_{11}\alpha$ from membranes to a high-speed supernatant 250 000 x *g* (Svoboda *et al.* 1996, Drmota *et al.* 1998, 1999). Agonist-induced translocation of $G_q\alpha/G_{11}\alpha$ immunoreactivity from plasma membranes to cytosol fraction was also reported in MDCK cells (Arthur *et al.* (1999). The soluble forms of $G_i\alpha$ and $G_s\alpha$ proteins were identified in the brain (Ihnatovych *et al.* 2001) and heart-muscle (Novotný *et al.* 2001) and they were found to be altered by physiological state of the tissue such as ontogenetic development.

In this work, we decided to resolve members of $G_q\alpha/G_{11}\alpha$ family by 2D electrophoresis and analyze the time-course of agonist effect on solubilization of these G proteins.

## Materials and Methods

*Materials*

All materials for tissue cultures were supplied by Sigma-Aldrich (Poole, U.K.) and Invitrogen (Paisley, U.K.). The complete protease inhibitor cocktail was from Roche Diagnostics Ltd. (Lewes, U.K.). Immobiline DryStrips, IPG buffer and secondary anti-rabbit antibody marked with horseradish peroxidase were purchased from Amersham Biosciences (Chalfont St. Giles, U.K.). Duracryl (30 % acrylamide, 0.8 % bisacrylamide) was from Genomic Solutions (Huntingdon, U.K.). All other chemicals and materials were from Sigma-Aldrich and Merck (Darmstadt, Germany) and were of the best grade available. The rabbit anti-$G_q\alpha/G_{11}\alpha$ antiserum (CQ) was oriented against C-terminus decapeptide of $G_q\alpha$ and $G_{11}\alpha$ and was prepared in the Prague laboratory.

*Stable transfection of HEK-293 cells*

Clone E2M11 of HEK-293 cells stably expressing high levels of the rat TRH receptor and murine $G_{11}\alpha$, was prepared as described previously by Svoboda *et al.* (1996). Briefly, a full-length rat (long isoform) TRH receptor cDNA (2.2 kb) (Sellar *et al.* 1993) was subcloned into the eukaryotic expression vector pcDNA1 (Invitrogen), which is driven by the cytomegalovirus (CMV) promoter. HEK-293 cells were co-transfected with linearized pcDNA1/TRH receptor (800 ng) and pSPneo (200 ng, Invitrogen) using Lipofectin reagent (30 mg, Life Technologies, Inc., Paisley, Strathclyde, UK) in serum-free Dulbecco's modified Eagle's medium (DMEM). Resultant geneticin-resistant clones were picked, and TRH receptor containing clones were identified as those in which TRH produced a rise in total inositol phosphate production. Expression of the TRH receptor in membranes from these clones was assessed by the specific binding of [$^3$H]TRH. Clone E2, which expresses some 14 pmol of the receptor/mg of membrane protein (Kim *et al.* 1994), was selected for further transfection with plasmid pCMV, into which a cDNA encoding murine $G_{11}\alpha$ was inserted, and with the plasmid pBABE hygro, which allows expression of resistance to the antibiotic hygromycin B. Clones were selected on the basis of resistance to hygromycin B, and the continued expression of the TRH receptor and novel expression of murine $G_{11}\alpha$ were examined (Kim *et al.* 1994, Svoboda *et al.* 1996).

*Cell culture*

HEK-293 cells (clone E2M11) were maintained in Dulbecco's modified Eagle's medium supplemented with 0.55 mg/ml L-glutamine and 10 % (v/v) heat-inactivated newborn calf serum and with selection markers geneticin (0.8 mg/ml) and hygromycin B (0.2 mg/ml) at 37 $^{o}$C in a 5 % $CO_2$ humidified atmosphere. E2M11 cells were cultivated in cell culture dishes according to the protocol of Svoboda *et al.* (1996).

*Cell fractionation*

E2M11 cells were grown to 70-80 % confluence and the day before the experiment the medium was replaced by fresh medium. Cells were incubated with 10 μM TRH in growth medium at the following time points – 0 min (control, naive, hormone-unexposed cells), 10 min, 30 min, 1 h, 2 h, 4 h and 16 h at 37 ºC in a 5 % $CO_2$ humidified atmosphere prior to the harvest. Cells were harvested from cell culture dishes (152 cm$^2$ each, 1.96 x 10$^6$ cells per dish); 7 dishes were used per each time point. Agonist stimulation was arrested by placing dishes on ice and by aspirating the medium. Cells were scraped in ice-cold phosphate-buffered saline solution, PBS (138 mM NaCl, 2.8 mM KCl, 1.5 mM $KH_2PO_4$,

10 mM $Na_2HPO_4$, pH 7,4) and washed twice in this solution by spinning at 3000 rpm for 5 min, 4 ºC. The final cell sediment was frozen at –80 ºC for at least 1 h.

The resulting sediment was resuspended by repeated pipetting in 3500 µl (500 µl per dish) of 250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA (STE buffer) containing freshly added 1 mM PMSF and protease inhibitors. The cell suspension was homogenized by hand in a glass-glass homogenizer for 7 min on ice. Each sample was then spun at 1000 rpm for 5 min at 4 ºC and the resulting supernatant (post-nuclear supernatant) was spun again at 250 000 x *g* for 2 h at 4 ºC. The resulting supernatant represented soluble (cytosol) fraction and was stored at –80 ºC until use.

*TCA precipitation and extraction in diethylether/ethanol*

Post-nuclear supernatant and soluble (cytosol) fractions were prepared as described in the previous paragraph and precipitated with ice-cold 7.5 % (w/v) TCA on ice for 1 h. The mixture was then centrifuged at 16 000 x *g* for 10 min at 4 ºC. The supernatant was discarded and the pellet was treated with 1 ml of ice-cold 5 % (w/v) TCA on ice for 10 min. The sample was then centrifuged at 16 000 x *g* for 5 min at 4 ºC. The supernatant was aspirated and the pellet was washed twice with ice-cold diethylether/ethanol (1:1, v/v) by mixing end-over-end for 30 min at room temperature. The pellet was regained in each step by centrifugation at 16 000 x *g* for 10 min at 4 ºC. For 2D electrophoresis the pellet was air-dried for a couple of minutes and dissolved in denaturating 2D sample buffer containing 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 1 % (v/v) IPG buffer, pH gradient 4-7, 1 % (w/v) DTT by shaking for 1 h at room temperature. The sample was cleared by centrifugation at 16 000 x *g* for 10 min at room temperature. The supernatant arising from this centrifugation was referred to as fraction 1. The remaining pellet was vortexed with freshly added 2D sample buffer for 1 h at room temperature, spun at 16 000 x *g* for 10 min at room temperature. The supernatant was added to fraction 1.

*Isoelectric focusing (IEF)*

The 18 cm linear gradient pH 4-7 ready-to-use Immobiline DryStrips were loaded with 400 µg of protein in 360 µl of 2D sample buffer with trace of bromophenol blue per strip by passive rehydration overnight at room temperature. Strips were transferred into coffins and covered with thin layer of mineral oil to prevent evaporation. Isoelectric focusing was performed on an Amersham IPGphor device, using a step-and-hold protocol of increasing voltage as follows: 30 V for 15 Vh, 150 V for 1000 Vh, 500 V for 500 Vh, 1500 V for 1 500 Vh, 3 000 V for 3 000 Vh, 8 000 V for 85 000 Vh, and 2 000 V for 24-48 h. The electrofocused strips were stored at –20 ºC before the second dimension.

*Two-dimensional SDS-polyacrylamide gel electrophoresis*

The IPG strips were rinsed thoroughly with distilled water, dried quickly on filter paper and equilibrated prior to the second dimension electrophoresis in 4 ml of equilibration buffer [30 % (v/v) glycerol, 2 % (w/v) SDS, 6 M urea, 50 mM Tris-HCl, pH 6.8] in Immobiline Dry Strip Re-swelling Tray. Subsequently, disulphide bridges present in protein molecules and other oxidized groups were reduced in equilibration buffer containing 2 % (w/v) DTT and alkylated in equilibration buffer containing 4.5 % (w/v) IAA, for 20 min each. After this, the IPG strips were rinsed thoroughly with distilled water, dried quickly on filter paper and treated with Laemmli solubilization buffer (SLB) [25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS)] for 10 min. The 14 cm x 16 cm x 1 mm gels were cast with 10 % (v/v) polyacrylamide mixture with double amount of SDS [0.2 % (w/v) SDS final concentration], using Hoefer SE 600 unit (2 x 16 cm x 18 cm). Equilibrated strips were cut 3 cm from left side and 2 cm from right side and loaded horizontally on the top of the gel. Molecular weight markers were loaded onto a piece of filter paper and placed close to the acidic side of the strip. The strip and molecular weight markers were sealed in place with 0.5 % (w/v) agarose in SLB containing bromophenol blue. Gels were run vertically in SLB at a constant voltage of 30 V for 30 min and then at constant voltage of 50 V overnight till the bromophenol blue dye reached the end of the gel (the apparatus was cooled to 20 ºC throughout electrophoresis).

*Production of antisera*

$G_q\alpha/G_{11}\alpha$ protein oriented antibody was rabbit polyclonal antipeptide serum raised in rabbits obtained from VELAZ (Prague). Immunization was performed with a glutaraldehyde conjugate of keyhole limpet hemocyanin and synthetic peptide QLNLKEYNLV representing the C-terminal decapeptide, which is conserved between $G_q\alpha$ and $G_{11}\alpha$. This antiserum identifies both polypeptides equally (Mitchell *et al.* 1991).

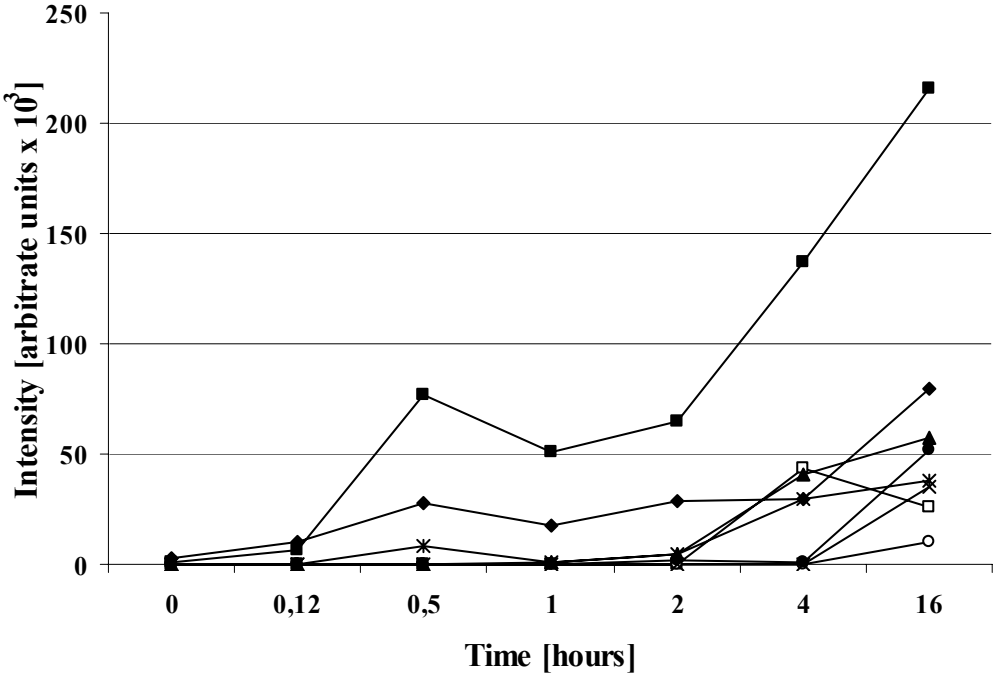


**Fig. 1.** The time-course of TRH-induced solubilization of $G_q\alpha/G_{11}\alpha$ proteins. E2M11-HEK-293 cells were either untreated (0) or exposed to 10 μM TRH for 10 min (0.12 h), 30 min (0.5 h), 60 min (1 h), 2 (2 h), 4 (4h) or 16 hours (16 h). Cell harvest and homogenization was performed as described in Methods. The soluble (cytosol) cell fraction was prepared by centrifugation of post-nuclear supernatant at 250 000 x *g* for 2 h. Cytosolic (soluble) proteins were concentrated by TCA precipitation, resolved by 2D electrophoresis and $G_q\alpha/G_{11}\alpha$ proteins identified by immunoblotting with CQ antiserum oriented against C-terminal decapeptide of $G_q\alpha$ and $G_{11}\alpha$. The results show the average intensity of immunoblot signals (expressed in arbitrary units) collected from 4 independent experiments at each time interval of agonist exposure. Spot 1, open squares; spot 2; open circles; spot 3, stars, spot 4, crosses, spot 5, full triangles; spot 6, full circles; spot 7, full squares, spot 8, full rhombuses.

*Immunoblotting*

After the second dimension, proteins were transferred to nitrocellulose membrane by semi-dry blotting. Transfer was performed at 21 V (constant voltage) for 90 min. Membranes were blocked with 5 % fat-free milk in PBS with 0.05 % (v/v) Tween 20 for 1 h at room temperature. Subsequently, the blots were incubated in 1 % fat-free milk, 0.05 % (v/v) Tween 20 in PBS with appropriate primary antibody for 2 h at room temperature. Rabbit antiserum against $G_q\alpha/G_{11}\alpha$ (84/1) was used at a 1:5000 dilution. After extensive washing with 0.05 % (v/v) Tween 20 in PBS (3x10 min), the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated with secondary anti-rabbit antibody which was diluted 20 000 x in 1 % fat-free milk in PBS with 0,05 % (v/v) Tween 20. After 3x 10 min washes with 0.05 % (v/v) Tween in PBS, the blots were developed by ECL Plus Kit (Amersham).

*Image Analysis*

The immunoblot signals were visualized on films, subjected to densitometric scanning and analyzed by PDQuest.

## Results

HEK293 cells expressing large amount of TRH-receptors and exogenous, mouse isoform of $G_{11}\alpha$ (clone E2M11) were incubated with 10 μM TRH for increasing periods of time – 0 min (control), 10 min, 30 min, 60 min, 120 min, 4 h and 16h. Harvested control and hormone-treated cells were subjected to homogenization and low-speed centrifugation in order to isolate post-nuclear supernatant from nuclear fraction. Subsequently, the cytosol fraction was prepared by high-speed centrifugation of post-nuclear supernatant (250 000 x *g*, 2 h, 4 °C) as described in Methods. The cytosol fraction was concentrated by TCA precipitation, extracted in diethylether/ethanol and resolved in the first dimension according to isoelectric points (1st D). The standard SDS-PAGE in 10 % polyacrylamide gel was used afterwards for resolution in the second dimension according to relative molecular weights (2nd D). $G_q\alpha/G_{11}\alpha$ proteins were then detected by Western blotting and ECL with specific anti-$G_q\alpha/G_{11}\alpha$ antibodies. The immunoblots were quantified by

**Table 1.** Quantitative analysis of the time-course of TRH-induced solubilization of $G_q\alpha/G_{11}\alpha$ proteins. The same legend as in Fig. 1. The results show the average intensity of immunoblot signals collected from 4 independent experiments at each time interval of agonist exposure. The total signal in control, hormone-unexposed cells (100 %) was compared with the total signal of all spots resolved at a given time interval of 0.12, 0.5, 1, 2, 4 and 16 hours.

| Time [h] | 0 | 0.12 | 0.5 | 1 | 2 | 4 | 16 |
|---|---|---|---|---|---|---|---|
| *Spot 1* | ND | ND | ND | ND | ND | 43349 | 25902 |
| *Spot 2* | ND | ND | ND | ND | ND | ND | 10009 |
| *Spot 3* | ND | ND | 8004 | 1284 | 4693 | 30034 | 37508 |
| *Spot 4* | ND | ND | ND | ND | ND | ND | 35439 |
| *Spot 5* | ND | ND | ND | 813 | 4920 | 40712 | 57748 |
| *Spot 6* | ND | ND | ND | ND | 1797 | 951 | 51661 |
| *Spot 7* | 1096 | 6489 | 76636 | 50915 | 64409 | 136745 | 215707 |
| *Spot 8* | 2368 | 9884 | 27987 | 17678 | 28849 | 29987 | 79901 |
| *Total* | 3463 | 16373 | 112626 | 70689 | 104668 | 281778 | 513876 |
| *%* | 100 | 473 | 3252 | 2041 | 3022 | 8136 | 14837 |

**Table 2.** The relative proportion among signals corresponding to $G_q\alpha/G_{11}\alpha$ proteins resolved by 2D electrophoresis and detected by immunoblotting in supernatant 250 000 x *g* prepared from e2m11-HEK293 cells stimulated by TRH for 16 hours.

| Spot | Average signal | % |
|---|---|---|
| *1* | 25902 | 5 |
| *2* | 10009 | 2 |
| *3* | 37508 | 7 |
| *4* | 35440 | 7 |
| *5* | 57749 | 11 |
| *6* | 51661 | 10 |
| *7* | 215707 | 42 |
| *8* | 79901 | 16 |
| *Total* | 513876 | 100 |

densitometric scanning and analyzed by PDQuest.

Small signal of the two soluble $G_q\alpha/G_{11}\alpha$ proteins was already detected in control, hormone-unexposed cells (spot 7 and 8, Fig. 1, time zero). Hormone stimulation for 10, 30, 60 and 120 min resulted in an increase of both intensity and number of immunoreactive signals/spots of these G proteins (Fig. 1, 0.12-2 h); at long time intervals (>2 h), significant increase of $G_q\alpha/G_{11}\alpha$-related immunoreactivity was detected (Fig. 1, 2-16 h). Comparison of the data collected from various time intervals indicated that the maximal increase of $G_q\alpha/G_{11}\alpha$ in soluble fraction was reached after 16 h of incubation with TRH. At this time period, eight distinct immunoreactive proteins were clearly resolved and the total intensity of these spots was 15 times higher than in control cells (Table 1, see the next paragraph for further details).

Various numbers of immunoblot signals of $G_q\alpha/G_{11}\alpha$ proteins were observed in the course of the hormone stimulation. The two signals were already detected in control cells (spot 7 and 8) and exposure to TRH for 10 and 30 min resulted in increased intensity of these spots and appearance of the third one (spot 3). Two major and three minor spots were identified after 2 h of stimulation and, as already mentioned in the previous paragraph, eight immunoreactive signals were identified after 16 h of hormone stimulation (Fig. 1). The relative proportion among these spots was 15:42:10:11:7:7:2:5 (Table 2). It is reasonable to assume that the two major signals correspond to exogenously (over)expressed mouse isoform of $G_{11}\alpha$ and endogenous human $G_q\alpha$. Our previous studies indicated that the presence of human $G_{11}\alpha$ was very low (Kim *et al.* 1994, Matoušek *et al.* 2004, 2005). It may be assumed that the minor signals, which were also clearly distinguished, represent covalently modified forms of the two major G proteins exhibiting the same relative molecular weight but different isoelectric points.

The isoelectric points of the two major forms of $G_q\alpha/G_{11}\alpha$ (estimated from the isoelectric focusing data) were at pH 5.7 and 5.9 (Fig. 2). Previously, we studied resolution of $G_q\alpha/G_{11}\alpha$ proteins in E2M11-HEK-293 cells by two-dimensional electrophoresis, but in membrane preparations (Matoušek *et al.* 2004, 2005). Five different signals were detected indicating either resolution of multiple members of $G_q\alpha/G_{11}\alpha$ family or some post-

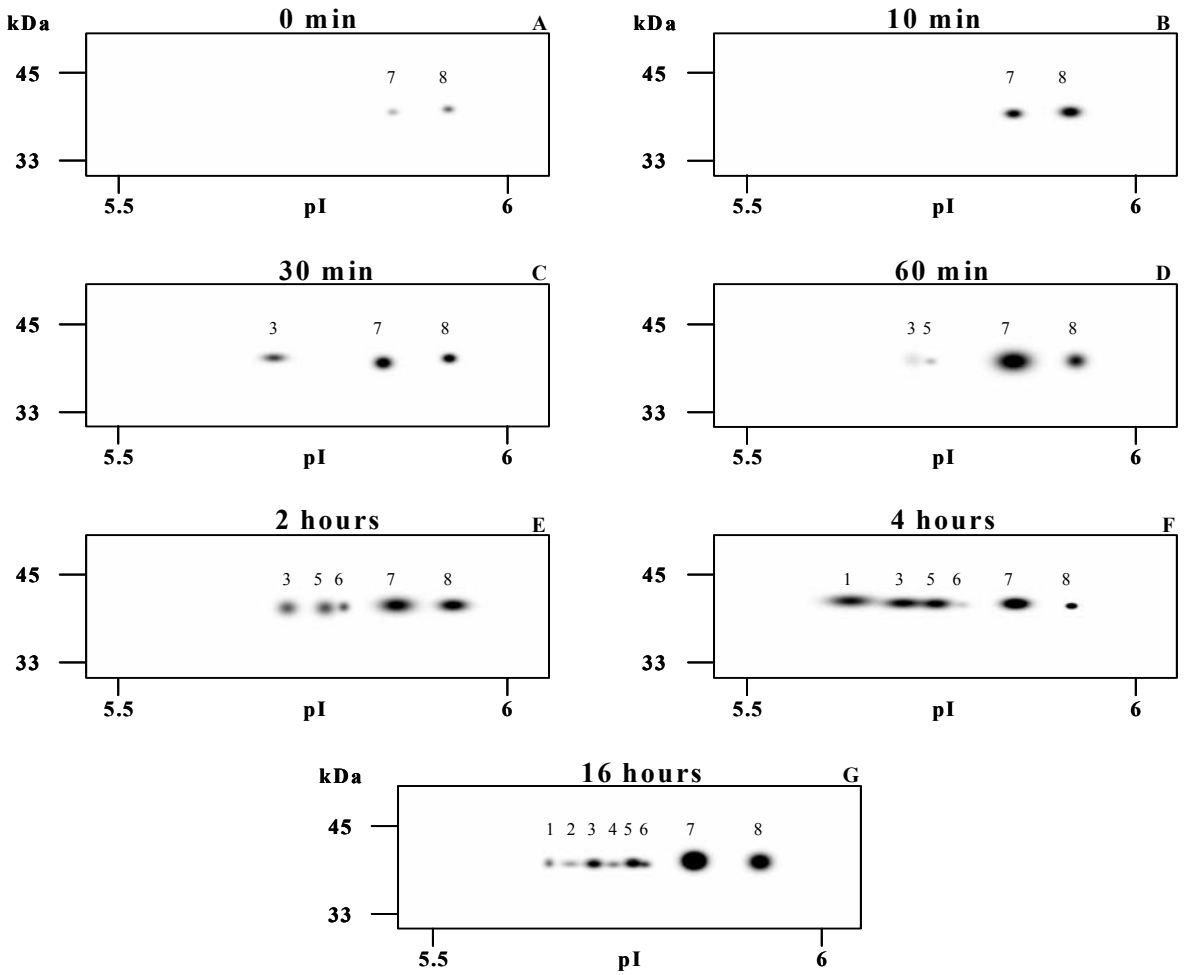


**Fig. 2.** PDQuest images of the soluble $G_q\alpha/G_{11}\alpha$ proteins resolved at various time intervals of TRH-exposure. Samples collected from hormone-unexposed, naive cells (0 min) and TRH-treated cells (10 min, 30 min, 60 min, 2, 4 and 16 hours) were resolved by 2D electrophoresis and analyzed by PDQuest program.

translational modification of the dominant proteins of $G_q\alpha/G_{11}\alpha$ family. In membranes, the two major spots corresponding to exogenously (over) expressed $G_{11}\alpha$ and endogenous $G_q\alpha$ were reduced; the minor spots were diminished to zero level (after 16 h of hormonal stimulation). Both lines of experimental evidence thus converge to each other and indicate that prolonged agonist stimulation of TRH-R in cells expressing large amount of these receptors results in a dramatic but slow decrease of membrane-bound complement of $G_q\alpha/G_{11}\alpha$. This decrease is accompanied by an increase of these proteins in the soluble, cytosol fraction, which is also very slow.

## Discussion

Post-translational modification of Gβγ subunits is regarded as an important cause of membrane localization of trimeric G-protein complex in hormonally non-stimulated cells. Gγ subunits are prenylated (Mumby *et al.* 1990a) and α subunits of $G_i$ family are myristoylated (Mumby *et al.* 1990b). In addition, nearly all Gα subunits are acylated on cysteine residues near amino termini by formation of thioesters, usually with palmitate (Parenti *et al.* 1993, Linder *et al.* 1993). Interaction with Gβγ is required for membrane targeting and palmitoylation of $G_s\alpha$ and $G_q\alpha$ (Evanko *et al.* 2000) and Gβγ isoforms selectively rescue plasma membrane localization and palmitoylation of mutant $G_s\alpha$ and $G_q\alpha$ (Evanko *et al.* 2001). Although there is general agreement that activation of G-proteins causes an increased turnover of palmitate on Gα, the topological fate of these proteins is not settled. It was proposed that activation and depalmitoylation of Gα promote its release from the membrane (Wedegaertner and Bourne 1994, Wedegaertner *et al.* 1995). It has also been reported that functional interaction between the $\alpha_{1b}$-adrenoreceptor and $G_{11}\alpha$ is reduced by de-palmitoylation of the G protein

(Novotný *et al.* 2006). On the other hand, data of Huang *et al.* (1997, 1999) supported a different conclusion: membrane association of Gα persists whether the protein is activated and/or depalmitoylated.

Hormonal or neurotransmitter stimulation results in an uncoupling of Gα from Gβγ subunits. Though evidenced mainly from a functional point of view (Gilman 1987), it is reasonable to assume that in hormonally stimulated cells, membrane attachment of Gα is weakened when compared with unstimulated cells. Aliphatic chains of fatty acids such as palmitic and myristic acid represent the only sites, which in the presence of hormone are linking Gα subunits to the membrane and protecting Gα from detachment from the membrane, i.e. solubilization (Iiri *et al.* 1996, Evanko *et al.* 2000, 2001). Potential for detachment from the membrane and solubilization of Gα subunits is further increased under the conditions of sustained agonist stimulation and/or in cells expressing high amount of corresponding receptors. It is reasonable to assume that under such conditions, equilibrium between Gα-GTP and Gα-GDP is shifted to „free" Gα-GTP, which is more susceptible to detachment from the membrane.

With the aim to bring a more detailed picture of agonist-induced release of G proteins from the membrane, the time course of TRH (thyrotropin-releasing hormone) solubilization of $G_q\alpha/G_{11}\alpha$ was analyzed in HEK-293 cells stably expressing a large amount of TRH-R. The soluble (cytosolic) forms of $G_q\alpha/G_{11}\alpha$ were resolved by 2D electrophoresis. In spite of the large number of receptors activated by continuous agonist presence, $G_q\alpha/G_{11}\alpha$ proteins were almost undetectable in soluble fraction till the 10th minute of continuous agonist exposure. The significant increase was observed after 0.5 to 2 h of stimulation and prolongation of hormone stimulation up to 16 h was reflected by the further increase of $G_q\alpha/G_{11}\alpha$ immunoreactivity in the soluble fraction. Membrane association of various species of $G_q\alpha/G_{11}\alpha$ thus persists under drastic conditions of hormonal stimulation and the release of these Gα subunits does not seem to follow rather fast palmitoylation/ depalmitoylation cycles and/or interaction(s) with Gβγ subunits.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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