Physiol. Res. 53: 295-303, 2004

Resolution of $G_s\alpha$ and $G_q\alpha/G_{11}\alpha$ Proteins in Membrane Domains by Two-Dimensional Electrophoresis: The Effect of Long-Term Agonist Stimulation

P. MATOUŠEK[1,2], J. NOVOTNÝ[1,2], P. SVOBODA[1,2]

[1]Institute of Physiology, Academy of Sciences of the Czech Republic, [2]Laboratory of Molecular Pharmacology, Department of Physiology, Faculty of Natural Sciences, Charles University, Prague, Czech Republic

Received April 23, 2003
Accepted August 7, 2003

Summary

Low-density membrane-domain fractions were prepared from S49 lymphoma cells and clone e2m11 of HEK293 cells expressing a large number of thyrotropin-releasing hormone receptor (TRH-R) and $G_{11}\alpha$ by flotation on sucrose density gradients. The intact cell structure was broken by detergent-extraction, alkaline-treatment or drastic homogenization. Three types of low-density membranes were resolved by two-dimensional electrophoresis and analyzed for $G_s\alpha$ (S49) or $G_q\alpha/G_{11}$ (e2m11) content. Four individual immunoblot signals of $G_s\alpha$ protein were identified in S49 lymphoma cells indicating complete resolution of the long $G_s\alpha$L±ser and short $G_s\alpha$S±ser variants of $G_s\alpha$. All these were diminished by prolonged agonist (isoprenaline) stimulation. In e2m11-HEK cells, five different immunoblot signals were detected indicating post-translational modification of G proteins of $G_q\alpha/G_{11}\alpha$ family. The two major spots corresponding to exogenously (over)expressed $G_{11}\alpha$ and endogenous $G_q\alpha$ were reduced; the minor spots diminished by hormonal stimulation. Parallel analysis by silver staining of the total protein content indicated that no major changes in protein composition occurred under these conditions. Our data thus indicate that agonist-stimulation of target cells results in down-regulation of all different members of G_s and G_q/G_{11} families. This agonist-specific effect may be demonstrated in crude membrane as well as domain/raft preparations and it is not accompanied by changes in overall protein composition.

Key words
G-Proteins • Two-dimensional electrophoresis • Desensitization • Membrane domains • Rafts

Introduction

GTP-binding regulatory proteins (G-proteins) constitute a family of proteins that convey signal transmission from hormone- or neurotransmitter-activated receptors to a various effector systems. They consist of three subunits α, β and γ, but only the α subunit defines each G-protein (Rodbell 1980, Gilman 1987, Kaziro *et al.* 1991, Simon *et al.* 1991). The stimulatory G-protein (G_s) transduces signals from stimulatory hormone receptors, among which the β-adrenergic or glucagon receptor are of the highest physiological importance, to all types of mammalian adenylyl cyclases (Stryer and Bourne 1986, Tang and

Gilman1992). The $G_s\alpha$ was identified in plasma-membrane fractions, hepatic microsomes and (canine) sarcoplasmic reticulum (Haga *et al.* 1977, Codina *et al.* 1988). The $G_s\alpha$ was also found in soluble cytosol fraction (supernatant 250 000 x*g*) and redistributed between membranes and cytosol after prolonged agonist stimulation (Ransnas *et al.* 1989). Because of alternative splicing of the precursor mRNA, four distinct isoforms of α subunit of $G_s\alpha$ exist: long $G_s\alpha$L±Ser and short $G_s\alpha$S±Ser with apparent molecular masses of 45 000 Da ($G_s\alpha$S) and 52 000 Da ($G_s\alpha$L) as calculated from SDS-PAGE (Kozasa *et al.* 1988, Robishaw *et al.* 1986). To study the β-adrenergic receptor-$G_s\alpha$-adenylyl cyclase pathway, we used the S49 lymphoma cells as a widely used model.

The $G_{11}\alpha$ protein falls into the G_q/G_{11} family that activates phosphoinositidase C (Aragay *et al.* 1992). We have produced a clonal cell line e2m11, which is derived from human embryonic kidney cells HEK293 that stably express a high amount of rat thyrotropin-releasing hormone (TRH) receptor and murine $G_{11}\alpha$ protein (Svoboda *et al.* 1996). Using a combination of confocal immunofluorescence microscopy and subcellular fractionation followed by immunoblots we found out that short-term agonist treatment of cells results in transfer of a significant portion of $G_{11}\alpha$ protein from plasma membranes to light-vesicular and cytosolic fractions, but long-term agonist treatment of cells results in an overall decrease of $G_{11}\alpha$ protein content. This proceeds on a much slower time scale than internalization of the corresponding receptor (Drmota *et al.* 1998, 1999, Pešanová *et al.* 1999). The stimulation of S49 lymphoma cells by isoprenaline or mastocytoma cells by iloprost also resulted in a transfer of a significant portion of $G_s\alpha$ from membrane to cytosol (Kvapil *et al.* 1994, Ransnas *et al.* 1989).

In this work we have studied the $G_s\alpha$-mediated cascade initiated by β-adrenergic receptor and compared it with the G_q/G_{11}-mediated cascade stimulated *via* TRHR-1. G-protein levels have been examined in distinct regions of the cell membranes – membrane domains or low density membrane fractions isolated by the flotation in sucrose density gradient (Pešanová *et al.* 1999, Bouřová *et al.* 2003, Song *et al.* 1996a,b) after the detergent extraction, alkaline treatment and drastic homogenization of the harvested cells. So-called "membrane domains" or "lipid rafts" are part of the plasma membrane of size of about 100 nm, which are resistant to the treatment of non-ionic detergent such as Triton X-100 at low temperatures. They are enriched in cholesterol, sphingolipids and GPI-proteins mainly engaged in the signal transduction (Shenoy-Scaria *et al.* 1993, Solomon *et al.* 1996, Cinek and Hořejší. 1992, Černý *et al.* 1996). However, membrane domains can also be prepared by detergent-free methods. Our second aim was to investigate the effect of long-term agonist treatment on the protein composition/reorganization of the membrane domains. We demonstrated that this process is highly specific as the agonist treatment was not accompanied by non-specific reorganization of membrane domains.

Methods

Tissue culture reagents and media were supplied by Gibco BRL (Renfrewshire, UK) or Sevac (Prague, Czech Republic). All other chemicals and drugs, including geneticin and hygromycin, were purchased from Sigma (St. Louis, MO, USA) and were of highest purity available. $G_s\alpha$-oriented polyclonal antiserum G-5040 was also from Sigma. Complete protease inhibitor cocktail was from Roche Diagnostic, Mannheim, Germany (1697498).

Cell cultures

Human embryonic kidney cells (clone e2m11) that stably express high amounts of TRH-R and $G_{11}\alpha$, were cultivated in Dulbecco´s modified Eagle´s medium supplemented with 10 % (v/v) heat-inactivated newborn calf serum, geneticin (800 μg/ml) and hygromycin B (200 μg/ml) at 37 °C under 5 % CO_2 atmosphere. The "treated" cells were incubated at 37 °C with 10^{-5} M TRH for 16 h. S49 lymphoma cells were cultivated in Dulbecco´s modified Eagle´s medium supplemented with 10 % (v/v) heat-inactivated horse serum at 37 °C under 5 % CO_2 atmosphere. The "treated" cells were incubated at 37 °C with 10^{-5} M (-)-isoproterenol (isoprenaline) for 16 h to which superoxide dismutase and catalase (both 10 μg/ml) were added, which blocked the degradation of (–)-isoproterenol and maintained the cell viability.

Isolation of crude membrane fraction

Cells were harvested from 6 x 80 cm^2 flasks (e2m11) or 150 ml culture medium (S49, 0.8-1.2 x 10^6 cells/ml) by centrifugation for 10 min at 1800 rpm, homogenized in 250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA (STE medium) containing freshly added 1 mM PMSF and complete protease inhibitors cocktail (1 tablet per 50 ml) by using tight teflon-glass homogenizer (7 min at 1700 rpm; Brown, Germany) and centrifuged at low-speed for 5 min at 1000 rpm. The crude membrane preparation was

sedimented from arising supernatant (post-nuclear supernatant) by centrifugation for 2 h at 250 000 x g. The resulting pellet was re-homogenized mildly by hand in STE medium (10-15 mg protein per ml), snap frozen in liquid nitrogen and stored at –80 °C until use. The 250 000 x*g* supernatant represented the soluble, cytosol cell fraction.

Isolation of membrane domains

e2m11-HEK cells were harvested from 15 flasks (15 x 80 cm^2); S49 cells were harvested at a concentration of 0.8-1.2 x 10^6 cells/ml. Cells were collected by centrifugation at 1800 rpm for 10 min. The cellular pellet was used as a starting material for the following methods of membrane domains isolation.

A. Detergent extraction procedure: Detergent-insensitive domains (DIMs) were prepared as follows: the cellular pellet was resuspended up to 2 ml of 1 % v/v Triton T-100, 20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride and was left 45 min on ice (0 °C). The resulting cell lysate (2 ml) was subjected to flotation gradient centrifugation. It was mixed with ice-cold 80 % (w/v) sucrose, transferred into a Beckman SW41 rotor centrifuge tube and overlaid with 35, 30, 25, 20, 15, 10 (1 ml each) and 5 % (w/v) sucrose (1.5 ml). The gradients were centrifuged at 39 000 rpm at 4 °C for 24 h. Sucrose density gradient fractions were collected manually from the meniscus by 0.5 ml (the first fraction) and 1 ml (the remaining fractions). Fractions 3 to 6 from sucrose gradient were mixed and precipitated with 6 % TCA w/v (final concentration) on ice for 1 h. The mixture was centrifuged at 10 000 rpm for 10 min at 4 °C. The pellet was washed with ethanol (96 %) and centrifuged again. The precipitate was stored at –20 °C.

B. Alkaline-treatment procedure: Alkaline-treated domains were prepared as follows: the cellular pellet was resuspended up to 1 ml of 20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride, then mixed with 1 ml of 1 M Na_2CO_3 (pH 11) and sonicated 3 times for 10 s on ice (0 °C) at low energy output. The resulting cell lysate was subjected to gradient ultracentrifugation and TCA precipitation as described in *A.*

C. Drastic homogenization procedure: Low-density membrane fragments were prepared as follows: the cellular pellet was resuspended up to 2 ml of 20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride and homogenized in the Elvehjm-Potter glass-teflon homogenizer for 10 min on ice (0 °C) at 1800 rpm. The resulting cell lysate was subjected to gradient ultracentrifugation and TCA precipitation as described in *A.*

Isoelectric focusing (IEF) and two-dimensional (2D) electrophoresis

IEF on immobilized pH gradients (pH 4-9) was used in this study. IPG strips were prepared according to the manufacturer protocol (Pharmacia) and stored at –20 °C. IEF was performed on a Multiphore II system (Amersham Pharmacia Biotech). Sample precipitates were resolved in 7 M urea, 2 M thiourea, 4 % CHAPS w/v, 1 % (v/v) Pharmalyte 3-10 (Amersham Pharmacia Biotech), 1 % (w/v) DTT and trace of bromphenol blue for 1 h at room temperature. An aliquot (200 µl containing 350 µg of protein) of the solution was transferred into Immobiline DryStrip Reswelling Tray (Amersham Pharmacia Biotech). A pre-cast IPG dry strip was placed into the mixture gel-side down. After rehydration for 12 h at room temperature, IEF was performed with the following power supply settings: 150 V for 1 Vh, 150 V for 750 Vh, 500 V for 500 Vh, 3500 V for 10 kVh, 3500 V for 32 kVh. The electrofocused strips were stored at –20 °C before the second dimension.

SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used in the second-dimensional separation. Before the SDS-PAGE, the strips were equilibrated in 2 % (w/v) SDS, 6 M urea, 100 mM EDTA, 0.01 % (w/v) bromphenol blue, 50 mM Tris HCl (pH 6.8), 30 % (v/v) glycerol and 1 % (w/v) DTT for 15 min. Then the strips were equilibrated in the equilibration solution that contains 2.5 % (w/v) iodacetamide instead of 1 % DTT. Second-dimensional separation was performed with Hoefer SE 600 unit (1 x 180 x 160 mm; Amersham Pharmacia Biotech) on 10 % SDS polyacrylamide gels with 10 mA for 30 min and 80 mA for 3 h.

Silver staining

Silver staining was performed mainly as described by Blum *et al.* (1987). Briefly, the gels were fixed in 40 % ethanol/10 % acetic acid overnight and then washed twice by 30 % ethanol for 20 min and once by water for 20 min. The gels were sensitized with 0.02 % sodium thiosulfate for 1 min and than washed by a quick water rinse (3 x 20 s). The gels were submerged in 0.2 % silver nitrate/0.02 % formaldehyde (37 %) for 20 min, washed with water (3 x 20 s) and then developed in 3 %

sodium carbonate/0.05 % formaldehyde (37 %)/0.0005 % sodium thiosulfate until the desired intensity of spots was attained. Then they were washed with water (20 s). The staining was stopped by 0.5 % glycine solution (5 min). The gels were then incubated in 3 % glycerol/20 % methanol, covered with cellophane sheet and dried.

Image analysis

The silver-stained 2D gels were scanned and the spot detection and the comparison of the gels were performed with PDQuest software (Bio-Rad, version 7.0.0).

G-protein oriented 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. $G_s\alpha$ proteins were detected by commercially available antiserum G-5040 (Sigma), which is also oriented against the C-terminus decapeptide of these proteins.

Immunoblotting

After the second dimension, proteins were transferred to nitrocellulose membrane. The membrane was blocked for 1 h in 5 % fat-free milk in PBS-T buffer (PBS containing 0.05 % v/v Tween 20). Then the membrane was incubated for 2 h at room temperature in an appropriate primary antibody in 1 % fat-free milk in PBS-T buffer. After the primary antibody was removed, the blot was washed 3 times for 10 min in PBS-T buffer. Subsequently, the membrane was incubated for 1 h at room temperature in appropriate secondary antibody in 1 % fat-free milk PBS-T buffer. After the removing of secondary antibody the membrane was extensively washed 3 times 10 min in PBS-T buffer and the blot was visualized by ECL (Amersham Pharmacia, UK).

Results

Resolution of G_q/G_{11} protein family by two-dimensional electrophoresis

Subcellular membrane fractions of e2m11-HEK293 cells expressing TRH-receptor and $G_{11}\alpha$ protein were isolated by flotation on sucrose density gradients designed essentially in the same way as in our previous studies (Svoboda *et al.* 1992, Kvapil *et al.* 1994, Svoboda *et al.* 1996). There was no major difference between flotation and sedimentation modes of density gradient centrifugation – the bulk of plasma membranes was localized in 35/40 % sucrose density area (data not shown). Cells were grown in 30 x 75 cm^2 flasks to the same degree of confluence, 60-80 %. The cells in 15 flasks were then treated with 10^{-5} M TRH for 16 h. The control and hormone-treated cells were harvested by low-speed centrifugation and used as a starting material for fractionation on 5/15/20/25/30/35/40 type of sucrose density gradient as described in Methods. Gradient fractions were collected from the top to bottom of the tube after the centrifugation at 39 000 rpm for 24 h in a Beckman SW 41 rotor. The protein concentration in each fraction was determined by method of Lowry *et al.* (1951). Detergent-insensitive membrane domains (DIMs) were localized in 15/20 % sucrose density area as we have shown in our previous work when using panel of markers (Pešanová *et al.* 1999). The low-density membrane fragments (after homogenization) and alkaline-treated, detergent-free preparations of membrane domains were collected from the same area of density gradient – 15/20 % sucrose.

Samples of crude membranes and membrane domain preparations (density gradient fractions 3-6) were precipitated with 24 % TCA and resolved by 2D electrophoresis. In the first dimension, the isoelectric focusing was carried out on immobilized pH gradient 4-9. To increase the resolution and focus on different isoforms of G-proteins, we used immobilized pH gradient with the range from 5 to 7. SDS-PAGE was used in the second-dimension. The total protein content was visualized by MALDI-TOF compatible silver staining, as described by Blum *et al.* (1987). The G-protein content was analyzed by immunoblotting of these samples following 2D electrophoresis. When using 4-9 immobilized pH gradient, we could distinguish the two major spots corresponding to the exogenously expressed $G_{11}\alpha$ and endogenous $G_q\alpha$. When using 5-7 pH gradient and 350 μg of crude membrane protein, we could further resolve the three minor spots, probably corresponding to post-translationally modified $G_q\alpha$ or $G_{11}\alpha$ (Fig. 1A). The two major G-proteins from the $G_q\alpha/G_{11}\alpha$ family could be clearly distinguished not only in a sample of crude membranes but also in samples of membrane domains, in both "classical" detergent-insensitive domains (Fig. 1B) prepared by extraction with Triton X-100 and in membrane domains prepared by detergent-free methods, alkaline treatment (Figs. 1C) or drastic homogenization (Fig. 1D). The minor spots were not detected in "domain preparations", apparently due to very small amount of

protein recovered in low-density fractions (no more than 50 μg protein could have been applied for resolution in the first dimension). The isoelectric points of the major forms of $G_q\alpha/G_{11}\alpha$ were estimated from the isoelectric focusing data to be at pH 5.7 and 5.9.

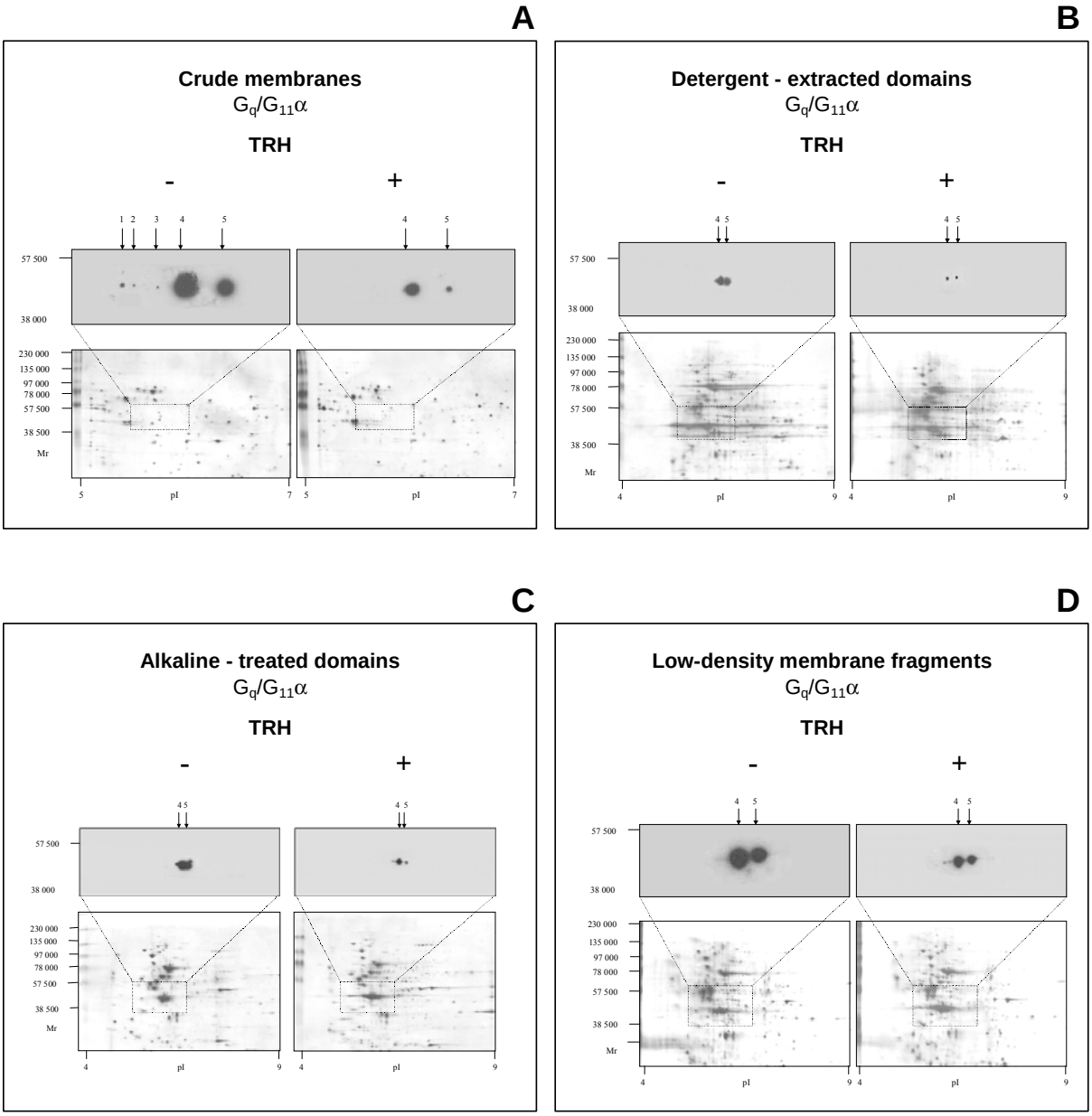

Fig. 1. 2D electrophoretic resolution of $G_q\alpha/G_{11}\alpha$ proteins in HEK293 cell. The cells were stimulated by 10^{-5}M TRH for 16 h or incubated for the same time without any additions (control). Crude membranes (**A**), detergent-insensitive domains (**B**), alkaline-treated domains (**C**) or low-density membrane fragments (**D**) were isolated from HEK293 cells expressing large number of TRH receptors and $G_q\alpha/G_{11}\alpha$ proteins (clone e2m11) as described in Methods. 350 μg (crude membranes), 50 μg (DIMs and alkaline-treated domains) or 150 μg (low-density membrane fragments) of protein were applied for the resolution in the first dimension; the immunoblot signals were developed by antiserum CQ oriented against the C-terminus decapeptide of $G_q\alpha/G_{11}\alpha$ (upper panels). Protein composition was analysed by MALDI-TOF compatible silver staining (lower panels). 1,2,3 - minor spots, 4 - $G_{11}\alpha$, 5 - $G_q\alpha$.

The resolution of G_s proteins by 2D electrophoresis

The S49 lymphoma cells (wild type) endogenously expressing β_2-adrenergic receptor and $G_s\alpha$ protein were grown in total 1000 ml of media. The cells in 500 ml of media were then treated with 10^{-5} M isoprenaline for 16 h. The control and agonist (isoprenaline)-treated cells were harvested at concentration of 0.8-1.2 x 10^6 cells/ml by low-speed centrifugation and were used as a starting material for isolation of crude membranes and membrane-domain

preparations. The same discontinuous sucrose gradient was also used to isolate detergent-insensitive micro-domains and detergent-free domains from ells subjected to alkaline treatment or drastic homogenization. The crude membranes (Fig. 2A) and membrane domain preparations (Figs. 2B-C) from S49 cells were used as a starting material for the 2D electrophoresis. The isoelectric focusing was carried out on immobilized pH gradient strips with the pH range from 4 to 9. It was not necessary to use another pH gradient for the better resolution in comparison with e2m11 cells. The resolution was sufficient to identify all variants of $G_s\alpha$ proteins that were found to be expressed in S 49 cells. The total protein content was visualized by MALDI-TOF compatible silver staining, as described by Blum *et al.* (1987). The 2D gel analysis by immunoblotting gave a consistent pattern with four spots of $G_s\alpha$ proteins, as we have separated serine+ and serine– variants of $G_s\alpha$ L and $G_s\alpha$S. The isoelectric points of $G_s\alpha$L were more acid than those of $G_s\alpha$S and were estimated from the isoelectric focusing to 5.3 and 5.4 for the $G_s\alpha$L variants and 5.75 and 5.85 for the $G_s\alpha$ S variants.

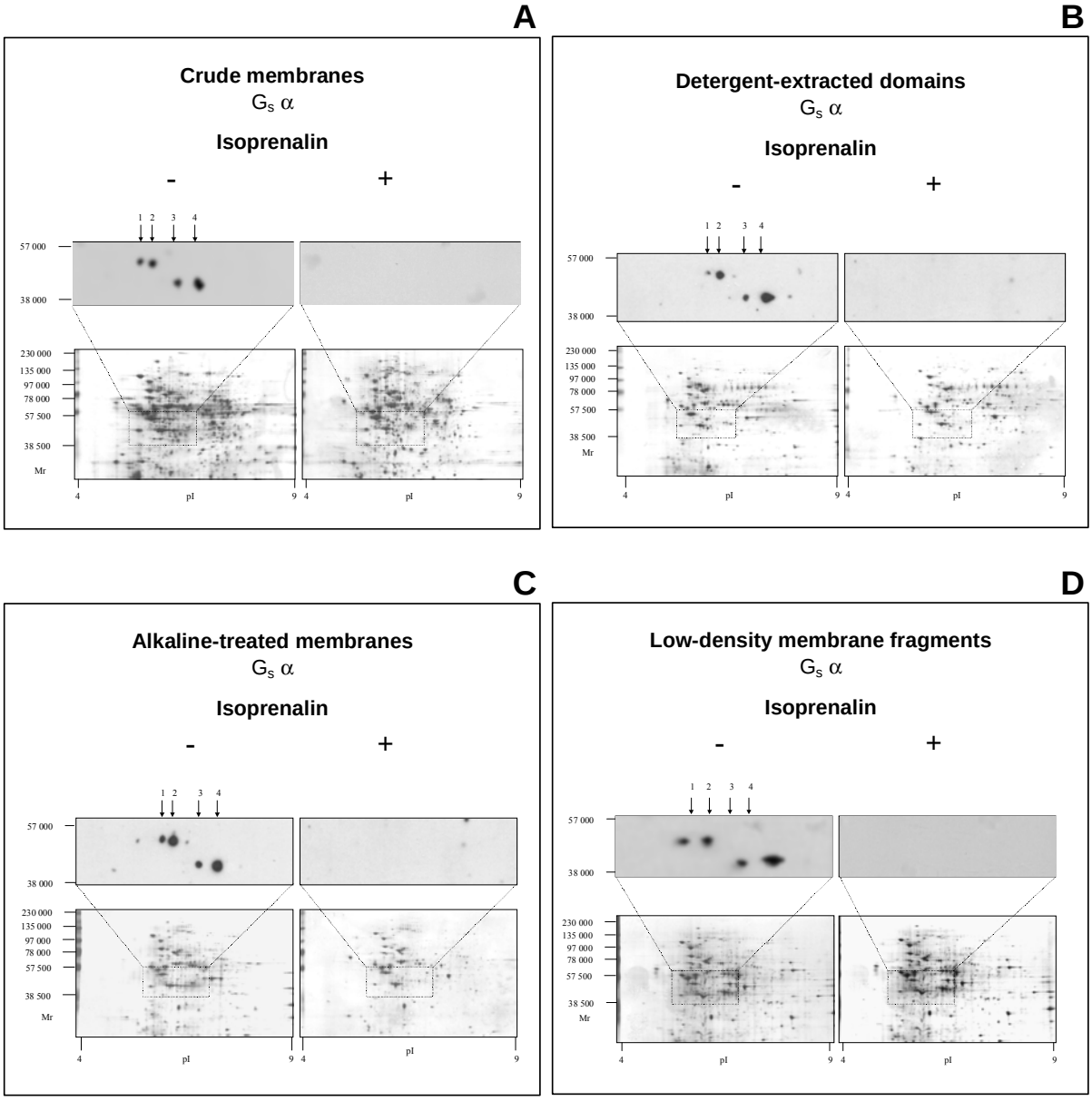

Fig. 2. 2D electrophoretic resolution of $G_s\alpha$ proteins in S49 lymphoma cells. Crude membranes (**A**), detergent-insensitive domains (**B**), alkaline-treated domains (**C**) or low-density membrane fragments (**D**) were isolated from control or isoprenaline (10^{-5} M, 16 h)-stimulated S49 lymphoma cells as described in Methods. 350 μg (crude membranes), 280 μg (DIMs and alkaline-treated domains) or 350 μg (low-density membrane fragments) of protein was applied for the resolution in the first dimension; the immunoblot signals were developed with commercially available antiserum prepared against C-terminus decapeptide of $G_s\alpha$ (G-5040, Sigma)(upper panels). Protein composition was analyzed by silver staining (lower panels). 1,2 - long variants of $G_s\alpha$ ($G_s\alpha$L); 3,4 - short variants of $G_s\alpha$ ($G_s\alpha$S).

Effect of long-term agonist treatment on the membrane domains composition

We have previously reported that agonist treatment results in the transfer of a significant portion of $G_{11}\alpha$ protein from plasma membranes to light-vesicular and cytosolic fraction or the long-term (> 4 h) agonist treatment of cells results in an overall decrease of $G_{11}\alpha$ protein content (Svoboda *et al.* 1996). The similar result was observed for $G_s\alpha$ protein in S49 cell line (Svoboda *et al.* 1992, Kvapil *et al.* 1994). All these changes were specifically restricted (specific) to the cognate G-protein(s); the levels of other, non-stimulated G-proteins were unchanged.

We addressed whether 16-h agonist treatment of the cells is not accompanied by changes of protein composition/reorganization of membrane domains. Therefore we have treated the e2m11 cells with 10^{-5} M TRH and the S49 cells with 10^{-5} M isoprenaline for 16 h. The membrane domains were isolated from control and treated cells of both cell lines, using detergent extraction, alkaline treatment and drastic homogenization procedure. The proteins of membrane domains were resolved by 2D electrophoresis, followed by silver staining of total proteins and immunoblotting to focus on fate of G-proteins. The results indicate a sharp decrease in the content of G_q/G_{11} proteins not only in crude membranes and membrane domains but also in all the variants of $G_s\alpha$ proteins (Figs 1 and 2, upper panels). The 2D maps of total protein composition in membrane domains indicate that the long-term agonist treatment is highly specific and it does not change the composition of membrane domains, both detergent-resistant and detergent-free (Figs 1 and 2, lower panels). Using PDQuest software, we did not find any distinct changes in about 170 major protein spots on the 2D map, when compared control and treated cells.

Discussion

Desensitization of hormone action is one of the autoregulatory mechanisms that protect the cell from pathological effects which may be caused by permanent activation of some signal transduction pathway. As the signal is transmitted through the highly organized parts of the plasma membrane called “membrane microdomains” or “lipid rafts”, much effort has been done to study the effect of hormones and neurotransmitters (generally agonists) on the membrane domains composition in recent years. Many of these effects have been studied using immunoblotting techniques, suggesting that the G-proteins and G-proteins coupled receptors (GPCR) are moving in and out of the membrane domains dependent on the agonist stimulation *via* appropriate receptor (Lasley *et al.* 2000, Rybin *et al.* 2000, Dessy *et al.* 2000). It was observed that the stimulation of S49 cells by isoprenaline (agonist) leads to a transfer of significant portion of $G_s\alpha$ protein from membrane to cytosol (Kvapil *et al.* 1994, Ransnas *et al.* 1989). The study of TRH action *via* TRH receptor revealed that short-term agonist treatment cause a transfer of significant portion of $G_{11}\alpha$ protein to light-vesicular and cytosolic fraction, whereas the long-term (4 h) agonist treatment is accompanied by overall drop of level of $G_{11}\alpha$ protein. The internalization of TRH receptor runs within minutes, but several hours of agonist stimulation are required for the $G_q\alpha/G_{11}\alpha$ internalization (Drmota *et al.* 1998, Pešanová *et al.* 1999). We separated all G-proteins from G_q/G_{11} and G_s family. In the first case we could identify five G-proteins with two major ones with pI 5.7 and 5.9 corresponding to G_q and G_{11}. The two-dimensional electrophoresis of S49 domains gave the consistent pattern of $G_s\alpha$. Longer variants of $G_s\alpha$ have a more acidic pI that is in a perfect accordance with the fact that the residues encoded by the 45 bp of exon 3 include four acidic and only one basic amino acid. Our interest in determining all variants of $G_s\alpha$ stemmed from a possibility that there might be a different efficiency in coupling G-protein to adenylyl cyclase and that the ser+ variants may be phosphorylated. However, the phosphorylation was never described *in vivo* (Bray *et al.* 1988). We found out that long-term agonist treatment for 16 h caused a depletion of all variants of G-proteins from membrane domains, but it did not influence the protein composition of the membrane domains. This phenomenon was demonstrated in two cell lines of widely different origin and physiological specialization – in epithelial embryonic kidney cells and in human lymphoma cells. In epithelial cells, the membrane domains are thought to be involved in sorting out of apical and basolateral proteins (Brown *et al.* 1992, Fiedler *et al.* 1993). In lymphocytes, the membrane domains are closely linked with the Src kinases signalization pathway (Hsi *et al.*1989, Robinson and Hederer 1994). Membrane domains were isolated using different procedures, involving classical detergent extraction with 1 % Triton X-100 at low temperature and detergent-free methods such as alkaline treatment and drastic homogenization procedure with similar results. We confirmed that membrane domains are genuine parts

of plasma membrane and are not an artefact of the detergent isolation method, as they can by isolated regardless of the method used.

Abbreviations

DIMs/DRMs, detergent-insensitive/detergent-resistant membrane domains; DTT, dithiothreitol; G-proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; $G_q\alpha/G_{11}\alpha$, G-proteins stimulating phosholipase C in pertussis-toxin independent manner; $G_s\alpha$, G-protein stimulating adenylyl cyclase activity; GPCR, G-protein-coupled receptor; GPI proteins, glycosyl-phosphatidylinositol-anchored proteins; HEK cells, human embryonic kidney cells; IEF, isoelectric focusing; IPG, isoelectric pH gradient; MALDI-TOF, matrix assisted laser desorption ionization – time of flight; SDS-PAGE, sodium dodecyl sulphate – polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TRH-R, receptor for thyrotropin-releasing hormone; TRH, thyrotropin-releasing hormone

Acknowledgements

The authors thank Ms. Ivana Švandová for valuable methodological advises and writing the essential parts of the manuscript. We also thank Mrs. Alena Povolná for valuable technical help. This work was supported by The Welcome Trust (UK), projects LN00A026 and 113100003 of Ministry of Education of the Czech Republic and by the Grant Agency of the Czech Republic (309/01/0255).

References

ARAGAY AM, KATZ A, SIMON MI: The Gαq and Gα11 proteins couple the thyrotropin-releasing hormone receptor to phospholipase C in GH3 rat pituitary cells. *J Biol Chem* **267**: 24983-24988, 1992.

BLUM H, BEIER H, GROSS HJ: Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93-99, 1987.

BOUŘOVÁ L, KOSTRNOVÁ A, HEJNOVÁ L, MORAVCOVÁ Z, MOON HE, NOVOTNÝ J, MILLIGAN G, SVOBODA P: Delta-opioid receptors exhibit high efficiency when activating trimeric G proteins in membrane domains. *J Neurochem* **85**: 34-49, 2003.

BRAY PF, BARSH G, ROSA JP, LUO XY, MAGENIS E, SHUMAN MA: Physical linkage of the genes for platelet membrane glycoproteins IIb and IIIa. *Proc Natl Acad Sci USA* **85**: 8683-8687, 1988.

BROWN DA, ROSE JK: Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**: 533-544, 1992.

ČERNÝ J, STOCKINGER H, HOŘEJŠÍ V: Noncovalent associations of T lymphocyte surface proteins. *Eur J Immunol* **26**: 2335-2343, 1996.

CINEK T, HOŘEJŠÍ V: The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. *J Immunol* **149**: 2262-2270, 1992.

CODINA J, KIMURA S, KRAUS-FRIEDMANN N: Demonstration of the presence of G-proteins in hepatic microsomal fraction. *Biochem Biophys Res Commun* **150**: 848-852, 1988.

DESSY C, KELLY RA, BALLIGAND JL, FERON O: Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alteration in NO signalling. *EMBO J* **19**: 4272-4280, 2000.

DRMOTA T, NOVOTNÝ J, KIM GD, EIDNE KA, MILLIGAN G, SVOBODA P: Agonist-induced internalisation of the G protein $G_{11}\alpha$ and thyrotropin-releasing hormone receptors proceed on different time scales. *J Biol Chem* **273**: 21699-21707, 1998.

DRMOTA T, NOVOTNÝ J, GOULD GW, SVOBODA P, MILLIGAN G: Visualisation of distinct patterns of subcellular redistribution of the thyrotropin-releasing hormone receptor-1 and $G_q\alpha/G_{11}\alpha$ induced by agonist stimulation. *Biochem J* **340**: 529-538, 1999.

FIEDLER K, KOBAYASHI T, KURZCHALIA TV, SIMONS K: Glycosphingolipid-enriched, detergent-insoluble complexes in protein sorting in epithelial cells. *Biochemistry* **32**: 6365-6373, 1993.

GILMAN AG: G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**: 615-649, 1987.

HAGA T, HAGA K, GILMAN AG: Hydrodynamic properties of the beta-adrenergic receptor and adenylate cyclase from wild type and variant S49 lymphoma cells. *J Biol Chem* **252**: 5776-5782, 1977.

HSI ED, SIEGEL JN, MINAMI Y, LUONG ET, KLAUSNER RD, SAMELSON LE: T cell activation induces rapid tyrosine phosphorylation of a limited number of cellular substrates. *J Biol Chem* **264**: 10836-10842, 1989.

KAZIRO Y, ITOH H, KOZASA T, NAKAFUKU M, SATOH T: Structure and function of signal-transducing GTP-binding proteins. *Annu Rev Biochem* **60**: 349-400, 1991.

KOZASA T, ITOH H, TSUKAMOTO T, KAZIRO Y: Isolation and characterization of the human $G_s\alpha$ gene. *Proc Natl Acad Sci USA* **85**: 2081-2085, 1988.

KVAPIL P, NOVOTNY J, SVOBODA P, RANSNAS LA: The short and long forms of the alpha subunit of the stimulatory guanine-nucleotide-binding protein are unequally redistributed during (–)-isoproterenol-mediated desensitisation of intact S49 lymphoma cells. *Eur J Biochem* 226: 193-199, 1994.

LASLEY RD, NARAYAN P, UITTENBOGAARD A, SMART EJ: Activated cardiac adenosine A_1 receptors translocate out of caveolae. *J Biol Chem* **275**: 4417-4421, 2000.

LOWRY OH, ROSENBROUGH NJ, FARR A, RANDALL RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275, 1951.

PEŠANOVÁ Z, NOVOTNÝ J, ČERNÝ J, MILLIGAN G, SVOBODA P: Thyrotropin-releasing hormone-induced depletion of $G_q\alpha/G_{11}\alpha$ proteins from detergent-insensitive membrane domains. *FEBS Lett* **464**: 35-40, 1999.

RANSNAS LA, SVOBODA P, JASPER JR, INSEL PA: Stimulation of beta-adrenergic receptors of S49 lymphoma cells redistributes the alpha subunit of the stimulatory G protein between cytosol and membranes. *Proc Natl Acad Sci USA* **86**: 7900-7903, 1989.

ROBINSON P, HEDERER R: Early molecular signals induced by antibodies to GPI-anchored proteins. *Braz J Med Biol Res* **27**: 263-267, 1994.

ROBISHAW JD, SMIGEL MD, GILMAN AG: Molecular basis for two forms of the G protein that stimulates adenylate cyclase. *J Biol Chem* **261**: 9587-9590, 1986.

RODBELL M: The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* **284**: 17-22, 1980.

RYBIN VO, XU X, LISANTI MP, STEINBERG SF: Differential targeting of beta-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signalling pathway. *J Biol Chem* **275**: 41447-41457, 2000.

SHENOY-SCARIA AM, GAUEN LK, KWONG J, SHAW AS, LUBLIN DM: Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases p56lck and p59fyn mediates interaction with glycosyl-phosphatidylinositol-anchored proteins. *Mol Cell Biol* **13**: 6385-6392, 1993.

SIMON MI, STRATHMANN MP, GAUTAM N: Diversity of G proteins in signal transduction. *Science* **252**: 802-808, 1991.

SOLOMON KR, RUDD CE, FINBERG RW: The association between glycosylphosphatidylinositol-anchored proteins and heterotrimeric G protein alpha subunits in lymphocytes. *Proc Natl Acad Sci USA* **93**: 6053-6058, 1996.

SONG KS, LI S, OKAMOTO T, QUILLIAM LA, SARGIACOMO M, LISANTI MP: Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J Biol Chem* **271**: 9690-9697, 1996a.

SONG KS, SCHERER PE, TANG Z, OKAMOTO T, LI S, CHAFEL M, CHU C, KOHTZ DS, LISANTI MP: Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J Biol Chem* **271**: 15160-15105, 1996b.

STRYER L, BOURNE HR: G proteins: a family of signal transducers. *Annu Rev Cell Biol* **2**: 391-419, 1986.

SVOBODA P, KVAPIL P, INSEL PA, RANSNAS LA: Plasma-membrane-independent pool of the α subunit of the stimulatory guanine-nucleotide-binding regulatory protein in low-density-membrane fraction of S49 lymphoma cells. *Eur J Biochem* **208**: 693-698, 1992.

SVOBODA P, KIM GD, GRASSIE MA, EIDNE KA, MILLIGAN G: Thyrotropin-releasing hormone-induced subcellular redistribution and down-regulation of $G_{11}\alpha$: analysis of agonist regulation of co-expressed $G_{11}\alpha$ species variants. *Mol Pharmacol* **49**: 646-655, 1996.

TANG WJ, GILMAN AG: Adenylyl cyclases. *Cell* **70**: 869-872, 1992.

Reprint requests

Doc. Petr Svoboda, PhD, DSc, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic. E-mail: svobodap@biomed.cas.cz