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Protein Alterations Induced by Long-Term Agonist Treatment of HEK293 Cells Expressing Thyrotropin-Releasing Hormone Receptor and $G_{11}\alpha$ Protein

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ABSTRACT

This study aimed to determine whether sustained stimulation with thyrotropin-releasing hormone (TRH), a peptide with important physiological functions, can possibly affect expression of plasma membrane proteins in HEK293 cells expressing high levels of TRH receptor and $G_{11}\alpha$ protein. Our previous experiments using silver-stained two-dimensional polyacrylamide gel electrophoretograms did not reveal any significant changes in an overall composition of membrane microdomain proteins after long-term treatment with TRH of these cells (Matousek et al. [2005] Cell Biochem Biophys 42: 21–40). Here we used a purified plasma membrane fraction prepared by Percoll gradient centrifugation and proteins resolved by 2D electrophoresis were stained with SYPRO Ruby gel stain. The high enrichment in plasma membrane proteins of this preparation was confirmed by a multifold increase in the number of TRH receptors and agonist stimulated G-protein activity, compared to postnuclear supernatant. By a combination of these approaches we were able to determine a number of clearly discernible protein changes in the plasma membrane-enriched fraction isolated from cells treated with TRH (1×10^{-5} M, 16 h): 4 proteins disappeared, the level of 18 proteins decreased and the level of 39 proteins increased. Our concomitant immunochemical determinations also indicated a clear down-regulation of $G_{q/11}\alpha$ proteins in preparations from hormone-treated cells. In parallel, we observed decrease in caspase 3 and alterations in some other apoptotic marker proteins, which were in line with the presumed antiapoptotic effect of TRH. J. Cell. Biochem. 109: 255–264, 2010.

KEY WORDS: THYROTROPIN-RELEASING HORMONE; $G_{q/11}$ PROTEIN; 2D ELECTROPHORESIS; PROTEOMICS

Thyrotropin-releasing hormone (TRH; pyro-Glu-His-ProNH_2) was originally identified as a crucial hypothalamic factor regulating the thyroid axis [Hajjar et al., 1973; Azizi et al., 1974]. Nevertheless, TRH is also produced by peripheral organs and it exerts central, as well as peripheral biological effects that are mediated by TRH receptors [Nillni and Sevarino, 1999]. TRH has been shown to control glycemia and it can also influence cardiac performance in heart failure [Duntas et al., 1998; Jin et al., 2004]. Despite its important physiological roles, the detailed molecular mechanisms of the effects of TRH have not yet been fully elucidated. In particular, there is not much information about the impact of prolonged TRH stimulation on protein expression.

TRH receptor belongs to the family of heptahelical G-protein-coupled receptors (GPCRs) and the initial response triggered by TRH binding to the receptor involves activation of phospholipase C-β (PLC-β) through heterotrimeric $G_{q/11}$ proteins [Straub et al., 1990; Hsieh and Martin, 1992]. The PLC-mediated stimulation of inositol trisphosphate (IP_3) production leads to opening of IP_3-sensitive calcium channels in the endoplasmic reticulum and a transient increase in cytosolic calcium [Taylor et al., 1991]. However, recent evidence suggests that many of cellular actions of G-proteins from the $G_{q/11}\alpha$ class can be independent of inositol lipid signaling [Hubbard and Hepler, 2006]. Reported binding partners of these G-proteins distinct from PLC-β include novel candidate effector

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proteins, various regulatory proteins, and a growing list of scaffolding/adaptor proteins. Ubiquitously distributed $G_{q/11}\alpha$ proteins thus seem to regulate different signaling pathways and their action may have a large impact on cell physiology. It can be expected that, especially under sustained hormone stimulation, considerable alterations would occur in affected cells, including changes in protein expression. Indeed, recent microarray analyses indicated that long-term administration of TRH has a profound effect on gene expression in pancreatic cells [Yano and Luo, 2004; Luo and Yano, 2005]. Changes in the transcriptional profiles observed in these studies, however, were not verified by parallel analyses of the proteins.

We have previously reported that long-term treatment with TRH of HEK293 cells, which express high levels of TRH receptor and $G_{11}\alpha$ protein (E2M11 cell line), dramatically down-regulated trimeric $G_{q/11}\alpha$ proteins but did not significantly change an overall composition of proteins in crude membrane or plasma membrane domain preparations [Matousek et al., 2004, 2005]. These results were achieved by 2D electrophoretic resolution of proteins followed by a standard silver staining and subsequent differential analyses of 2D maps of control and hormone-treated cells. In order to be able to detect the presumed changes in minor proteins, we decided to use a purified fraction of plasma membranes from E2M11 cells in the present study. In addition, we applied SYPRO Ruby fluorescent protein dye, which is as sensitive as silver staining procedures, but it has much better linear dynamic range and provides a more accurate estimation of quantitative changes in proteins resolved by 2D electrophoresis [Yan et al., 2000]. By a combination of these approaches we were able to determine a number of clearly discernible changes in plasma membrane proteins isolated from TRH-treated cells. These changes occurred in parallel with specific decrease in the amount of $G_{q/11}\alpha$ proteins detected by an immunoblot and decrease in the functional activity of these G-proteins measured as agonist-stimulated high-affinity [^{35}S]GTPγS binding.

MATERIALS AND METHODS

MATERIALS

All materials for tissue culture were supplied by Sigma–Aldrich (Milwaukee, WI), Gibco-BRL (Gaithersburg MD), and NUNC (Rochester, NY). Complete Protease Inhibitor cocktail was from Boehringer Mannheim (Mannheim, Germany). Immobiline Dry-Strips, Pharmalyte buffer, and secondary anti-rabbit antibody labeled with horseradish peroxidase were purchased from GE Healthcare (Piscataway, NJ). SYPRO Ruby stain was from Molecular Probes (Eugene, OR), trypsin from Promega (Madison, WI), acrylamide-bis-acrylamide and *N*,*N*,*N*',*N*'-tetramethylethylenediamine from Bio-Rad (Hercules, CA), nitrocellulose membrane from Schleicher-Schuell (Erdmannhausen, Germany) and Whatman GF/C filters from Whatman Ltd. (Oxford, UK). All other chemicals and materials were from Sigma–Aldrich and were of the best quality available. Preparation and characterization of rabbit polyclonal antiserum against $G_{q/11}\alpha$ protein was described previously [Bourova et al., 1999]. All other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

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°C in a 5% CO_2 humidified atmosphere [Svoboda et al., 1996].

ISOLATION OF THE PLASMA MEMBRANE-ENRICHED FRACTION BY PERCOLL^R DENSITY GRADIENT

Cells were harvested from 15 flasks (80 cm^2 each), collected by centrifugation at 1,800 rpm for 10 min, washed once in PBS and re-suspended on ice by repeated pipetting in 250 mM sucrose, 50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$, pH 7.5 (STEM medium) supplemented with Complete Protease Inhibitor cocktail. Fresh phenylmethylsulfonyl fluoride (1 mM final concentration) was added shortly before homogenization (5 min at 1,800 rpm, on ice) in a Teflon-glass homogenizer. Homogenate was centrifuged for 3 min at 1,500 rpm (1,000*g*) at 4°C in Hettich centrifuge. Resulting postnuclear supernatant (PNS) was applied on the top of 30% w/v Percoll^R in STEM medium. Centrifugation for 30 min at 30,000 rpm (65,000*g*) in Beckman Ti70 rotor resulted in separation of the two clearly visible layers. Whereas the upper layer represented the plasma membrane-enriched fraction (PM), the lower layer contained higher density membranes and mitochondria. The upper layer was diluted 1:3 in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA (TE medium) and centrifuged in Beckman Ti70 rotor for 90 min at 50,000 rpm (175,000*g*). Membrane sediment was removed from a stiff jellylike sediment of Percoll^R, homogenized in small volume of 150 mM NaCl, 20 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4, snap frozen in liquid nitrogen and stored at −80°C.

LIGAND BINDING STUDY OF TRH RECEPTORS

[^{3}H]TRH binding experiments were carried out as described before [Kim et al., 1994; Lee et al., 1995] in 50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4 (TM medium) for 90 min at 0°C. The specific radioactivity of a single concentration of [^{3}H]TRH (∼10 nM) was gradually decreased by addition of TRH in the range of 10^{-10}–10^{-4} M and the B_{max} (maximum binding capacity) and K_d (dissociation constant) values calculated according to the algorithm of DeBlasi et al. [1989]. The binding reaction was terminated by rapid filtration through Whatman GF/B filters. Filters were subsequently washed 3 times with 3 ml of ice-cold TM medium and 3 times with 0.9% NaCl. Radioactivity retained on the filters was determined by liquid scintillation counting using CytoScint cocktail (GE Healthcare).

AGONIST-STIMULATED [^{35}S]GTPγS BINDING ASSAY

Agonist-stimulated [^{35}S]GTPγS binding was measured according to Bourova et al. [2003]. Briefly, aliquots of PNS or PMs (10 μg protein per assay) were incubated with (total) or without (basal) TRH in a final volume of 100 μl of reaction mixture containing 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 2 μM GDP, 0.2 mM ascorbate and [^{35}S]GTPγS (about 100,000 dpm per assay) for 30 min at 37°C. The binding reaction was discontinued by dilution with 3 ml of ice-cold HM buffer (20 mM HEPES and 3 mM $MgCl_2$, pH 7.4) and

immediate rapid filtration through Whatman GF/C glass-fiber filters under vacuum in Brandel cell harvestor. The filters were washed twice with 5 ml ice-cold HM buffer and radioactivity remaining on the membranes on the filters was determined by liquid scintillation counting using BioScint cocktail (National Diagnostics, Hessle Hull, UK). In routine analysis, TRH-stimulated [^{35}S]GTPγS binding was determined by a one-point assay using 5 nM [^{35}S]GTPγS ± 10 μM TRH. When analyzing dose-response curves, TRH concentration was gradually increased from 1×10^{-11} to 1×10^{-4} M. Non-specific binding was determined by parallel assays containing 10 μM unlabeled GTP and it was subtracted from total bound radioactivity. The EC_{50} values were calculated from the dose-response curves by the GraphPad Prism 3.02 software (San Diego, CA).

ELECTROPHORESIS AND IMMUNOBLOTTING

Samples of PNS or PMs were analyzed by standard SDS–PAGE followed by Western blot using enhanced chemiluminiscence. All these procedures were performed at room temperature. Briefly, proteins were resolved on 10% polyacrylamide gels using a Mini-Protean II system (Bio-Rad) and electrotransferred onto nitrocellulose membranes. After blocking with 5% fat-free milk in PBS-T (PBS buffer containing 0.05% Tween-20) for 1 h, nitrocellulose membranes were incubated with appropriate primary antibodies for 2 h, with constant rocking on a shaker. At the end of the incubation, the blots were washed 3 times, 10 min each, with PBS-T buffer, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. After three 10-min washes, the blots were developed employing the SuperSignal detection system (Pierce Biotechnology, Rockford, IL) and exposed to Kodak MXB film.

TREATMENT OF CELLS WITH CAMPTOTHECIN

To assess the presumed antiapoptotic effect of TRH, control and hormone-treated (10 μM, 12 h) E2M11 cells were exposed to camptothecin (1×10^{-6} M) for 4 h. After harvesting the cells and homogenization, the distribution of caspase 3, PARP, Bcl-2 and Bax was determined in 50-μg samples by SDS–PAGE and immunoblotting as described above.

SAMPLE PREPARATION FOR ISOELECTRIC FOCUSING

Samples of PNS or PMs were disposed of salts and other interfering compounds by TCA precipitation followed by ethanol washing. Briefly, a sample containing 100–400 μg protein was diluted with H_2O to the final volume of 90 μl and precipitated with 30 μl 24% TCA for 1 h on ice. After centrifugation at 16,000*g* for 10 min at 4°C, the supernatant was discarded and the pellet washed by a vigorous mixing with 200 μl of ice-cold 96% ethanol. The mixture was centrifuged at 16,000*g* for 10 min at 4°C and the resulting pellet (after aspiration of the supernatant) was air-dried and solubilized with 250 μl 2DE sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% Pharmalyte buffer (pH 3-10), 1% DTT and 0.1% bromophenol blue) for 3 h at room temperature. Finally, after removal of the unsolubilized material by centrifugation (16,000*g*, 1 min), the sample was pipetted into a groove of the Immobiline DryStrip Reswelling Tray (GE Healthcare).

2DE PROCEDURE

Separation of proteins by isoelectric focusing (IEF) was performed using IPG gels (Immobiline DryStrip, pH 4–7; GE Healthcare). Prior to the IEF electrophoresis, ready-to-use Immobiline DryStrips (linear pH gradient 4–7, length 18 cm) were placed into the Immobiline DryStrip Reswelling Tray containing solubilized protein samples and rehydrated overnight. IEF electrophoresis was performed using the Multiphor II system (GE Healthcare) at 14°C with a stepwise increase of voltage in the following manner: 150 V for 5 h, 500 V for 1 h, and 3,500 V for 12 h. The focused strips were stored at −20°C until second-dimensional electrophoresis was performed.

Before carrying out the second-dimensional SDS–PAGE, the strips were incubated in equilibration buffer E (50 mM Tris–HCl (pH 6.8), 6 M urea, 0.1 mM EDTA, 2% SDS, 30% glycerol and 0.01% bromophenol blue) containing 1% DTT for 15 min in order to reduce the –S-S– bridges and other oxidized groups present in protein molecules. The strips were subsequently alkylated in the same equilibration buffer E containing 2.5% iodoacetamide for 15 min. The equilibrated strips were then transferred onto 10% SDS–polyacrylamide slab gels (14 cm × 16 cm × 1 mm) and the gels were run vertically in Laemmli running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) cooled to 14°C using the Hoefer SE 600 unit (GE Healthcare). SDS–PAGE was carried out at a constant current of 10 mA for 20 min and then at 80 mA for 2 h till the bromophenol blue dye reached the end of the gel.

STAINING OF POLYACRYLAMIDE GELS

The proteins in polyacrylamide gels were visualized with SYPRO Ruby according to the manufacturer's instructions. Briefly, the gel was incubated with gentle agitation in 250 ml SYPRO Ruby dye staining solution overnight in the dark and washed in 250 ml of 10% methanol and 7% acetic acid for 30 min. For MS analysis, the gels were stained by colloidal Coomassie Blue (17% ammonium sulphate, 34% methanol, 3% orthophosphoric acid, and 0.1% Coomassie G-250). After staining, the gels were kept in 1% acetic acid at 4°C.

IMAGE ACQUISITION AND ANALYSIS

After staining, the 2DE protein patterns were imaged using Molecular Imager FX ProPlus (Bio-Rad, Hercules) at 100 μm resolution. The SYPRO Ruby stained gels were scanned at an excitation wavelength of 532 nm and an emission wavelength of 555 nm. The gel images were saved as 8-bit TIFF images and subsequently processed and analyzed using the PDQuest software (Bio-Rad). In order to reveal irregular spots occurring only on some 2D maps, master gel was constructed as a mutual intersection of all spots obtained by analyzing all the maps of control and hormone-treated samples. Sporadically occurring irregular spots were excluded from further analyses. Following spot detection, the original gel scan was filtered and smoothed to clarify the spots, and then 3D Gaussian spots were created from the clarified spots. In this way, three separate sets of images were obtained: the original raw 2D scans, the filtered images and the Gaussian images. All quantitations and other analyses were performed on the Gaussian images. A match set, that can compare one or many gels, was created. Gel images were aligned and automatically overlayed. The

program identified matched and unmatched spots from all gels. All matched and unmatched spots were then checked visually. The gels were normalized according to the total quantity in valid spots (the raw quantity of each spot in a member gel is divided by the total quantity of all the spots in that gel that have been included in the Master gel). Protein levels increased or decreased at least twofold were taken into account as altered.

IN-GEL DIGESTION, MASS SPECTROMETRY, AND DATA PROCESSING

Individual spots cut from 2D gel were placed in microtubes and covered with 100 μl of 50 mM ammonium bicarbonate (ABC) buffer in 50% acetonitrile (ACN) with 50 mM DTT. The samples were subjected to sonication in an ultrasonic bath for 5 min. After 15 min the supernatant was discarded and the gel was covered with 100 μl of 50 mM ABC/50% ACN with 50 mM iodoacetamide and sonicated for 5 min. After 25 min, the supernatant was discarded and exchanged for 100 μl 50 mM ABC/50% ACN with 50 mM DTT and sonicated for 5 min to remove excess iodoacetamide. Supernatant was discarded and samples were sonicated for 5 min in 100 μl of ultra-pure water for HPLC. The water was then discarded and samples sonicated for another 5 min in 100 μl of ACN. ACN was discarded and microtubes with samples were left open for a couple of minutes to allow the rest of ACN to evaporate. Five nanogram of trypsin in 10 μl of 50 mM ABC were added to the gel. Samples were incubated at 37°C overnight. Trifluoroacetic acid (TFA) and ACN were added to reach final concentration of 1% TFA and 30% ACN. Samples were sonicated for 10 min and 0.5 μl drop was transferred onto MALDI target and let to dry. Dried droplets were covered with 0.5 μl drop of alpha-cyano-hydroxycinnamic acid solution (2 mg/ml in 80% ACN) and let to dry. Samples were measured using a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems/MDS Sciex) equipped with a Nd:YAG laser (355 nm, firing rate 200 Hz).

Peak lists from the MS spectra were generated by 4000 Series Explorer V 3.5.3 (Applied Biosystems/MDS Sciex) without smoothing, peaks with local signal to noise ratio greater than 5 were picked and searched by local Mascot v. 2.1 (Matrix Science) against non-redundant NCBI database of protein sequences (6573034 sequences; 2244863856 residues). Database search criteria were as follows—enzyme: trypsin, taxonomy: human (118921 sequences), fixed modification: carbamidomethylation, variable modification: methionine oxidation, peptide mass tolerance: 120 ppm, one missed cleavage allowed. Only hits that were scored as significant ($P < 0.001$) were included.

MISCELLANEOUS

Protein was determined according to Lowry method. If not stated otherwise, all data were obtained from at least three independent PNS or PM preparations. The numbers represent the average (±SEM) values of radioligand ([^{3}H]TRH or [^{35}S]GTPγS) binding assays carried out in triplicates. Master gels for 2D proteome analyses were obtained by averaging the data of 16 replicas of each experimental group.

RESULTS

CHARACTERIZATION OF TRH RECEPTORS AND $G_{q/11}$ ACTIVITY IN POSTNUCLEAR SUPERNATANT AND IN THE PLASMA MEMBRANE-ENRICHED FRACTION

Subcellular fractionation and isolation of purified plasma membrane-enriched fraction on Percoll^R density gradient is schematically depicted in Figure 1A. High enrichment with plasma membranes of the upper gradient fraction was corroborated by analyzing the distribution of the protypical PM marker protein Na,K-ATPase (Fig. 1B). Centrifugation of postnuclear supernatant (PNS) in PercollR density gradient enabled to obtain the plasma membrane-enriched fraction (PM), which was further evidenced by comparison of TRH receptor distribution in PNS and PM (Fig. 2A). Basic characteristics of TRH receptors in these two preparations from control cells were determined by analysis of [^{3}H]TRH/TRH displacement curves according to the method of DeBlasi et al. [1989]. The affinity of [^{3}H]TRH binding to TRH binding sites in PNS and PM was almost the same (K_D (PNS) = 21.4 ± 4.5 nM, K_D (PM) = 28.9 ± 6.2 nM), but the number of these sites was markedly increased in PM (B_{max} (PNS) = 2.2 ± 0.8 pmol/mg protein, B_{max} (PM) = 11.3 ± 1.5 pmol/mg protein).

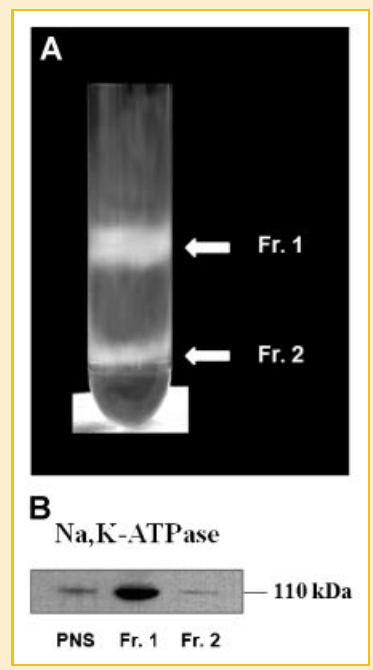

Fig. 1. Isolation of the plasma membrane-enriched fraction on Percoll^R density gradient. A: Postnuclear supernatant (PNS) prepared from homogenized E2M11 cells grown in 15 culture flasks (80 cm^2 each) was centrifuged on Percoll^R density gradient as described in Materials and Methods Section. Whereas the opaque upper layer (Fr. 1) was highly enriched with plasma membranes, the lower layer (Fr. 2) contained membranes and mitochondria with higher buoyant density. The plasma membrane-enriched fraction (the upper layer) was collected and used for subsequent experiments. B: Samples (50 μg protein) of PNS, Fr. 1 and Fr. 2 were separated by standard SDS–PAGE and immunoblotted with anti-Na,K-ATPase (α) antibody. The strongest signal is clearly visible in Fr. 1 (plasma membranes).

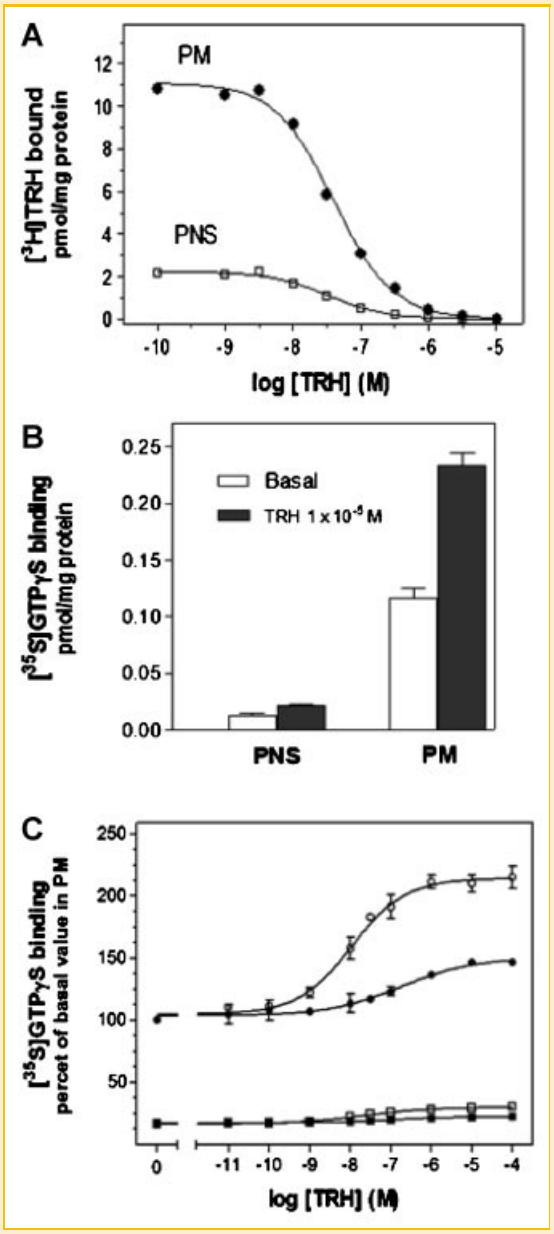

Fig. 2. [^{3}H]TRH and [^{3}H]GTPγS binding in the postnuclear supernatant and plasma membrane-enriched fractions. A: Postnuclear supernatant (PNS) and Percoll^R-purified plasma membranes (PM) were prepared from the same harvest of E2M11 cells as described in Methods. [^{3}H]TRH/TRH competition binding experiments were performed in triplicates and calculated according to DeBlasi et al. [1989]. Data are presented as agonist-displacement curves of specific [^{3}H]TRH binding. B: Basal (open bars) and TRH-stimulated (solid bars) [^{3}H]GTPγS binding was measured by a one-point assay using 5 nM [^{3}H]GTPγS in the presence of 2 μM GDP in samples of PNS and PM from control naïve E2M11 cells. Data are representative of a typical experiment performed in triplicates. C: [^{3}H]GTPγS binding was measured in samples of PNS (squares) and PM (circles) isolated from control (hormone-unexposed) cells (open symbols) or cells exposed to TRH for 16 h (closed symbols) using increasing concentrations of TRH in the concentration range of 1×10^{-11}–1×10^{-4} M in the presence of 2 μM GDP. Non-specific GTPγS binding was determined as that remaining in the presence of 100 μM GTPγS. [^{3}H]GTPγS binding curves were expressed as percentage of maximum binding in the absence of unlabeled TRH (100%).

To assess the presumed effect of Percoll^R purification procedure on the functional activity of G-proteins, both basal and agonist-stimulated [^{35}S]GTPγS binding were compared in samples of postnuclear supernatant and the plasma membrane-enriched fraction. Samples of PM exhibited almost 10 times higher basal as well as TRH-stimulated GTPγS binding than samples of PNS (Fig. 2B). TRH in the range of its most effective concentrations (1×10^{-6}–1×10^{-4} M) roughly doubled the value of GTPγS binding in both types of preparations, but the hormone-induced increment was much easier discernible and more significant in PM than in PNS. To show the specificity of agonist-stimulated GTPγS binding, TRH-stimulated GTPγS binding was also measured in preparations isolated from cells treated with the hormone for 16 h. We have previously observed that TRH receptors are desensitized and $G_{q/11}$ proteins down-regulated under these conditions [Svoboda et al., 1996]. Accordingly, TRH-stimulated GTPγS binding was substantially diminished in samples from TRH-treated cells, compared to the corresponding controls (Fig. 2C). Hence, isolation of the plasma membrane-enriched fraction represents an advantageous methodological tool for characterization of the binding parameters of TRH receptors as well as functional activity of their cognate G-proteins.

ASSESSMENT OF PROTEIN COMPOSITION OF POSTNUCLEAR SUPERNATANT AND THE PLASMA MEMBRANE-ENRICHED FRACTION BY 2DE

To compare the major protein composition of samples of postnuclear supernatant and the plasma membrane-enriched fraction, both these preparations were resolved on linear IPG strips (pH 4–7) and then by SDS–PAGE on 10% acrylamide gels, which were subsequently stained with Sypro Ruby. Representative 2D maps of PNS and PM clearly differed from one another in most major proteins (Fig. 3). There were only 200 identical proteins detectable in these two preparations, all the other proteins (229 in PNS and 166 in PM) were different. This marked difference indicated a great effect of purification of PNS by Percoll^R density gradient centrifugation on major protein composition of the resulting plasma membrane-enriched fraction. Our preliminary experiments aiming to compare protein composition of PNS from control and TRH-treated cells revealed only four changed proteins in these preparations. Therefore, all next experiments exploring the presumed effect of TRH on protein expression were performed on samples of PM.

EFFECT OF LONG-TERM TREATMENT WITH TRH ON PROTEIN LEVELS IN THE PLASMA MEMBRANE-ENRICHED FRACTION

Proteins in PM derived from control E2M11 cells and those treated with TRH (1×10^{-5} M, 16 h) were resolved by 2D electrophoresis and 2DE analyses revealed 61 qualitative or quantitative protein changes in preparations from control and hormone-treated cells (Fig. 4). In samples from TRH-treated cells, 4 proteins disappeared, the level of 18 proteins decreased and the level of 39 proteins increased. All the changed proteins (spots) are indicated on the Master gel displayed in Figure 4C. Most of these changed proteins were subsequently identified by mass spectrometry. Some changed proteins (and their different isoforms or split products) occurred in several different spots. All the individual identified proteins were assigned numbers and basic characteristics of these proteins are listed in Table I. Many

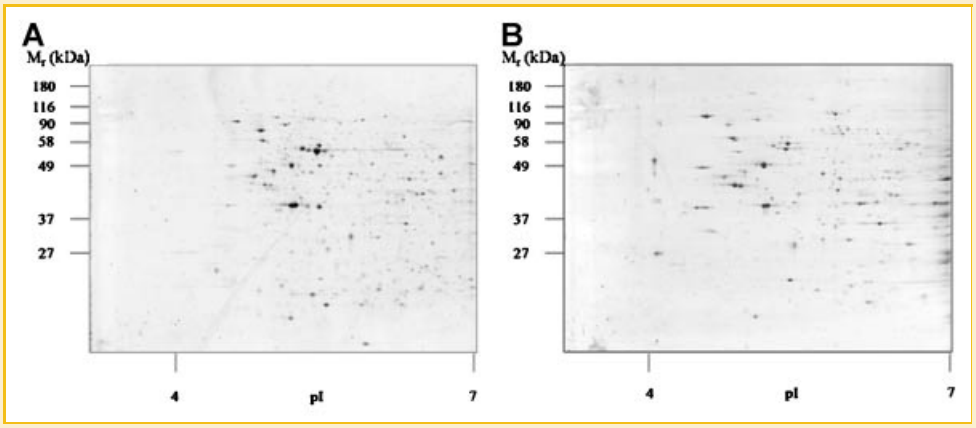

Fig. 3. Representative 2D maps showing differences in major protein composition of postnuclear supernatant and the plasma membrane-enriched fraction. The protein samples from the postnuclear supernatant (A) and plasma membrane-enriched fraction (B) were separated on linear IPG strips (pH 4–7) and then by SDS–PAGE on 10% acrylamide gels. The gels were stained using the fluorescent dye Sypro Ruby.

of them belong to the protein families whose expression is usually affected by stimulation of human cells, such as keratins, tubulins, vimentins, protein disulfide isomerases, T-complex polypeptide 1, peroxiredoxines, ATP synthases, elongation factors, subunits of proteasome and hnRNP [Petrak et al., 2008]. Sixteen altered proteins were from the mitochondria, 11 proteins from the cytoskeleton or cytoplasm, 2 proteins from the endoplasmic reticulum, 1 protein from the centrosome, 2 proteins from the nucleus, 1 protein from the peroxisome, 1 protein from the plasma membrane and 8 proteins might have originated from several different compartments.

Our concomitant 2D electrophoretic resolution of proteins in the lower density gradient fraction provided the same maps as those obtained for the PM-enriched fraction (data not shown). Hence, both the upper and lower gradient fractions contained high amounts of proteins originated from mitochondria broken by intensive homogenization. The observed cross-contamination of these two fractions could also partly originate from some larger membrane or other cellular fragments and associated proteins, which could come down to the lower gradient fraction because of their higher buoyant density, and represent the major components detectable by 2DE/MS.

EFFECT OF LONG-TERM TREATMENT WITH TRH ON THE LEVELS OF SELECTED G-PROTEIN SUBUNITS AND APOPTOTIC MARKERS

Our earlier observations indicated that sustained stimulation of E2M11 cells with TRH caused subcellular redistribution and down-regulation of the cognate $G_{q/11}\alpha$ proteins [Drmota et al., 1998]. Here we examined the effect of TRH on the $G_{q/11}\alpha$ levels in PNS and in PM by immunoblotting after resolution of these protein samples by standard SDS–PAGE or 2DE, respectively (Fig. 5). Both these analyses confirmed profound down-regulation (>90%) of $G_{q/11}\alpha$ proteins. Results of our concomitant determination of Gβ subunit indicated slight down-regulation (~20%) of this protein in preparations from TRH-treated cells (Fig. 5A).

Beside others, our current 2DE/MS analyses revealed a significantly increased expression of some antiapoptotic proteins (peroxiredoxin III, prohibitin, and MTHSP75) in samples from TRH-treated cells. Accordingly, analogous changes in the distribution of these proteins were also found by subsequent immunoblot analyses (Fig. 6A). In order to confirm the presumed antiapoptotic effect of prolonged TRH treatment on E2M11 cells, we also assessed several other crucial apoptotic marker proteins. Whereas a highly pronounced (roughly 10-fold) decrease was determined in the amount of proapoptotic enzyme caspase 3, the level of PARP (poly(ADP-ribose) polymerase) was not altered in samples from TRH-treated cells (Fig. 6B). The antiapoptotic effect of TRH was also confirmed by a markedly increased ratio (from 1 to 3.9) of the antiapoptotic protein Bcl-2 to the proapoptotic protein Bax in samples from hormone-treated cells (Fig. 6B). Our subsequent experiments with camptothecin, a drug causing apoptosis, further corroborated the antiapoptotic effect of TRH. TRH increased the ratio Bcl-2/Bax from 0.6 to 1.6 in cells treated with camptothecin (Fig. 6B).

DISCUSSION

We reported previously that prolonged stimulation of E2M11 cell with TRH did not change an overall composition of membrane microdomain proteins [Matousek et al., 2005]. In the present study we have revisited this model and used the plasma membrane-enriched fraction of E2M11 cells for determination of the possible hormone-induced changes in the proteins resolved by 2D electrophoresis and stained with the fluorescent dye SYPRO Ruby. The high effectiveness of Percoll^R gradient centrifugation used for isolation of the plasma membrane-enriched fraction was indicated by a multifold increase in the number of TRH receptors and basal, as well as agonist stimulated G-protein activity in this fraction, compared to the initial postnuclear supernatant. The subsequent comparison of 2D maps of PNS and PM further confirmed a big difference between these two preparations: there were only 200 same proteins present in both PNS and PM, whereas all the other proteins were different.

Isolation of PM along with application of a highly sensitive staining with SYPRO Ruby, a fluorescent dye with a broad linear dynamic range, enabled us to detect several clearly discernible changes in protein composition after prolonged treatment of E2M11

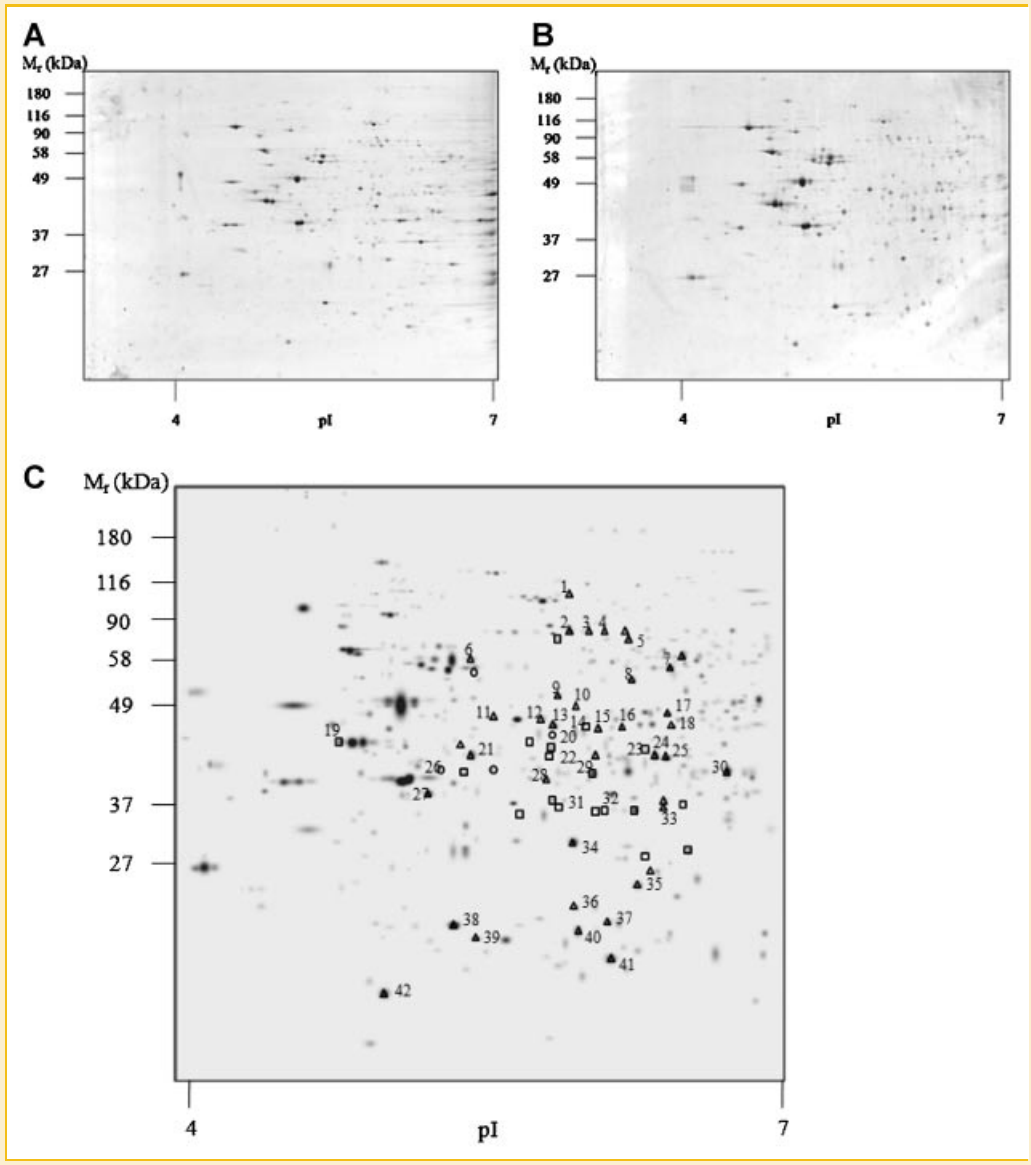

Fig. 4. Effect of long-term treatment with TRH on the levels of proteins in the plasma membrane-enriched fraction. Representative 2D maps showing the difference in protein composition of plasma membrane-enriched fraction derived from control (A) and TRH-treated (B) cells were prepared by separation of protein samples on linear IPG strips (pH 4–7) and then by SDS–PAGE on 10% acrylamide gels. The gels were stained using the fluorescent dye Sypro Ruby. C: Master gel (2D map) of major proteins in the plasma membrane-enriched fraction of E2M11 cells was derived from 16 replicas gels. Differences between samples from control and TRH-treated cells are indicated by circle (qualitative change, not detected after TRH treatment), square (quantitative change, down-regulation of proteins after TRH treatment), and triangle (quantitative change, up-regulation of proteins after TRH treatment). The spot numbers correspond to the proteins listed in Table I.

cells with TRH. Overall, 4 proteins disappeared, the level of 18 proteins decreased and the level of 39 proteins increased. In contrast, there were only 4 detectable changes in PNS because this preparation apparently did not contain sufficient amount of the relevant proteins. These 4 changed proteins were present neither in PM from control nor in PM from TRH-treated cells. Out of all identified changed proteins only 1 increased protein (phospholipase C-α) originated from the plasma membrane, all the others were from different cellular organelles or compartments. Interestingly, up-regulation of PLC-α transcript was also observed during prolonged stimulation of Jurkat cells and the rat uterus or hypothalamus [Goldfien et al., 1991; Kaplitt et al., 1993]. To date, there is no information about localization of PLC-α in membrane rafts and, on the contrary, it has been demonstrated to associate with angiotensin II receptors [Mah et al., 1992], which are normally not located in caveolar microdomains [Ishizaka et al., 1998]. Hence, these findings are basically in line with the results of our earlier experiments showing no alterations in an overall composition of plasma membrane microdomain proteins of E2M11 cells after stimulation with TRH [Matousek et al., 2005]. Another important conclusion which can be drawn from these observations is that the plasma membrane-enriched fraction did not contain only membrane proteins but it was relatively highly contaminated with proteins from different cellular organelles and compartments. Despite that,

TABLE I. List and Basic Characteristics of the Proteins Identified in the Plasma Membrane-Enriched Fraction From E2M11 Cells That Were Altered by Long-Term Treatment With TRH

Spot	Accession no.	Protein name	Subcellular localization	Change (fold)
1	gi|2274968	Glucosidase II	ER	↑ (2.0)
2	gi|516764	Motor protein	Inner mitochondrial membrane	↑ (2.2)
3	gi|48145703	Inner membrane mitochondrial protein (mitofilin)	Inner mitochondrial membrane	↑ (3.0)
4	gi|62414289	Vimentin	Cytoskelet	↑ (2.7)
5	gi|46249758	Ezrin	Cell membrane, cytoplasm, cytoskelet	↑ (2.0)
6	gi|292059	MTHSP75	Mitochondrion	↑ (2.7)
7	gi|156416003	Succinate dehydrogenase complex, subunit A, flavoprotein precursor	Inner mitochondrial membrane	↑ (2.5)
8	gi|4506753	RuvB-like 1	Nucleus, cytoplasm, membrane	↑ (4.6)
9	gi|36796	T-complex polypeptide 1	Cytoplasm	↑ (2.4)
10	gi|403456	26S protease (S4) regulatory subunit	Cytoplasm, nucleus	↑ (2.6)
11	gi|860986	Protein disulfide isomerase	ER, cell membrane	↑ (2.2)
12	gi|303618	Phospholipase C alpha	Cell membrane	↑ (2.0)
13	gi|6137677	Chain A, human mitochondrial aldehyde dehydrogenase complexed with NAD^+ and Mn^{2+}	Mitochondrion	↑ (3.4)
14	gi|1710279	Dihydrolipoamide acetyl transferase	Mitochondrion	↓ (5.2)
15	gi|18605506	PMPCA protein	Mitochondrion	↑ (2.2)
16	gi|5453603	Chaperonin containing TCP1, subunit 2	Cytoplasm	↑ (2.0)
17	gi|7657381	PRP19/PSO4 pre-mRNA processing factor 19 homolog	Nucleus	↑ (2.3)
18	gi|5771523	3-Phosphoglycerate dehydrogenase	Cytoplasm	↑ (2.2)
19	gi|32189394	ATP synthase, H^+ transporting, mitochondrial F1 complex, beta subunit precursor	Inner mitochondrial membrane	↓ (2.5)
20	gi|14124984	Chaperonin containing TCP1, subunit 3	Cytoplasm	↓ (4.5)
21	gi|46593007	Ubiquinol-cytochrome c reductase core protein I	Inner mitochondrial membrane	↑ (2.0)
22	gi|5031753	Heterogeneous nuclear ribonucleoprotein H1	Nucleus	↓ (3.8)
23	gi|31543831	Tubulin, gamma 1	Centrosome	↓ (5.0)
24	gi|10835067	Autoantigen La	Nucleus, cytoplasm	↑ (3.7)
25	gi|2697005	Cell cycle protein p38-2G4 homolog	Cytoplasm, nucleus	↑ (2.7)
26	gi|30311	Cytokeratin 18	Cytoplasm	↓ (7.1)
27	gi|7305503	Stomatin (EPB72)-like 2	Cell membrane, inner mitochondrial membrane, cytoplasm	↑ (2.1)
28	gi|386854	Type II keratin subunit protein	Cytoskelet	↑ (2.5)
29	gi|39644794	EEF1G protein	Cytoplasm	↓ (2.2)
30	gi|1706611	Elongation factor Tu, mitochondrial precursor	Mitochondrion	↑ (2.4)
31	gi|181969	Elongation factor 2	Cytoplasm	↓ (2.5)
32	gi|11935049	Keratin 1	Cytoskelet	↓ (5.0)
33	gi|9910244	Mitochondrial ribosomal protein S22	Mitochondrion	↑ (2.0)
34	gi|12654583	Ribosomal protein, large, P0	Cytoplasm	↑ (3.0)
35	gi|16924265	Enoyl coenzyme A hydratase 1, peroxisomal	Peroxisome	↑ (2.0)
36	gi|110349780	ATP synthase mitochondrial F1 complex assembly factor 1 isoform 1 precursor	Inner mitochondrial membrane	↑ (2.0)
37	gi|5803013	Endoplasmic reticulum protein 29 isoform 1 precursor	ER	↑ (2.8)
38	gi|4505773	Prohibitin	Inner mitochondrial membrane, cell membrane, nucleus	↑ (2.2)
39	gi|4758788	NADH dehydrogenase (ubiquinone) Fe-S protein 3	Inner mitochondrial membrane	↑ (2.4)
40	gi|12707570	Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor	Mitochondrial membrane	↑ (2.5)
41	gi|32483377	Peroxiredoxin III isoform b	Mitochondrion	↑ (2.7)
42	gi|51479152	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit d, isoform b	Inner mitochondrial membrane	↑ (2.0)

PM proved to be a very useful and advantageous material for precise determination of TRH receptors and $G_{q/11}$ protein activity, as well as for assessment of hormone-induced alterations in other proteins, when compared to PNS.

Our analyses of the upper (PM-enriched) and lower density gradient fraction provided the same protein maps and identified the same altered proteins after TRH treatment. These data indicate that the major protein components of mitochondria, as well as other membranes were distributed in both analyzed gradient fractions.

Interestingly enough, roughly about 40% of the identified proteins with altered expression originated from the mitochondria, suggesting an important effect of TRH on mitochondrial processes. Long-term treatment with TRH significantly increased the level of succinate dehydrogenace complex subunit A flavoprotein precursor, ubiquinol-cytochrome c reductase core protein I, and NADH dehydrogenase (ubiquinone) Fe-S protein 3 (components of the respiratory chain). Among other elevated mitochondrial proteins were functionally closely related prohibitin, mitofilin and stomatin, which support proper functioning of mitochondria [Arnold and Langer, 2002; John et al., 2005; Merkwirth et al., 2008]. The amount of PMPCA protein, a peptidase that cleaves the N-terminal peptide of mitochondrial protein precursors during their transport from the cytoplasm to mitochondria [Ito, 1999], was increased as well. These data indicate that TRH can not only affect the expression of mitochondrial proteins but also the production of mature proteins from appropriate precursors. In this context it can be worth to mention that chronic administration of TRH to neonatal rats altered activities of several mitochondrial enzymes [Miyamori et al., 1988].

TRH has been demonstrated to exert antiapoptotic activity in neurons, as well as in pancreatic β-cells [Luo et al., 2002; Luo and Yano, 2005] and it seems to function as an important neurotropic factor, because its removal leads to a predictable cell death [Luo

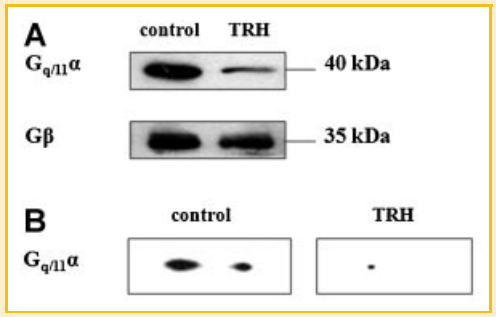

Fig. 5. Effect of long-term treatment with TRH on the levels of selected G-protein subunits. A: The distribution of $G_{q/11}\alpha$, and Gβ was determined in samples (50 μg protein) from control and TRH-treated (10 μM, 16 h) E2M11 cells by standard SDS–PAGE and immunoblotting using specific antibodies as described in Materials and Methods Section. The relative levels of $G_{q/11}\alpha$ proteins in the plasma membrane-enriched fractions from control and hormone-treated cells were also determined by immunoblotting after separation of these proteins by 2DE (B). The larger spot (to the left) corresponds to the dominant $G_{11}\alpha$ protein.

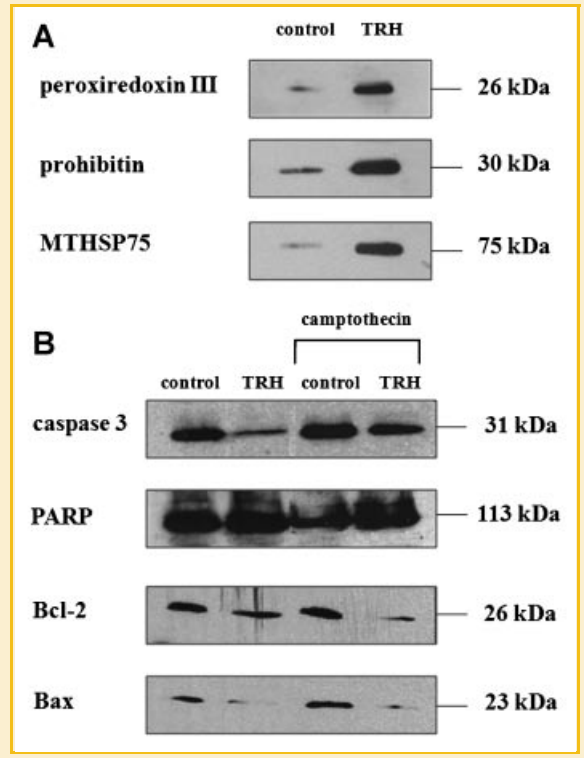

Fig. 6. Effect of long-term treatment with TRH on the levels of selected apoptotic markers. A: The TRH-induced changes identified by 2DE/MS analysis in the content of antiapoptotic proteins peroxiredoxin III, prohibitin, and MTHSP75 were corroborated by SDS–PAGE and immunoblotting using specific antibodies as described in Materials and Methods. For these analyses, 5 μg protein from control and TRH-treated (10 μM, 16 h) E2M11 cells were applied on the gel. B: The distribution of caspase 3, PARP, Bcl-2 and Bax was determined in samples (50 μg protein) from control and TRH-treated (10 μM, 12 h) E2M11 cells, which were subsequently exposed to camptothecin (1×10^{-6} M) for 4 h. SDS–PAGE and immunoblotting was conducted as described in Materials and Methods. The ratio of Bcl-2/Bax signals was calculated for each sample. Values were expressed as relative amount of protein expression, with the mean density in the control group (lane 1) being 1. The Bcl-2/Bax ratio in the TRH (lane 2), camptothecin/control (lane 3) and camptothecin/TRH (lane 4) group was 3.9, 0.6, and 1.6, respectively.

et al., 2001]. Our current observations of increased levels of mitochondrial proteins with presumed antiapoptotic activity, such as peroxiredoxine III, prohibitin, mitofilin and MTHSP75 [John et al., 2005; Merkwirth et al., 2008], in samples from TRH-treated cells support the notion about antiapoptotic effects of the hormone. The antiapoptotic effect of TRH on E2M11 cells was also corroborated by our finding of substantial decrease in the content of proapoptotic enzyme caspase 3. Accordingly, the level of PARP, an enzyme engaged in repair of DNA damage, which is cleaved during early apoptosis by caspase 3 [Decker et al., 2000], was not changed. The results of our subsequent analyses of other crucial apoptotic markers, Bcl-2 and Bax, as well as experiments with the apoptosis-inducing drug camptothecin further supported the notion about marked antiapoptotic effects of TRH.

We have previously found out that prolonged treatment of E2M11 cells with TRH led to marked down-regulation of the cognate $G_{q/11}\alpha$ proteins [Drmota et al., 1998]. Our present analyses using standard SDS–PAGE or 2DE followed by immunoblotting of PNS and PM from TRH-treated cells confirmed a substantial down-regulation of $G_{q/11}\alpha$ proteins. Moreover, we also observed a significant decrease in the content of Gβ subunit. It has been reported previously that the level of expression of G-protein α and β subunits can be at least to some extent co-regulated [Hermouet et al., 1993]. Hence, it can be assumed that the reduction of Gβ might have accompanied the down-regulation of $G_{q/11}\alpha$ apparently in order to maintain an approximately equimolar ratio between the G-protein α and β subunits.

In summary, our present findings of changed expression of several different proteins in preparations from E2M11 cells treated in the long term with TRH suggest that this peptide may exercise profound effects on gene expression and thus on cell physiology. This was also evidenced by cessation of growth and division of the cells during sustained presence of TRH. Out of all identified altered proteins only one was suspected to originate from the plasma membrane, which is basically in line with the results of our earlier study showing no changes in protein composition of membrane microdomains [Matousek et al., 2005]. Perhaps rather surprisingly, majority of the herein identified altered proteins did not match previously reported data of microarray analyses dealing with gene expression in pancreatic cells affected by TRH [Yano and Luo, 2004; Luo and Yano, 2005]. This incongruity can be attributed to different cell models and experimental conditions. In addition, the data from the microarray hybridization indicating an altered gene expression cannot be taken as a guarantee for actual changes in protein levels.

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