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Cell signalling and molecular complexes of the TRH receptor

PhD. Thesis

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List of author´s publications

Original papers related to the thesis:

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Abbreviations

2DE	Two-dimensional electrophoresis
ABC	Ammonium bicarbonate
AC	Adenylyl cyclase
ACN	Acetonitrile
ADP	Adenosine diphosphate
AGS	Activator of G-protein signalling
AKAP	A-kinase anchor protein
AR	Adrenergic receptor
ARF	ADP-ribosylation factor
ARNO	ARF nucleotide-binding site opener
ASK	Apoptosis signal-regulating kinase
ATP	Adenosine triphosphate
BRET	Bioluminescence resonance energy transfer
BTK	Bruton's tyrosine kinase
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CCR	CC chemokine receptor
cGMP	Cyclic guanosine monophosphate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CK	Casein kinase
CN-PAGE	Clear native polyacrylamide gel electrophoresis
CREB	cAMP response element-binding
CXCR	CXC chemokine receptor
DAG	Diacylglycerol
DAMGO	[D-Ala^2, N-$MePhe^4$, Gly-ol]-enkephalin
DEP	Disheveled/EGL-10/Plextrin homology
DH	Dbl homology
DMEM	Dulbecco's modified eagle medium
DPDPE	D-Penicillamine(2,5)-enkephalin
DTT	Dithiotreitol
E2	Cell line HEK293 expressing exogenous TRH receptor
E2M11	Cell line HEK293 expressing exogenous TRH receptor and $G_{11}\alpha$ protein
EBP50	ERM-binding phosphoprotein 50
EC	Extracellular loop
ECL	Electrochemiluminiscence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
ERM	Ezrin-radixin-moesin
FERM	4.1 protein/ezrin/radixin/moesin
FGF	Fibroblast growth factor
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
FSH	Follicle-stimulating hormone

GABA	Gamma-aminobutyric acid
GAP	GTPase-activating protein
GASP	GPCR-associated sorting protein
GDI	GTPase dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGL	Gγ-like
GIPC	GAIP-interacting protein C terminus
GIRK	G protein-coupled inwardly-rectifying potassium channel
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GRP1	General receptor for phosphinositides-1
GRP75	Glucose-regulated protein 75
GTP	Guanosine triphosphate
GTPγS	Non-hydrolyzable analog of GTP
HA	Hemagglutinin
HEK293	Human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAA	Iodoacetamide
IC	Intracellular loop
IEF	Isoelectric focusing
IGF	Insulin-like growth factor
IP_3	Inositol triphosphate
JAK/STAT	Janus protein kinase/signal transducers and activators of transcription
JNK	*c-jun* NH_2-terminal kinase
LDH	Lactate dehydrogenase
LHCG	Luteinizing hormone/choriogonadotropin receptor
LM	Lauryl maltoside
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen-activated protein kinase
MKK	Mitogen-activated protein kinase kinase
MS	Mass spectrometry
MTHSP75	Mitochondrial heat shock protein 75
NDPK	Nucleoside diphosphate kinase
NFAT	Nuclear factor of activated T-cells
NHERF	Na^+/H^+ exchanger regulatory factor
NP-40	Nonidet P-40
NSF	N-ethylmaleimide-sensitive factor
PAF	Platelet-activating factor
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated protein kinase
PAR	Protease-activated receptor
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDZ	PSD-95/Discs large/Zone occludens-1
PH	Pleckstrin homology

PI	Phosphatidyl inositol
PI3K	Phosphoinositide-3 kinase
PI-(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PM	Plasma membrane
PNS	Postnuclear supernatant
PP2A	Protein phosphatase 2A
PTB	Phosphotyrosine-binding
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RGS	Regulator of G-protein signalling
RH	RGS homology
RKIP	Raf kinase inhibitor protein
RTK	Receptor tyrosine kinase
SDS	Sodium dodecylsulfate
SH2	Src homology 2
siRNA	Small interfering ribonucleic acid
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TH	Tec homology
TNF	Tumor necrosis factor
TOF	Time-of-flight mass spectrometer
TRAF	TNF receptor-associated factor
TRH	Thyrotropin-releasing hormone
TRH-R	TRH receptor
TSH	Thyroid-stimulating hormone

1. Introduction

Signal transduction is one of the most important processes in cells enabling that cells can communicate with each other and respond to stimuli from extracellular environment. The communication between cells is realized by action of chemical signals such as hormones, neurotransmitters, neuromodulators and sensory stimuli. The signals are transferred from extracellular environment into cell interior by interaction of signalling molecules with receptors, which are transmembrane proteins localized in the plasma membrane. Response of cells can differ dependently on the type of a particular receptor as well as the type of effectors to which signal is subsequently transferred. The sensitivity of cells to a particular signal can be raised by increase of receptor number on the cell surface or reduced due to desensitization (reduction of biological response).

One of the main classes of membrane-bound receptors is a class of G-protein coupled receptors (GPCRs), which transfer the signal to effectors (e.g., adenylyl cyclase, phospholipase C, phosphodiesterase and ion channels) via heterotrimeric proteins binding guanine nucleotides, G-proteins. The regulation of effector activity by G-proteins induces changes in concentrations of second messengers such as cAMP, Ca^{2+}, DAG and IP_3, which trigger many cell processes resulting in subsequent physiological response.

The prolonged or repeated stimulation of cells with hormones leads to desensitization, a process in which biological response to some stimulus is decreased or eliminated in order to protect cells against damage. Desensitization on the level of receptors is realized by receptor uncoupling, redistribution or degradation. Similar mechanisms can be also observed on the level of G-proteins.

The transfer of signal is carried out by interactions between specific proteins. The protein-protein interactions of signalling molecules determine which signalling pathways will be turned on. Actual cell response is then defined by combination of cell processes induced by triggering of different signalling pathways. Interactions of signalling proteins are mediated by specific protein modules determining which proteins will interact with each other. Signalling pathways can be then interconnected into protein networks. This complex mechanism co-ordinates individually received signals into one a unique cell response.

2. Aims of the thesis

The primary objective of the thesis was to investigate signal transduction via thyrotropin-releasing hormone (TRH) receptor and $G_{q/11}$ protein in HEK293 cell line exogenously expressing long isoform of rat TRH receptor (TRH-R1) and mouse $G_{11}\alpha$ protein (clone E2M11). There were two main aims to explore. The first one was preoccupied with determination of the impact of prolonged TRH exposure on the expression of membrane-bound proteins and the second was focused on identification of signalling molecular complexes of TRH receptor and its cognate $G_{q/11}$ protein.

Specific aims:

1) To isolate the plasma membrane-enriched fraction from HEK293-E2M11 cells
2) To investigate the effects of prolonged TRH treatment on the expression and distribution of proteins in the plasma membrane-enriched fraction by 2D electrophoresis
3) To identify the altered proteins by mass spectrometry
4) To identify and characterise molecular complexes of the TRH receptor and $G_{q/11}$ protein
5) To assess the effect of TRH on the stability of TRH-R and/or $G_{q/11}$ protein molecular complexes

3. Literature review

3.1. G-protein-coupled receptors

A family of G-protein coupled receptors (GPCRs) is the largest family of receptors involving more than one thousand members and represents 1-5% of the invertebrate or vertebrate genomes (Bockaert *et al.*, 2003). It comprises 3-4% of the human genome (about 1,000 genes) with 1,200 - 1,300 members and 5% of the *Caenorhabditis elegans* genome with approximately 1,100 members. The Drosophila genome involves at least 160 members of GPCRs (Lefkowitz, 2004; Tuteja, 2009). Human genes coding GPCRs represent mainly intronless genes (Gentles and Karlin, 1999).

The structure of ligands which bind to GPCRs is highly variable from large molecules like proteins and peptides to small molecules like nucleotides or even cations. The largest group of ligands is represented by proteins and peptides which naturally bind to approximately 50% of GPCRs. Next groups are biogenic amines (26% of GPCRs), lipid-like ligands (15%), amino acids (4%), nucleotides (2%) and cations (2%) (Bjarnadottir *et al.*, 2006; Lagerstrom and Schioth, 2008). Nevertheless, only a small number of human GPCRs have been successfully targeted for therapeutic purposes. For example, about 17% of receptors interacting with peptides and proteins, 29% of receptors interacting with biogenic amines and 20% of receptors interacting with lipid-like ligands are currently used for curing different health problems (Lagerstrom and Schioth, 2008). From another point of view, about 50-60% of the investigated prescription drugs, which are marketed, act on GPCRs (Tuteja, 2009). Although many GPCRs-targeted medicines remain to be investigated, these receptors represent the most useful therapeutic family.

The history of the observation of GPCRs started as far back as the first decade of the 20th century when Langley and his student Dale first stated the idea of a ‘receptive substance’ on reactive cells when they studied receptors which are now known as adrenergic receptors, muscarinic acetylcholine receptors and nicotinic acetylcholine receptors. In the next decades, several scientists, e.g. Ariens and Furchgott, performed the experiments based on the dose-dependent activity of hormones, neurotransmitters and drugs and introduced the conceptions of the efficacy and the affinity in the drug action. In the 1960s and 1970s, Krebs, Sutherland and Rodbell and Gilman discovered the elements of the signaling cascade of GPCRs such as cAMP-dependent protein kinase, the second messenger cAMP and a guanine nucleotide-binding regulatory protein, respectively (Jacoby *et al.*, 2006; Lefkowitz, 2004). The next studies could be performed only with the development of modern biochemical methods including radioligand binding, affinity labelling techniques, affinity chromatography purification and lipid re-constitution. Many primal experiments were accomplished to study the nicotinic acetylcholine receptor and rhodopsin because they are abundant in electric organs of electric fish and retinal rods, respectively, and thus their purifications were not required. The next

receptor which was studied at this time was β_2-adrenergic receptor which was the first GPCR to be purified (Caron *et al.*, 1979; Lefkowitz, 2004). Although rhodopsin was one of few receptors which were deeply studied, it was not viewed as a receptor model until it was found that its structure is homologous to the structure of the β_2-adrenergic receptor (Jacoby *et al.*, 2006).

More recent studies investigate not only the receptor structure, efficacy, kinetics, ligand binding and their downstream signaling pathways but also processes concerning the desensitization (reduction of biological response to long-term or repeated stimulus), internalization (transfer of molecules from the plasma membrane into the intracellular membrane compartments) and down-regulation (protein degradation).

3.1.1. Classes of GPCRs

The classical division of GPCRs is represented by the A-F classification system which involves all receptors coupled to heterotrimeric G-proteins.

The class A, the rhodopsin receptor family, is the largest family of GPCRs with approximately 700 members and is divided into four groups (α, β, γ and δ). Members of the α-group are e.g. histamine, dopamine, serotonin and muscarinic receptors as well as adrenergic receptors and rhodopsin. These receptors often bind ligands through the binding site embedded in the cavity formed by the seven transmembrane domains. The β-group involves receptors for peptide and protein ligands such as endothelin, gonadotropin-releasing hormone or oxytocin, which are bound to the pocket in the transmembrane domains, extracellular loops and the N-terminus of a receptor. The members of the γ-group are receptors for peptides and lipid-like compounds such as opioids, somatostatin or angiotensin. The important cluster of the γ-group represents the chemokine receptors participating in acute and chronic inflammation. The δ-group involves olfactory receptors activated by a broad range of odorants, glycoprotein-binding receptors (FSH receptor, TSH receptor and LHCG receptor) or protease-activated receptors (PARs) (Lagerstrom and Schioth, 2008; Strotmann *et al.*, 2011).

The class B is constituted by the secretin receptor family and the adhesion receptor family. The secretin receptor family is comprised of 15 different receptors, e.g. calcitonin receptor, corticotropin-releasing hormone receptor and glucagon receptor, which bind peptide hormones. The N-terminal domain is the most variable part of these receptors but it is important for ligand binding. Members of the adhesion receptor family differ from the members of the secretin receptor family in their N-terminal domain architecture. The N-terminal domain may include cadherin, lectin, laminin, olfactomedin, immunoglobulin and thrombospondin domains. Contrary to the secretin receptor family, these receptors have a GPCR proteolytic site (GPS) domain and bind extracellular matrix molecules as ligands (Lagerstrom and Schioth, 2008; Strotmann *et al.*, 2011).

The class C represents the glutamate receptor family with metabotropic glutamate receptors, $GABA_B$ receptors and the calcium-sensing receptor. These receptors contain large ectodomains responsible for ligand binding. The class D, fungus pheromone receptor family, includes pheromone receptors coupled with G_i-proteins. The class E, the cAMP receptor family, has been found only in slime mold *Dictyostelium discoideum*. The members of the class F, the frizzled/smoothened receptor family, are involved in embryonic development, cell polarity or segmentation (Tuteja, 2009).

3.1.2. Evolution of GPCRs

By phylogenetic analyses, the seven-transmembrane core was assessed to be highly conserved during GPCR evolution. Although these proteins are typical mainly for eukaryotic organisms, they were also identified in prokaryotic organisms such as archea and bacteria. Light-sensitive proteo-, bacterio- and halorhodopsins structurally similar to eukaryotic organisms are involved in non-photosynthetic energy harvesting or may control phototaxis. Nevertheless, bacteriorhodopsin was shown not to couple to the G-proteins. It is worth pointing out that only a few GPCRs have been found in plants and fungi genomes and the number of these receptors has increased several times from nematode to mammals. Some receptor groups expanded at different stage of evolution while other receptors can be considered ancient. The highest sequence similarity of bacteriorhodopsin and mammalian GPCR was observed between non-homologous helices, which could be caused by exon shuffling or gene duplication of an ancestral three-transmembrane module. Different receptor families as well as receptors in different species vary in their exon and intron composition, e.g. mammalian receptors genes represent intronless genes or genes with a lower density of introns in comparison with their cognate receptors in invertebrates (Fridmanis *et al.*, 2007; Strotmann *et al.*, 2011). Two opposite concepts can explain this fact. The first notion presumes a decrease of an intron number by gene multiplication through retroposition (Gentles and Karlin, 1999). The second notion suggests a major loss of introns. An example represents the Rhodopsin family in which loss of an ancient intron located in the DRY motif occurred (Bryson-Richardson *et al.*, 2004). On the other hand, introns are frequent in receptors of the secretin family or in metabotropic glutamate-receptor in both invertebrate and vertebrate species (Strotmann *et al.*, 2011).

Phylogenetic classification of GPCRs based on differences of functional domains within the N- and C-terminal regions and membrane-spanning region distinguishes receptors into five basic families. This classification is called GRAFS according to the names of individual groups: Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F) and Secretin (S) (Schioth and Fredriksson, 2005).

The Glutamate receptor-like family represents the class C in the classification. It is present in the parazoon *Geodia cydonium* and even in the slime mold *Dictyostelium discoideum* whose

phylogenetic origin is dated 600 million years ago. The Venus fly trap module which has the sequence similarity to the bacterial periplasmic amino acid-binding proteins is characteristic for this group. This group is related to the Rhodopsin-like family due to a number of amino acids highly conserved in their transmembrane domains. Members of the Rhodopsin receptor family have been identified in bilateria and cnidaria which suggests that they evolved before the protostome-deuterostome split. The expansion of their evolution occurred mainly in vertebrate compared to invertebrate. The Adhesion family of GPCRs can be found not only in most metazoan species but even in fungal and plant genomes. These receptors show high expansion in mouse and humans which could be caused by rapid gene duplication. Members of the Frizzled-like receptor family, which are the most highly conserved, have been found e.g. in sponges, cnidaria and placozoa. The last group, the Secretin receptor family, forms a phylogenetic group which shares a similar transmembrane core and the N-terminal domain with the Adhesion family and is likely to be descended of the group V adhesion receptors. All members of the Secretin receptor family contain conserved cysteine residues in the first and second extracellular loops of the transmembrane domains (Lagerstrom and Schioth, 2008; Strotmann *et al.*, 2011). In conclusion, many groups of GPCRs evolved before metazoan evolution and thus these receptors might have played an important role in the evolution and development of many different species.

3.1.3. Structure of GPCRs

In many experiments concerned with the structure of GPCRs, the structure of rhodopsin has been studied. The first experiment performed in 1978, which was based on circular dichroism (a method used to observe secondary structure of proteins), provided a model for rhodopsin as a series of transmembrane helical segments connected by random-coil regions (Albert and Litman, 1978). Using sequencing experiments, the primary structure of rhodopsin was revealed in 1982-1983 (Hargrave *et al.*, 1983; Ovchinnikov *et al.*, 1982). Nowadays, more than two hundred members of the Rhodopsin family from different species have been sequenced (Lagerstrom and Schioth, 2008).

The first two-dimensional crystal structure of rhodopsin was obtained using cryo-electron microscopy in 1993 when a bundle of seven transmembrane helices was seen (Schertler *et al.*, 1993). The bovine rhodopsin was also the first receptor whose three-dimensional crystal structure was solved using diffraction data extending to resolution 2.8 Å (Palczewski *et al.*, 2000). The crystal structure was resolved for other receptors in their structural modifications with different ligands of heterotrimeric G-protein such as β_1-adrenergic (Warne *et al.*, 2008), β_2-adrenergic (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2011), adenosine A2 (Jaakola *et al.*, 2008), dopamine D3 (Chien *et al.*, 2010), histamine H1(Shimamura *et al.*, 2011) and chemokine CXCR4 receptors (Wu *et al.*, 2010).

GPCRs are proteins with seven transmembrane α-helical domains (TM I-VII) connected by three extracellular loops (E1-E3) and three intracellular loops (I1-I3). The N-terminal region of these receptors is oriented to the extracellular side of the plasma membrane while the C-terminal region is oriented to the intracellular side. The members of GPCRs can differ in their lengths or in the lengths of their N-terminus, the C-terminus or intracellular loop I3 (Baldwin, 1993).

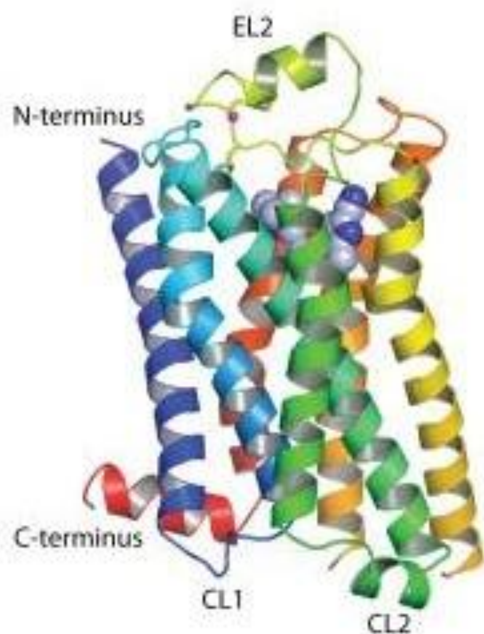


Fig. 3.1
A three-dimensional model of the β_1-adrenergic receptor (N-terminus blue, C-terminus red) (Warne *et al.*, 2008). EL2 (extracellular loop 2), CL1 and CL2 (intracellular loops 1 and 2)

The length of members falling into different classes of GPCRs as well as the length of a particular receptor parts can be greatly variable. In case of family A, the length is between 290 and 951 amino acid residues. These receptors incorporate several areas of conserved residues in the transmembrane α-helical domains. The conserved residues Gly and Asn are in TM I, Leu and Asp in TM II, Cys and AspArgTyr (DRY-motif) in TM III, Trp and Pro in TM IV, Pro and Tyr in TM V, Phe, Trp and Pro in TM VI and Asn, Pro and Tyr of the NPXXY motif in TM VII (Mirzadegan *et al.*, 2003).

However, the common feature of all GPCRs is that the individual transmembrane α-helices are packed into a ring-shaped bundle forming a strongly hydrophobic core of receptor. The observation of arrangement of rhodopsin transmembrane α-helices by cryo-electron microscopy revealed that TM IV, TM VI and TM VII are not significantly tilted whereas TM I, TM II, TM III and TM V are tilted up to an angle of 30 degrees. TM I has a tilt angle of 28 degrees and probably makes a short cytoplasmic connection to TM II, which is tilted by an angle of 27 degrees, and interacts with TM I, TM III and

TM VII on the extracellular side and with TM IV on the intracellular side. The most tilted α-helix is TM III with an angle of 30 degrees. It is buried in the centre of the molecule and interacts with TM II, TM IV, TM V and TM VI on the intracellular side. On the other hand, TM IV is the least tilted α-helix and appears to be the shortest in the structure. It interacts with TM II and TM III on the intracellular side where is separated from TM VI and TM VII by TM II and TM III. On its extracellular side, it is in contact with TM III and TM V. TM V has an angle of 23 degrees and connects to TM III towards to the intracellular side. TM VI is nearly perpendicular and is in contact with TM V and TM VII on the extracellular side and with TM III, TM V and TM VII on the intracellular side. TM VII is almost perpendicular as well and is in contact with TM I, TM II and TM VII on both sides. The transmembrane α-helices are closely packed on the intracellular side and open towards the extracellular side where a cavity for binding of retinal (vitamin A aldehyde) is formed by TM III, TM IV, TM V, TM VI and TM VII (Unger *et al.*, 1997).

Using multiple sequence analyses of many members of receptor class A (Rhodopsin family), the N-terminal region was found to be highly variable at a number of amino acid residues (4-50 amino acids). The E1 loop which connects TM II and TM III is the most consistent loop in its size and can range from 3 to 8 amino acid residues. The E2 and E3 loops are more variable in their size. In most members of receptor family A, the E2 loop involving two twisted β-hairpins is connected to the Cys residue on the extracellular end of the TM III by disulfide bridge which takes part in the stabilization of an activated state (Lu *et al.*, 2002; Mirzadegan *et al.*, 2003; Peeters *et al.*, 2011). On the contrary, the E2 loop in the β_2-adrenergic receptor is formed by α-helix which is locked in its position by two disulfide bonds and several hydrophobic packing interactions (Weis and Kobilka, 2008).

The N-terminus and extracellular loops constitute the area which the ligands are bound to. The area of receptor taking part in ligand binding varies dependently on the size and character of ligands. Small molecules like catecholamines bind in a cavity formed by TM III and TM VI, short peptides, e.g. thyrotropin-releasing hormone (TRH), interact with extracellular loops, the N-terminal region as well as a cavity as in the case of catecholamines and large proteins are bound to the large N-terminal extracellular domain (Bockaert and Pin, 1999). Ligand binding causes a conformational change in the receptor and thereby its activation. This conformation change can be triggered by disruption of a salt-bridge interaction between TM III and TM VII and mainly by the changes in the relative positions of TM III and TM VI (Karnik *et al.*, 2003; Weis and Kobilka, 2008).

The C-terminus of many members of GPCRs involves an amphipathic helix 8 previously called an intracellular loop 4. It is connected to the cytoplasmic end of TM VII by a short linker sequence containing two cysteine residues, which can be palmitoylated and attach the proximal portion of the C-terminus to the plasma membrane. It is believed to be an important part of the receptor C-terminus participating in conformational change during receptor activation and

deactivation, receptor trafficking, internalization and dimerization and G-protein coupling and activation (Gehret *et al.*, 2010; Huynh *et al.*, 2009; Wess *et al.*, 2008).

Apart from a cytoplasmic C-terminus, a third intracellular loop is also an important intracellular part of GPCRs. Changes in the C-terminal region were found to inhibit receptor internalization and mutation of some amino acid residues in the third intracellular loop of the human somatostatin receptor 5 abolished or reduced arrestin association and a significantly reduced receptor internalization (Peverelli *et al.*, 2008). This loop plays an important role in binding of the α subunit of G-protein as well as receptor activation (Chakir *et al.*, 2003; Oldham and Hamm, 2008).

3.1.4. Dimerization and oligomerization of GPCRs

One of the most important characteristic of GPCRs is their ability to form dimers or higher-structure oligomers. If oligomers are formed by the specific receptor isoform, these complexes are called homodimers or homooligomers. On the other hand, oligomers comprised of different receptor isoforms are called heterodimers or heterooligomers. Theoretical consideration and experimental evidence of receptor dimer existence suggested that at least in the case of some receptors the classical concept of the interaction between the receptor and the G-protein with the 1 : 1 stoichiometry should be replaced with the 2 : 1 stoichiometry (Maggio *et al.*, 2007). Such pentameric complexes of the receptor dimer with the G-protein were proven for leukotriene B_4 (LTB_4) receptor BLT1 or serotonin 5-hydroxytryptamine 2C (5-HT_{2C}) receptor (Baneres and Parello, 2003; Herrick-Davis *et al.*, 2005). Although receptor homo- and heterodimers have been observed more often, it was shown that rhodopsin dimers in rod outer membranes or M2 muscarinic receptor in *Sf9* cells can form oligomeric linear arrays (Maggio *et al.*, 2007). It seems that distinct regions are crucial during dimerization and oligomerization of different receptors. These regions can be represented by extracellular Cys residues forming disulphide bonds, the C-terminal region or transmembrane domains (Pfleger *et al.*, 2004).

Dimerization can play an important role in trafficking as well as signalling. Constitutive dimers represent receptor complexes formed in the endoplasmic reticulum during the receptor biosynthesis. These preformed dimers can be subsequently exported to the cell surface and their activation by ligand binding occurs only on the level of their conformational changes. Heterodimers of $GABA_{B1}$ and $GABA_{B2}$ receptors exemplify constitutive dimerization (Bulenger *et al.*, 2005). On the contrary, the ligand-induced dimerization represents the formation of receptor dimers after ligand binding and receptor activation. Using immunoblot, FRET and BRET, it was observed that chemokine receptors CCR2, CCR5 and CXCR4 and gonadotropin-releasing hormone (GnRH) receptor form homodimers after agonist binding (Angers *et al.*, 2002).

The formation of heterodimers can strongly influence the coupling with different interacting partners and the signalling efficiency or cause a crosstalk between different signalling pathways. For

example, the interaction of M2/M3 heterodimers with β-arrestin 1 is more efficient in comparison with the interaction of M3 sole receptor or M3/M3 homodimer with β-arrestin 1 (Novi *et al.*, 2005). The next example is represented by isoforms of the α_2-adrenergic receptor. The α_{2A}-adrenergic receptors as well as α_{2C}-adrenergic receptors are able to form homodimers but when they are co-expressed, they prefer to form heterodimers. Small and co-workers observed that the formation of heterodimers was accompanied by a marked reduction in the level of GRK2-mediated α_{2A}-AR phosphorylation and subsequent attenuation of β-arrestin recruitment. These processes were found to result in a decrease of Akt activation whereas the p44/p42 (ERK1/ERK2) MAP kinase pathway was unaffected (Small *et al.*, 2006).

The formation of heterodimers can also lead to the alteration of selectivity, e.g. the co-expression of δ- and κ-opioid receptors resulted in a loss of binding to selective δ- and κ- opioid ligands but does not affect the binding of non-selective ligands. Nevertheless, the simultaneous adding of the both δ- and κ-selective agonists caused the restoration of their binding what suggests that the mechanism of their binding corresponds to the positive co-operativity (Angers *et al.*, 2002). In the case of μ- and δ-opioid receptors, their heterodimers formed a novel signal entity with lower affinity for μ- a δ-selective agonists DPDPE and DAMGO in comparison with the individual isoforms of opioid receptors (George *et al.*, 2000). When cells co-expressing these both these receptors were treated with δ-opioid ligands such as TIPP*ψ* or deltorphin II, the increase of the positively co-operative binding of μ-opioid ligand DAMGO occurred (Angers *et al.*, 2002). Interestingly, contrary to the observed selective coupling of separately expressed D_1 and D_2 dopamine receptors to $G_i\alpha$ or $G_s\alpha$ proteins, respectively, the co-expression of these receptors in COS-7 resulted in activation of $G_{q/11}\alpha$ protein and stimulation of the phospholipase C/calcium pathway (Lee *et al.*, 2004).

Heterodimerization can cause the modulation of endocytotic properties what was observed e.g. for internalization of opioid receptors. The etorphine treatment of cells caused the efficient internalization of δ-opioid receptors whereas it did not affect the κ-opioid receptors. Co-expression of these two receptors resulted in decrease of internalization of δ-opioid receptors as a likely consequence of heterodimerization (Jordan and Devi, 1999). Contrary to the expression of δ-opioid receptors alone, co-expression of μ- and δ-opioid receptors increased the internalization of receptors after DAMGO treatment whereas DPDPE treatment reduced desensitization as well as internalization compared to the expression of μ-opioid receptors alone (George *et al.*, 2000).

Taken all these findings together, dimerization and oligomerization play a very important role in modulation of many cellular processes not only concerned with signal transduction across the plasma membrane but also with triggering of different signalling pathways, which are critical in determining the cellular responses.

3.1.5. Posttranslational modifications

The posttranslational modifications of receptors play a crucial role in their regulation. Some members of GPCRs can be N-glycosylated in their N-terminal region, e.g. GnRH receptor, LH receptor, FSH receptor, V_{1a} vasopressin receptor, oxytocin receptor or bradykinin B2 receptor (Michineau *et al.*, 2006; Wheatley and Hawtin, 1999). O-glycosylation is not such a common posttranslational modification of GPCRs as in case of N-glycosylation although this modification has been observed for V_2 vasopressin receptor or human δ-opioid receptor (Petaja-Repo *et al.*, 2000; Sadeghi and Birnbaumer, 1999).

Another next type of posttranslational modification of GPCRs is acylation such as palmitoylation or isoprenylation. Anchoring of fatty acids to peripheral membrane proteins ensures tight membrane attachment. This kind of modification of transmembrane proteins has a functional significance because it is important for receptor trafficking, desensitization, signalling and interaction with regulatory proteins.

Isoprenylation was identified only in the C-terminal region of the prostacyclin receptor. On the other hand, palmitoylation is a very frequent kind of thio-acylation whereby the 16-carbon palmitate is attached to one or more cysteine residues by a thioester bond. The process in which protein can be palmitoylated during its synthesis or shortly after it is called the constitutive palmitoylation. Contrarily, the term dynamic palmitoylation is used when palmitate is incorporated into a molecule of mature protein (Escriba *et al.*, 2007).

The common sites of palmitoylation are cysteine residues in the cytoplasmic C-terminal region which were found in about 80% of GPCRs. Nevertheless, the evidence that cysteine residues in other receptor domains can be palmitoylated has been provided by experiments in which all cysteine residues in the C-terminal region of rat μ-opioid receptor or V_{1a} vasopressin receptor were mutated but it did not affect or eliminate the palmitate incorporation (Hawtin *et al.*, 2001; Chen *et al.*, 1998). Palmitoylation of receptors plays an important role in signal transfer and activation of heterotrimeric G-proteins because the elimination of palmitoylated sites can result in a receptor inability to couple to some G-protein α subunits (Okamoto *et al.*, 1997).

Phosphorylation of receptors represents another way how receptors can be regulated. The amino acids undergoing phosphorylation are serine or threonine residues mainly in the C-terminal region and the third intracellular loop of a receptor but these residues localized in the first and second intracellular loops can by phosphoralated as well. Several different kinds of protein kinases can phosphorylate the receptors. The first group contains the protein kinases whose activities are regulated by intracellular second messengers, e.g. cAMP-regulated protein kinase A (PKA) or Ca^{2+}-regulated protein kinase C (PKC). These protein kinases phosphorylate appropriate sites in the C-terminal region

and the third intracellular loop of unoccupied as well as agonist-occupied receptors and play an important role in the process of receptor desensitization (Tobin *et al.*, 2008).

Next important group of protein kinases is represented by G-protein-coupled receptor kinases which phosphorylate the serine and threonine residues in the C-terminal region only in agonist-occupied receptors. This group involves seven isoforms of GRK (GRK1–GRK7). The phosphorylation by GRKs results in the uncoupling of the receptor and the G-protein and in the subsequent recruitment of arrestin which is able to uncouple the receptor from its G-protein-dependent signaling pathways (Tobin, 2008). Although at least two amino acid residues in close proximity are needed to be phosphorylated for the arrestin recruitment, it seems that this process occurs due to negative charge of the binding site rather than its specific amino acid sequence (Gurevich and Gurevich b, 2006).

The protein kinases which can also phosphorylate the serine or threonine residues are Akt/PKB (protein kinase B) and casein kinases CK1α and CK2. The sites phosphorylated by Akt/PKB differ from those phosphorylated by GRKs. It was observed that Akt/PKB can phosphorylate the serine residues Ser^{345}/Ser^{346} in the C-terminal region of the β_2-adrenergic receptor and the serine residue Ser^{412} in the C-terminal region of the β_1-adrenergic receptor and these phosphorylations then result in internalization of the receptors. Moreover, the CK1α protein kinase is responsible for the phosphorylation of the serine residues in the C-terminal region of the M_1- and M_3-muscarinic receptors, the subsequent mono-ubiquitination of the lysine residues downstream of the phosphorylation sites and the receptor internalization. Phosphorylation by CK1α can be probably connected with coupling of the receptor to the ERK-1/2 pathway (Tobin, 2008). On the other hand, the protein kinase CK2 which phosphorylates residues in the third intracellular loop of a receptor appears not to be involved in the process of the M_3-muscarinic receptor internalization or regulation of the ERK-1/2 pathway. However, it has been reported that the inhibition of receptor phosphorylation by CK2 increased the magnitude of the Jun-kinase response (Torrecilla *et al.*, 2007).

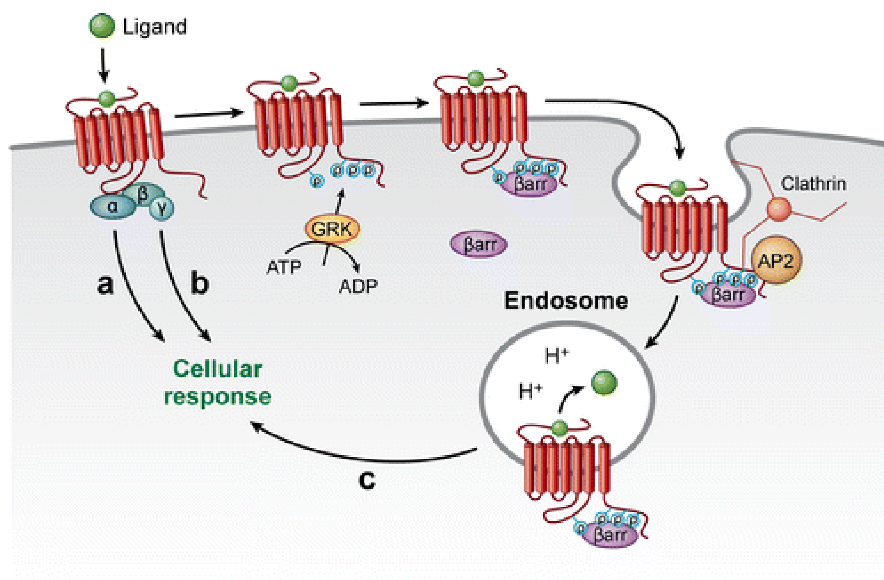
Some studies showed that the tyrosine residues in the β_2-adrenergic receptor can be phosphorylated. Insulin-like growth factor-1 stimulates phosphorylation of tyrosine residues Tyr^{132} and Tyr^{141} in the second intracellular loop whereas the activation of the insulin receptor results in phosphorylation to tyrosine residues Tyr^{350}, Tyr^{354} and Tyr^{364} in the C-terminal region (Karoor and Malbon, 1996). The tyrosine residue Tyr^{350} in the SH2-binding site was shown to be phosphorylated by c-Src tyrosine kinase (Fan *et al.*, 2001).

3.1.6. Desensitization and internalization of GPCRs

Desensitization is a process whereby prolonged agonist stimulation leads to reduction or elimination of biological response to the stimulus. In this way, the cells are protected against the excessive stimulation and possible damage. Two types of this mechanism can be distinguished – the

homologous (specific) and the heterologous (non-specific) desensitization. The homologous desensitization prevents overstimulation of cells by prolonged or repeated exposure to the same ligand whereas the heterologous one represents a decreased responsiveness to a variety of other ligands stimulating different receptors which use the same signalling pathways. During this process, the active receptor is phosphorylated by different protein kinases. GRKs and second messenger-regulated protein kinases (protein kinase A and protein kinase C) phosphorylate receptors during the homologous and heterologous desensitization, respectively.

After activation of a receptor by ligand binding, GRKs phosphorylate the serine and threonine residues in the third intracellular loop and the C-terminus of the active receptor, which mediates the arrestin recruitment to the cytoplasmic part of the phosphoreceptor. The selectivity of the particular isoform of arrestin for the active phosphoreceptor is given due to the activation and phosphorylation sensors in arrestin which are responsible for arrestin transition into its active receptor-binding conformation. Therefore, arrestin binding precludes the activation of Gα protein which suggests that a competition between G-protein and arrestin is the basis of the receptor desensitization (Gurevich and Gurevich b, 2006).



Hanyaloglu AC, von Zastrow M. 2008.
Annu. Rev. Pharmacol. Toxicol. 48:537–68

Fig. 3.2

Desensitization of GPCRs mediated by GRK and β-arrestin (Hanyaloglu 2008).

Arrestin has also properties of scaffolding proteins. It serves as an adaptor protein which binds the internalization machinery of the clathrin-coated pits via the interaction with clathrin and clathrin-associated protein AP-2 complex. Although this pathway of receptor endocytosis is predominant for

many receptors, β_2-adrenergic or adenosine A1 receptors were shown to be internalized through caveolae or other plasma membrane microdomains. Still another pathway of internalization which was observed in the case of M_2-muscarinic acetylcholine receptor in some cell lines appeared to be independent on clathrin and caveolae but dependent on dynamin and ADP-ribosylation factor 6 (ARF6) which may participate in clathrin-independent endocytic trafficking. Therefore, different mechanisms of receptor internalization may be described as arrestin- and clathrin-dependent, arrestin-dependent and clathrin-independent, arrestin-independent and clathrin-dependent or arrestin-independent and clathrin-independent (Marchese *et al.*, 2003; Prossnitz, 2004). In the clathrin- and dynamin-dependent pathway, the interaction of the β-subunit of adaptor protein AP-2 with arrestin promotes assembling of clathrin-coated pits and receptor recruitment to them. The clathrin-coated pits are invaginated and the subsequent recruitment of the GTPase dynamin mediates fission and a release of mature endocytic vesicles.

It is worth pointing out that some receptors such as thromboxane-$A_2\beta$ receptor and protease-activated receptor-1 exhibit the constitutive internalization which means that they are internalized without any stimulus. It seems that the mechanisms of constitutive and agonist-induced internalizations are different because the constitutive internalization of thromboxane-$A_2\beta$ receptor does not require GRK or arrestin binding whereas the agonist-induced one is GRK-, arrestin- and dynamin-dependent. The type of internalization can also determine different fate of the internalized molecules (Marchese *et al.*, 2003).

Two sorting fates of receptors in endosomes are known. The first is recycling pathway when receptors are dephosphorylated and restored to the plasma membrane (re-sensitization) and the second is the degradation pathway whereby receptors are transported into lysosomes and proteolyzed, which leads to the attenuation of signaling (down-regulation). Thus, receptors can be divided into two groups according to their sorting fate. For example, β_2-adrenergic receptors are efficiently recycled but δ-opioid receptors are rapidly degraded. The sorting signals which determine the receptor fate are located in the C-terminal region of a receptor. Some of them, which are short, linear peptide sequences including tyrosine-based motifs (NPXY and GDXY motifs, YXXphi-type motif), dileucine-basef motifs, PDZ ligands and NSF interaction motif, bind the sorting proteins such as ezrin-radixin-moesin(ERM)-binding phosphoprotein 50 (EBP50; also known as Na^+/H^+ exchanger regulatory factor NHERF1) containing a modular PDZ domain or N-ethylmaleimide-sensitive factor (NSF) containing NSF interaction motif. These sorting signals mediate recycling of receptors to the plasma membrane. Other interaction motifs can bind sorting proteins and this interaction may lead to the degradation pathway. The binding of GPCR-associated sorting protein (GASP) to β_2-adrenergic or δ-opioid receptor and binding of sorting nexin-1 to protease-activated receptor 1 result in targeting these receptors to lysosomes (Marchese *et al.*, 2008; Pandey, 2009).

Another important signal for receptor sorting is ubiquitination. The length of the ubiquitin chain and configuration of ubiquitin-ubiquitin linkages are the most significant factors which determine the fate of ubiquitinated proteins. Degradation of a protein by the 26S proteasome is determined by a chain of four or more ubiquitins in which the C-terminus of one ubiquitin is attached to Lys^{48} of the adjacent ubiquitin. On the contrary, mono-ubiquitination or attachment of ubiquitin to Lys^{63} of an adjacent ubiquitin may lead to different cellular processes such as endocytic sorting, but not to proteasomal degradation. Proteins are ubiquitinated through the activity of enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase). On the contrary, deubiquitinating enzymes are proteases which cleave the isopeptide bonds in the chain of ubiquitins. In yeast, ubiquitination is necessary for constitutive as well as agonist-induced receptor internalization but its role in the regulation of mammalian GPCRs is more variable. Protease-activated 1 receptor appears to be ubiquitinated under basal conditions and deubiquitinated after activation. On the other hand, the β_2-adrenergic receptor undergoes the agonist-dependent ubiquitination and degradation, which occur after receptor phosphorylation and arrestin binding. In addition to the β_2-adrenergic receptor, ubiquitination also leads to the degradation of chemokine receptor CXCR4, sst3 somatostatin receptor or vasopressin V_2 receptor. However, some GPCRs may be sorted through the endosomal-lysosomal system, which is independent on ubiquitination, as in the case of δ-opioid receptor (Marchese *et al.*, 2008). It is worth mentioning that polyubiquitination of receptors can be a signal for sorting of misfolded receptors to the endoplasmic reticulum-associated protein degradation pathway during biosynthesis, which was observed for TRH receptor, rhodopsin or δ-opioid receptor (Marchese *et al.*, 2008).

3.1.7. Thyrotrophin-releasing hormone receptor

The thyrotropin-releasing hormone (TRH) receptor (TRH-R) belongs to family A of GPCRs. This receptor has been found in many different organs, such as brain, pancreas, testis, uterus, thymus or adrenal gland. The gene encoding TRH receptor is localized to chromosome 8q23 by in situ hybridization (Morrison *et al.*, 1994).

This receptor is activated by thyrotropin-releasing hormone (TRH). This is a hypothalamic tripeptide, pGlu-His-$ProNH_2$, whose endocrine role is to stimulate the secretion of thyrotropin (thyroid-stimulating hormone, TSH) and prolactin (Yu *et al.*, 1998). It acts as a hormone, a paracrine regulatory factor and a neurotransmitter/neuromodulator. It was shown that it may act in the central nervous system and the cardiovascular system and has neuroprotective and anti-depressant effects (Pfleger *et al.*, 2004).

The TRH receptor has two isoforms. TRH-R1 and TRH-R2 represent the long isoform with 412 amino acids in the human genome and the short isoform with 387 amino acids found in rat

genome, respectively. These isoforms are generated by alternative splicing. The long isoform has not yet been found in human genome. These two isoforms are generated by alternative splicing (de la Pena *et al.*, 1992). They have similar binding affinities for TRH and activate similar signalling pathways via $G_{q/11}\alpha$ protein and phospholipase Cβ (PLCβ). Some of the differences between these two isoforms are that TRH-R2 exhibits higher basal signalling activity and more rapid internalization than TRH-R1. They bind TRH as well as TRH analog MeTRH with equal affinity probably due to the similar binding sites in both isoforms (O'Dowd *et al.*, 2000).

The TRH-R isoforms share only about 20% of sequence identity with bovine rhodopsin and are approximately 50% homologous to each other. The most conserved region is the C-terminal amphipathic helix 8 which is 90% homologous. The key patterns of TM domains of family A of GPCRs are conserved in TRH receptor. Most of loops are short and E2 loop is involved in recognition of TRH. The I3 loop which is important for binding of Gα protein or arrestin as well as internalization is longer compared to rhodopsin. The geometry of the disulfide bridge between the E2 loop and TM3 domain involving Cys residue is very similar to that in rhodopsin (Deflorian *et al.*, 2008). Amino acid residues Asn^{43}, Asp^{71} and Asn^{316} of TRH receptor, which are localized in TM helices I, II and VII and highly conserved in family A of GPCRs, appear to be important for G-protein coupling and were shown to interact with each other (Pfleger *et al.*, 2004). TRH-R contains the NPXXY motif at the end of TMVII and probably the amphipathic helix 8 with cysteine residues which can be palmitoylated (Du *et al.*, 2005).

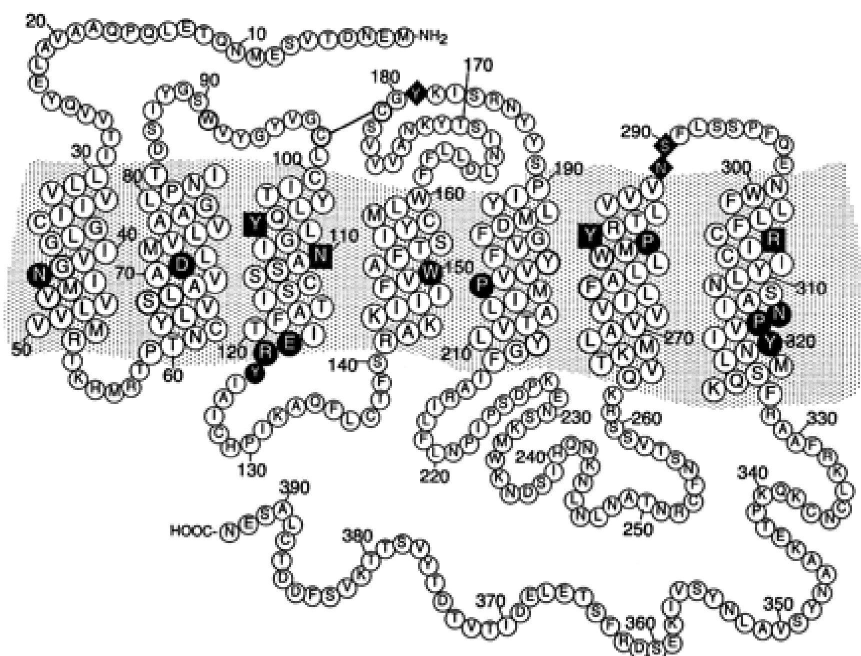


Fig. 3.3

A two-dimensional model of the TRH receptor with the extracellular space at the top and the intracellular space at the bottom. The residues highlighted in circles and squares are sites of high

conservation and sites representing the transmembrane binding pocket, respectively. Those highlighted in diamonds are the residues participating in the attachment of TRH to the receptor surface (Gershengorn and Osman, 2001).

The TRH receptor is able to form dimers and oligomers. These isoforms were observed as homodimers or TRH-R1/TRH-R2 heterodimers by BRET or co-immunoprecipitation. By immunoblotting using epitope-tagged TRH-R1, higher order oligomers were identified. Differential immunoprecipitation of FLAG-tagged and HA-tagged TRH-R1 revealed that when both epitope tagged receptors were co-expressed together, each of them was found in immunoprecipitates captured using the antibody against the other epitope. It seems that neither extracellular Cys residues nor the C-terminal region, which can play important role in oligomerization of some GPCRs, are involved in oligomerization of TRH-R1. Although both these isoforms form constitutive dimers or oligomers, TRH treatment results in additional increase in the formation of dimers or oligomers in a time- as well as dose-dependent manner. The TRH-R1/TRH-R2 heterooligomers exhibit similar pharmacological properties and signalling but different internalization properties and altered β-arrestin interactions compared to TRH-R1 and TRH-R2 when expressed alone (Angers *et al.*, 2002; Pfleger *et al.*, 2004).

The TRH receptor can be probably phosphorylated by different protein kinases. Although it is likely not to be phosphorylated by second-messenger protein kinases, it was shown that GRK2 and CK2 phosphorylate the serine and threonine residues in the C-terminal region of TRH-R as well as in regions outside the C-terminus (Gehret and Hinkle, 2010; Gehret *et al.*, 2010; Hanyaloglu *et al.*, 2001). It was shown that this receptor is rapidly desensitized within several minutes after agonist-induced phosphorylation by GRK2 and arrestin binding. It subsequently undergoes internalization in which the residues upstream of position 370 play an important role (Pfleger *et al.*, 2004). Moreover, it was shown that mutations in the TM II domain or I3 loop lead also to defects in internalization. The TRH-R/ligand complexes are internalized with the bound arrestin via clathrin-coated pits and targeted to lysosomes or recycled to the plasma membrane. The ligands can dissociate from receptors and be degraded in lysosomes or remain coupled with receptors and thus be recycled (Sun *et al.*, 2003).

3.2. G-proteins

G-proteins (guanine nucleotide-binding proteins) are a group of regulatory proteins which are involved in signal transduction. According to their structure, they are divided into small G-proteins, which exist as monomers, and heterotrimeric G-proteins, which are composed of three different subunits. They all exhibit GTP-binding features and GTPase activity and can be found in different organisms from bacteria to humans.

In 1971, Martin Rodbell proposed the idea that the signal is transferred from glucagon receptor to adenylyl cyclase via a guanine nucleotide regulatory protein (Rodbell *et al.*, 1971). The existence of this protein was verified by Alfred G. Gilman who successfully purified a G-protein later named $G_s\alpha$ (Gilman, 1987). It turned out that it was actually the subunit of a protein which consists of three subunits (α, β and γ) and that it is activated by GTP binding. The $G_s\alpha$ protein was named due to its stimulating effect on adenylyl cyclase.

Small G-proteins are similar to functional GTPase domain (G domain) of the α subunit of the heterotrimeric G-proteins. They both bind GDP under control conditions and GTP after their activation. The bound GTP is hydrolyzed to GDP due to their endogenous GTPase activity and in this way G-proteins are deactivated. Nevertheless, the GTPase activity is significantly lower in small G-proteins than in the α subunit of the heterotrimeric G-proteins and thus GTPase-activating protein (GAP) which accelerates GTPase activity is needed for deactivation of small G-proteins. Next regulatory proteins, which regulate the activity of small G-proteins, are GTPase dissociation inhibitors (GDI) keeping small G-proteins in an inactive state and guanine nucleotide exchange factors (GEF) accelerating the exchange of GTP for GDP.

Both types of G-proteins also differ in cell localization and function. Heterotrimeric G-proteins are localized near the plasma membrane due to their lipid attachments and are involved mainly in signal transduction while small G-proteins are in the cytosol or intracellular compartments and have many different functions in regulation of cell growth, protein secretion or endocytic processes. The family of small G-proteins involves Ras, Rab, Rap, Ran, Rho, ARF, Rad and Rheb proteins.

3.2.1. Heterotrimeric G-proteins

Heterotrimeric G-proteins represent an important segment at the beginning of signalling pathways. They activate appropriate effectors on the intracellular side of the plasma membrane and thus trigger more complicated intracellular regulatory processes. They consist of three functionally different polypeptides – α, β and γ subunits. The α and γ subunits contain lipid modifications which

attach them to the plasma membrane. The β and γ subunits form a heterodimer which can dissociate only under strong denaturing condition. All these subunits are in tight interactions under basal conditions. After activation of G-protein, they probably dissociate into α subunit and βγ heterodimer. However, some studies suggest that all subunits remain together and only their conformations are altered (Evanko *et al.*, 2005; Rebois *et al.*, 1997).

To this day, 23 Gα subunits coded by 16 genes, 7 Gβ subunits coded by 5 genes and 13 Gγ subunits coded by 14 genes have been identified (McIntire, 2009; Milligan and Kostenis, 2006). Because some subunits are expressed only in specific tissues and some Gβγ heterodimers are not functional, not all theoretical combinations of identified subunits can be found (Peng *et al.*, 1992; Yan *et al.*, 1996).

3.2.2. Classes of G-proteins

G-proteins are classified according to structural and functional features of the Gα subunits. Based on their amino acids sequences, they can be divided into four groups represented by $G_s\alpha$, $G_i\alpha$, $G_q\alpha$ and $G_{12}\alpha$ proteins (Simon *et al.*, 1991).

G_s class

The G_s class involves $G_s\alpha$ and $G_{olf}\alpha$ proteins, which are 88% homologous (Morris and Malbon, 1999). $G_s\alpha$ protein is encoded by the gene GNAS. Four different splice variants, two long isoforms $G_s\alpha1$ and $G_s\alpha2$ with molecular size of 52 kDa and two shorts isoforms $G_s\alpha3$ and $G_s\alpha4$ with molecular size of 45 kDa, can be expressed (Bray *et al.*, 1986). Due to the presence of promoter upstream of the $G_s\alpha$ promoter, next two transcripts $XL\alpha_s$ and Nesp55 are generated. The $XL\alpha_s$ has long N-terminal extension compared to $G_s\alpha$ protein but the rest of the molecule is structurally the same. It exhibits the same functions as well (Wettschureck and Offermanns, 2005).

$G_s\alpha$ protein is ubiquitously expressed while $G_{olf}\alpha$ protein is specific only for the olfactory epithelium. These proteins transfer signals from such GPCRs as β-adrenergic receptors, glucagon receptor, vasopressin receptor and some isoforms of serotonin, histamine and dopamine receptors. This class is named with letter “s” according to ability of all members to stimulate a particular effector adenylyl cyclase, which results in an increase of cAMP concentration. It is important to note that members of this class are substrates for ADP-ribosylation catalyzed by cholera toxin secreted by *Vibrio cholerae*. This modification inhibits the intristic GTPase activity and thus $G_{s/olf}\alpha$ protein remains permanently activated (Morris and Malbon, 1999).

G_i class

This class contains three isoforms of $G_i\alpha$ proteins expressed ubiquitously ($G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$ encoded by three different genes *GNAI1-3*), two isoforms of brain specific $G_o\alpha$ proteins ($G_{o1}\alpha$ and $G_{o2}\alpha$ encoded only by one gene *GNAO*), two isoforms of $G_t\alpha$ protein (transducin) expressed in retinal rods and cones ($G_{t\text{-}r}\alpha$ and $G_{t\text{-}c}\alpha$ encoded by two genes *GNAT1* and *GNAT2*), $G_{gust}\alpha$ protein (gustducin) expressed in taste cells (encoded by gene *GNAT3*) and $G_z\alpha$ protein expressed in neuronal tissue and platelets (encoded by gene *GNAZ*) (Wettschureck and Offermanns, 2005).

These proteins transfer signals from $GABA_B$ receptors, α_2-adrenergic receptors, chemokine receptors, some isoforms of muscarinic and dopamine receptors, *etc*. This class is named according to $G_i\alpha$ proteins because these proteins inhibit the activity of adenylyl cyclase and decrease cAMP concentration. The inhibiton of adenylyl cyclase can also be mediated by $G_o\alpha$ proteins and $G_z\alpha$ protein. $G_o\alpha$ proteins are able to affect the activity of some calcium channels. The transducins, which transfer signal from rhodopsins, activate retinal cGMP phosphodiesterase, while gustducin activates cGMP as well as cAMP phosphodiesterases (Wettschureck and Offermanns, 2005).

All members of this class except for $G_z\alpha$ protein can be modified by ADP-ribosylation at a conserved C-terminal cysteine residue. This modification is caused by pertussis toxin secreted by *Bordetella pertussis* and uncouples the G-protein from its activating receptor, which results in inability of the receptor to transfer signals to its signalling pathway (Morris and Malbon, 1999).

G_q class

This class is represented by ubiquitously expressed $G_q\alpha$ (encoded by the gene *GNAQ*) and $G_{11}\alpha$ (encoded by the gene *GNA11*), $G_{14}\alpha$ expressed in kidney, lung and spleen (encoded by the gene *GNA14*) and by two very close members $G_{15}\alpha$ and $G_{16}\alpha$ expressed in hematopoietic cells (encoded by the gene *GNA16*) (Wettschureck and Offermanns, 2005).

These proteins couple to bradykinin receptor, gastrin and ghrelin receptors, gonadotropin-releasing hormone receptor, thyrotropin-releasing hormone receptor, α_1-adrenergic receptors *etc*. All receptors which interact with $G_{q/11}$ proteins do not appear to discriminate between two these isoforms (Wettschureck and Offermanns, 2005). All members of this class are insensitive to cholera and pertussis toxins. They regulate the activity of phosphinositide-specific phospholipases Cβ1-4 (Smrcka *et al.*, 1991).

G_{12} class

This class contain two ubiquitously expressed members $G_{12}\alpha$ and $G_{13}\alpha$ encoded by the gene *GNA12* and *GNA13*, respectively (Wettschureck and Offermanns, 2005). They are often activated by the receptors which also couple to $G_{q/11}$ proteins, e.g. tromboxane A_2 receptor, lysophosphatidic acid receptor and thyrotropin receptor, and regulate the activity of different effectors such as RhoGEF,

phospholipase A2 (PLA2), *c-jun* NH_2-terminal kinase (JNK), Na^+/H^+ exchanger or ERK kinases (Wettschureck and Offermanns, 2005).

3.2.3. Structure of the subunits of heterotrimeric G-proteins

The α subunit of a heterotrimeric G-protein is composed of a GTPase domain and a helical domain, which are connected by two linkers L1 and L2. The GTPase domain, which is also conserved in small G-proteins and elongation factors, is responsible for hydrolysis of GTP and provides the binding surfaces for Gβγ heterodimers, GPCRs and effectors. This domain consists of six β-strands surrounded by five α-helices and has three flexible loops, named switches I, II and III which significantly alter their conformation during GTP exchange. The guanine nucleotide binding site called P loop connects the β1 sheet with the α1 helix. The second nucleotide binding site is the TCAT motif in β6/α5 loop. The GTP hydrolysis is triggered by the loss of two hydrogen bonds between the γ-phosphate and conserved threonine residue in switch I or glycine residue in switch II. Upon the conformational change of switch II, switch III changes its conformation as well (Hilgenfeld, 1995; Oldham and Hamm, 2008). The helical domain consists of six α-helix bundles in which one central α-helix is surrounded by five short α-helices. It aids to GTPase domain to form nucleotide-binding pocket for nucleotide binding (Oldham and Hamm, 2008).

The β subunit is composed of a seven-bladed β-propeller with seven WD-40 sequence repeats, which are characterized by a repeating motif of 27-45 amino acids with Trp-Asp (WD) dipeptide sequence at the C-terminal region. Its N-terminus with α-helical structure forms coiled-coil structure with the N-terminus of the γ subunit and blades five and six interact with α-helical C-terminus of the γ subunit (Oldham and Hamm, 2008). Four isoforms of β subunit (β_1-β_4) are highly homologous (about 80-90%) while β_5 is only 50% homologous to β_1-β_4. It was found that β_3 subunit exists in several truncated splice variants β_3s, β_3s2 and β_3v and β_5 has an N-terminal extension β_{5L}. Contrary to the β subunits, the γ subunits exhibit lower sequence identity (between 25% and 75%) (McIntire, 2009).

Two crystal structures of heterotrimeric G-proteins, $G\alpha_{t/i}\beta_1\gamma_1$ and $G\alpha_{i1}\beta_1\gamma_2$, were solved and revealed that the surface on Gα·GDP interacting with Gβγ heterodimers is a hydrophobic pocket formed by switches I and II and partly by the N-terminal helix (Oldham and Hamm, 2008).

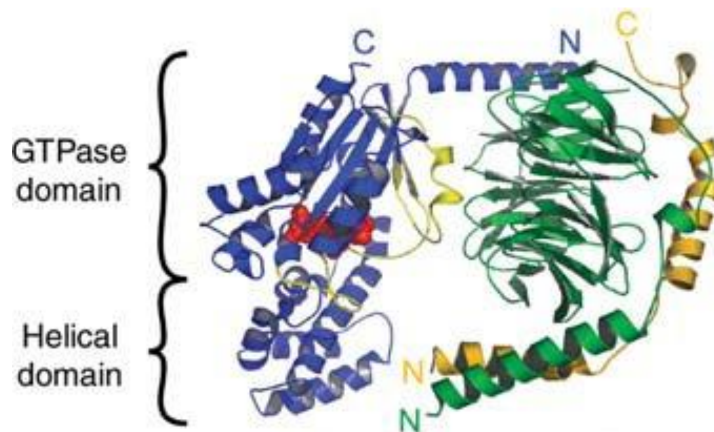


Fig. 3.4

Ribbon model of $G_{t/i}\alpha(GDP)\beta_1\gamma_1$ heterotrimer, where Gα subunit is blue, Gβ subunit is green and Gγ subunit is gold. The three switch regions in Gα are highlighted in yellow. GDP (red) is buried between the GTPase and helical domains of Gα (Oldham and Hamm, 2006).

3.2.4. Mechanism of activation and deactivation

Activation and deactivation of heterotrimeric G-proteins is based on the cyclic process in which GTP exchange for GDP leads to activation of α subunit followed by subsequent hydrolyzis of GTP of to GDP and phosphate due to GTPase activity of the α subunit.

In the absence of stimulation, α subunit binds GDP and is associated with the Gβγ dimer. The constitutively active receptors or receptors activated by agonist binding bind the β6/α5 loop with nucleotide binding TCAT motif of the α subunit and this interaction induces conformational changes of switch I and switch II (β3/α2 loop) and a release of GDP. The conformation of active Gα protein exhibits high affinity to GTP, which is in higher cellular concentrations than GDP. After GTP binding, all three switch regions undergo conformational changes which result in lower affinity of Gα protein for Gβγ dimers and their subsequent dissociation. Thereafter, separated Gα protein and Gβγ dimers can regulate different effectors and trigger various signalling pathways. Because Gα protein has intrinsic GTPase activity, the terminal phosphate of GTP is hydrolyzed to restore GDP in a nucleotide-binding pocket. The inactivated Gα protein with bound GDP re-associates with Gβγ dimers and the heterotrimeric G-protein is thus re-arranged for new activation by receptor (McIntire, 2009; Milligan and Kostenis, 2006; Oldham and Hamm, 2008).

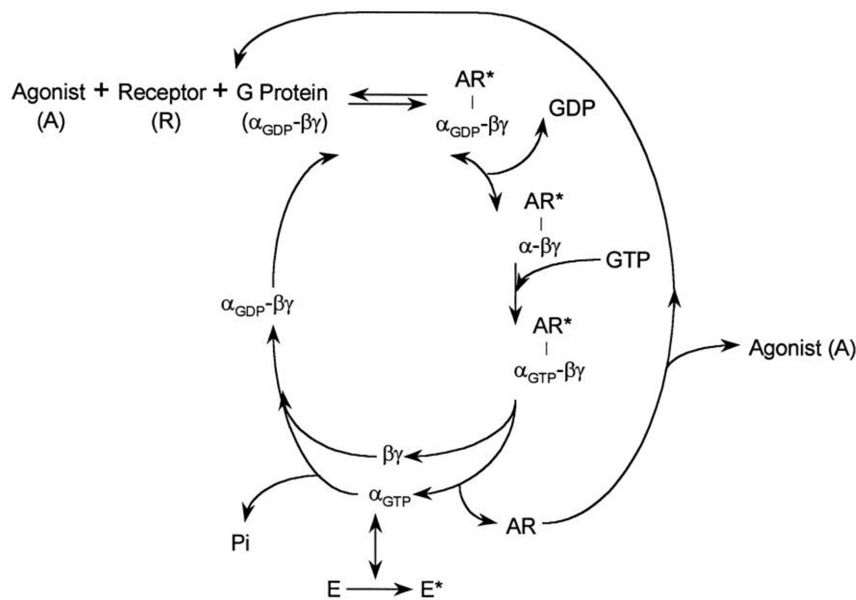


Fig. 3.5

The cycle of G-protein activation/deactivation after GPCR activation (Quock 1999).

This mechanism is considered as a classical model of the G-protein activation/deactivation cycle. Nevertheless, some elements in this model can be replaced by other possible ideas. The classical model of receptor coupling with G-proteins supposes that signalling molecules are freely diffusible in the plasma membrane and their interactions are results of random collisions. In the past several years, this model of the coupling of some receptors and particular G-proteins after receptor activation has been challenged with the idea in which both these signalling proteins are in a pre-coupled or pre-associated protein complex and the receptor activation causes the decomposition of this complex. This pre-coupling would provide the more rapid signal transfer across the plasma membrane.

The pre-associated complexes of receptors and G-proteins were investigated mainly by fluorescence imaging methods as FRET (fluorescence resonance energy transfer) and BRET (bioluminescence resonance energy transfer) and less by co-immunoprecipitation methods. These interactions under basal conditions were identified for α_1-, α_2- and β_2-adrenergic, muscarinic acetylcholine M4, dopamine D2, adenosine A1, prostacyclin, bradykinin 2 and chemokine receptors (Gales *et al.*, 2006; Lei *et al.*, 2009; Levoye *et al.*, 2009; Nobles *et al.*, 2005; Philip *et al.*, 2007). Nevertheless, other studies using FRET and BRET did not confirm the existence of pre-associated complexes at least in case of α_2-adrenergic receptor (Hein *et al.*, 2005; Qin *et al.*, 2008). Hence, the issue of pre-coupling complexes has not been resolved completely and it seems that different receptors can behave in different ways.

The dissociation of Gα protein and Gβγ dimer can be represented by a classical model involving physical separation of these proteins or by a new model involving conformational re-arrangements of G-protein subunits. Both models can be applied according to the combination of

observed interacting isoforms of subunits. Using FRAP (fluorescence recovery after photobleaching), $G_o\alpha$ and $G_{i3}\alpha$ proteins were shown to dissociate from $G\beta_1\gamma_2$ dimer after receptor activation while $G_s\alpha$ protein did not dissociate from the same Gβγ dimer and receptor activation resulted only in subunit re-arrangement (Digby *et al.*, 2006). It appears that G-protein behaviour after receptor activation is determined by the character of α subunits.

3.2.5. Posttranslational modifications

All subunits of heterotrimeric G-proteins can be posttranslationally modified although only the α and γ subunits contain lipid modifications which form lipid anchors to the plasma membrane. The β subunit is attached to the plasma membrane by firm interaction with the γ subunit and thus no lipid modification of this subunit is needed. Lipid modifications of the α and γ subunits play an important role not only in membrane localization of G-proteins but also in the regulation of interactions with other signalling proteins. The α and γ subunits can be palmitoylated, N-myristoylated or isoprenylated. Isoprenylation includes farnelysation or geranylgeranylation. Contrary to the palmitoylation, when palmitate is incorporated reversibly into the molecule, N-myristoylation and isoprenylation are irreversible processes. Isoprenoids farnesyl and geranylgeranyl are attached to the proteins by thioester bonds while myristate is attached to the N-terminal glycine residue by amide bond after removal of the initiator methionine of polypeptides within the sequence Met-Gly-Xaa-Xaa-Xaa-Ser (Milligan and Kostenis, 2006).

Gα subunits

The Gα subunit can undergo palmitoylation and/or N-myristoylation. All α subunits except for $G_t\alpha$ are posttranslationally modified with palmitate at the N-terminus while only members of $G_i\alpha$ are myristoylated (Oldham and Hamm, 2008). It is worth pointing out that Gα can be palmitoylated at several positions. For example, $G_q\alpha$ and $G_{11}\alpha$ have two cysteine residues at positions 9 and 10 that are palmitoylated and thus form two lipid anchors for these subunits while $G_s\alpha$ and $G_{12}\alpha$ are palmitoylated only at one site (Milligan and Kostenis, 2006).

It seems that N-myristoylation is the prerequisite for palmitoylation of $G_i\alpha$ and helps anchorage of $G_i\alpha$ proteins to the plasma membrane. When overexpressed, Gβγ heterodimers can also serve as signal for palmitoylation and also for targeting of Gα to a particular cellular compartment. Moreover, the mutation in $G_o\alpha$ which led to the increase of its affinity to Gβγ heterodimers enhanced palmitoylation simultaneously (Chen and Manning, 2001). Further evidence that Gβγ heterodimers are required for membrane attachment and palmitoylation was provided using point mutations which prevented binding of $G_s\alpha$ and $G_q\alpha$ to Gβγ heterodimers. As a result, $G_s\alpha$ and $G_q\alpha$ could not be palmitoylated and were localized to the cytosol. Another $G_q\alpha$ mutant, in which the N-myristoyl and

palmitoyl moieties could be attached, was localized back to the plasma membrane but it did not result in restoration of Gβγ heterodimer binding (Evanko *et al.*, 2000). Hence, it is possible to suppose that both N-myristoylation and binding of Gβγ heterodimers to Gα can prevent its depalmitoylation.

Next example how palmitoylation and N-myristoylation can regulate the localization of Gα was shown for $G_z\alpha$. When this subunit was mutated to prevent palmitoylation, it was found in the plasma membrane as well as in intracellular membranes. The mutation which stopped N-myristoylation led to prevention of palmitoylation and to the localization of $G_z\alpha$ in the cytosol and nucleus (Morales *et al.*, 1998).

As mentioned above, lipid modifications can significantly affect interactions between Gα protein and Gβγ heterodimer. This is supported by other studies with different isoforms of Gα protein. N-myristoylation strongly increased the affinity of $G_o\alpha$ protein to Gβγ heterodimer (Linder *et al.*, 1991). On the other hand, palmitoylation promoted the binding of $G_s\alpha$ protein to Gβγ heterodimer (Iiri *et al.*, 1996).

Because palmitoylation is reversible process, palmitoylation/depalmitoylation cycle can highly affect the fate of Gα protein as well as signalling. It is known that palmitoylation is modulated by activation of Gα protein. It was found that only a particular Gα conformation can enable access of depalmitoylation enzymes to this subunit (Chen and Manning, 2001). Wedegaertner and co-workers proposed a model in which depalmitoylation of $G_s\alpha$ protein upon activation leads to its translocation from the plasma membrane to the cytosol and thus limits the proximity to membrane-bound effectors. This translocation would cause the dampening of signal (Wedegaertner *et al.*, 1996).

An important posttranslational modification is phosphorylation. Gα proteins can be phosphorylated at their serine or tyrosine residues. Serine residues are phosphorylated by PKC or p21-activated protein kinase PAK and these phosphorylations block binding of Gβγ heterodimers and *vice versa*. Sites for phosphorylations by PKC were shown to be in N-terminal 53 amino acids. For example, preferred site of phosphorylation in $G_z\alpha$ protein is Ser^{27} and the second is Ser^{16}. $G_{12}\alpha$ protein is phosphorylated at site Ser^{38}. The kinase PAK was observed to phosphorylate $G_z\alpha$ protein at site Ser^{16} but not other tested subunits ($G_s\alpha$, $G_q\alpha$, $G_o\alpha$ and $G_i\alpha$) (Chen and Manning, 2001). $G_s\alpha$, $G_i\alpha$ and $G_{q/11}\alpha$ proteins can be phosphorylated at the C-terminal tyrosine residues (Tyr^{37} and Tyr^{377} in $G_s\alpha$ protein or Tyr^{356} in $G_{q/11}\alpha$ protein) by non-receptor tyrosine kinases such as insulin receptor kinase or proto-oncogene c-Src (pp60c-src) (Chen and Manning, 2001).

Gβ subunits

Although Gβ subunits are not modified with lipid anchors and their modifications are not so variable, they contain some uncommon modifications in comparison with other two subunits. Apart from N-acetylation also observed in Gγ subunit, they can be phosphorylated at a histidine residue or ADP-ribosylated. The N-terminus of $G\beta_1$ subunit has been reported to be N-acetylated at Ser^2 after a

removal of the first methionine residue Met^1 (Matsuda *et al.*, 1994). Nevertheless, the significance of N-acetylation is unclear.

The histidine residue His^{266} of $G\beta_1$ subunit was shown to be phosphorylated by nucleoside diphosphate kinase (NDPK). The His^{266}-phosphate can be transferred onto GDP leading to GTP formation and thus activation of Gα protein. Hence, this reaction may represent receptor-independent activation of G-proteins explaining under basal conditions (Maurer *et al.*, 2005).

Activated Gβ subunits can also undergo mono-ADP-ribosylation at Arg^{129} located in the effector-binding domain. ADP-ribosylation regulates the activity of Gβ subunits and this subunit becomes unable to activate its effectors such as phosphoinositide 3-kinase γ (PI 3-kinase) or phospholipase Cβ2 (PLCβ2) (Lupi *et al.*, 2002).

Gγ subunits

Gγ subunits can be modified at both N- and C-termini. Serine residue in the C-terminus is prenylated by farnesyl or geranylgeranyl moieties depending on the C-terminal region. If X residue in the C-terminal region CAAX is serine, glutamine or methionine as in case of γ_1, γ_8 and γ_{11} subunits, farnesyl is attached to the serine residue. The other γ subunits whose X residue in C-terminal CAAX is leucine are modified by the geranylgeranyl group. After prenylation, the C-terminal AAX regions of some γ subunit are cleaved by an endoprotease and C-terminal serine residues are carboxymethylated by a methyltransferase. This processing of γ subunits is considered to be important for maturation and subsequent assembly of Gβγ heterodimers (McIntire, 2009). However, the last three amino acids in the C-terminal CSFL region of the geranylgeranylated γ_5 subunit was found not to be cleaved (Kilpatrick and Hildebrandt, 2007). It seems that the unprocessed CAAX region can play an important role in the interaction with PDZ domain-containing proteins as observed in case of the γ_{13} subunit (Li *et al.*, 2006).

As mentioned above, one of the significant features of prenylation is to target proteins to the plasma membrane. The lack of γ_2 prenylation caused translocation of Gβγ heterodimer into nucleus and its increased regulation of transcription (Kino *et al.*, 2005).

The other observed modifications of Gγ subunit are serine phosphorylation of $G\gamma_{12}$ protein by PKC and N-acetylation of Met^2 in the N-terminus of $G\gamma_2$ subunits as in case of Gβ subunit (McIntire, 2009).

3.2.6. Desensitization of G-proteins

Prolonged treatment with agonist induces desensitization on the level of G-proteins which includes the decrease in the amount of G-proteins in the plasma membrane due to their translocation

into light vesicles (endosomes) or cytosol (solubilisation) and the decrease in the total amount of G-proteins in cells (down-regulation).

The redistribution of $G_s\alpha$ after agonist stimulation of the β_2-adrenergic receptor has been frequently observed although other some studies were focused on translocation of $G_q\alpha$ protein or $G_t\alpha$ protein and $G\beta_1\gamma_1$ (Marrari *et al.*, 2007).

One of the first studies investigated translocation of $G_s\alpha$ and $G_i\alpha$ proteins in rat adipocytes. Isoprotenerol treatment resulted in the translocation of both Gα proteins from the plasma membrane to a low-density microsomal fraction rich in endosomes and Golgi bodies (Haraguchi and Rodbell, 1990). The next studies based on fractionation, immunofluorescence microscopy and live cell imaging found out that the release of $G_s\alpha$ protein induced by stimulation of β_2-adrenergic receptors is a rapid process, observable within 1-5 min after agonist addition, and $G_s\alpha$ protein is localized throughout the cytoplasm in a soluble form. Nevertheless, it seems that at least a fraction of internalized $G_s\alpha$ protein is bound to vesicles (Marrari *et al.*, 2007).

$G_{q/11}\alpha$ proteins were shown to internalize after activation of the angiotensin II receptor or thyrotropin-releasing hormone receptor-1 (TRH-R1). It seems that the time course of $G_{q/11}\alpha$ internalization can differ dependently on which receptor is activated. Activation of the angiotensin II receptor resulted in $G_{q/11}\alpha$ internalization within 5-20 minutes while this process was much slower (2 hours) after activation of TRH-R1 (Marrari *et al.*, 2007).

The redistribution after receptor activation is noticeable not only for Gα proteins but for Gβγ dimers as well. $G\beta_1\gamma_7$ dimers were translocated with $G_s\alpha$ proteins from the plasma membrane into vesicles after activation of β_2-adrenergic receptors within minutes, $G\beta_1\gamma_{11}$ dimers into Golgi apparatus after activation of M2 muscarinic acetylcholine receptors within 20 seconds but $G\beta_1\gamma_5$ dimers showed less and slower internalization compared to $G\beta_1\gamma_{11}$ dimers (Akgoz *et al.*, 2004; Akgoz *et al.*, 2006; Hynes *et al.*, 2004).

It seems that the translocation of G-proteins is related to the presence of lipid modifications and is independent on GPCR endocytic pathways (Marrari *et al.*, 2007). Next, it was found out that $G_s\alpha$ and $G_q\alpha$ proteins undergo an active recycling process and recover localization to the plasma membrane after agonist removal. This recycling process is probably connected with interaction of Gα protein with Gβγ dimers (Cronin *et al.*, 2004; Kosloff *et al.*, 2003).

Altogether, G-protein internalization induced by receptor activation can contribute to the desensitization process and protection of cells against damage. It can also lead to triggering of other signalling pathways and affect cellular processes.

3.3. Signalling pathways and interactomes of GPCRs and G-proteins

The transfer of signal via GPCRs and G-proteins across the plasma membrane is the starting point of various signalling pathways which include diverse signalling, regulating and scaffolding proteins that provide the specificity of signalling pathways and thus determine the cell fate. After G-protein activation, the signal is transferred to different effectors which subsequently convey information to other members of signalling pathways formed by interacting protein chains. Such pathways can comprise many members whose engagement into signal transduction determines the final response of the cell.

Members of signalling pathways can be relay proteins which simply transfer the message to next component downstream in signalling pathway; messenger proteins which pass the signal from one part of cell to another; amplifier proteins which amplify the signal by activating many downstream signalling proteins or producing large number of second messenger; transducer proteins which convert the signal from one form to another; second messengers (mediators) which are small molecules relaying the signal; modulator proteins regulating the activity of other proteins; adaptor proteins linking the components of signalling pathway; anchor proteins arranging framework for enzymes and their substrates; scaffold proteins binding several components of signalling pathway into a complex; bifurcation proteins dividing the signal from one into two or more signalling pathways; and integration proteins which integrate the signals from two or more signalling pathways into one.

Components of signalling pathways convey the signal by interaction with each other, which can end in conformational change, enzymatic reaction and activation of protein or its modification, such as phosphorylation. The protein-protein interactions occur due to specific protein domains (modules) of 60-100 amino acids. Many signalling protein can contain several these domains mediating interactions with a variety of interacting partners and thus connecting different signalling pathways into protein networks.

3.3.1. Protein domains

The well-investigated protein domains, which participate in the interactions of signalling proteins with other partners, are SH2 and PTB domains, SH3 domain, PH domain, PDZ domain, WW domain and Armadillo repeat. The next domains are LIM domain, FERM domain, HOOK domain, RGS domain, leucine repeat, *etc*.

The SH2 (Src homology 2) domain exhibits sequence homology with a sequence of the Src tyrosine kinase. It is folded into β-sheet sandwiched by two α-helices. This domain binds to proteins with phosphorylated tyrosine residue (pY) when pY is inserted into a pocket of SH2 domain. The

proteins with SH2 domains are e.g. p85 subunit of PI3-kinase, Grb2 protein, Src kinase or phospholipase Cγ (Ladbury and Arold, 2000).

The phosphotyrosine binding (PTB) domain which comprises 100-150 amino acids consists of a β sandwich with 2 perpendicular anti-parallel β sheets capped on one edge by the C-terminal α-helix. It binds a ΦXNPXpY motif (Φ is a hydrophobic residue, X is any amino acid, pY is phosphotyrosine) in such proteins as Shc, Talin, Dok1 or Radixin (DiNitto and Lambright, 2006).

The SH3 domain forms a partly open β-barrel consisting of two β-sheets. It interacts with proline-rich section with PXXP motif, which is a consensus motif for SH3 ligands. Proteins with SH3 domain are Abl kinase, Fyn kinase, p85 subunit of PI3-kinase or Grb2 protein.

The PH (pleckstrin homology) domain corresponds to the 100 amino acid region of sequence homology occurring twice in pleckstrin, which is the substrate for PKC. It has a seven-stranded β-sandwich structure with β1-β2 loop which contains the sequence motif KX_n(K/R)XR. This motif possesses an interacting site for phosphoinositide and thus these domains are effectors of the lipid second messengers PI-(3,4,5)P_3 and PI-(3,4)P_2. Proteins with this domain include GRK2, Bruton's tyrosine kinase (BTK), general receptor for phosphoinositides-1 (GRP1), protein kinase B (PKB/AKT) and phospholipase Cδ1 (Lemmon, 2008).

The PDZ domain is a small globular module whose name is derived from the first three identified PDZ domain-containing proteins – the postsynaptic density protein PSD-95/SAP90, the Drosophila septate junction protein Discs-large and the epithelial tight junction protein ZO-1. It consists of six β-strands and two α-helices folded into a six-stranded sandwich. PDZ domains are able to recognize different protein sequences in their ligands such as the carboxyl-terminal amino acid sequences, cyclic peptides, spectrin-like motifs, ankyrin repeats, LIM domains or even another PDZ domain. The next proteins with this domain are ARHGEF11, LIMK1, RGS2, NHERF or InaD (van Ham *et al.*, 2003).

The WW domain is the smallest module of 35 amino acids whose name is derived from the occurrence of two tryptophan residues spaced 20-22 amino acids apart. It is compactly folded into a short anti-parallel β-sheet with conserved aromatic (mainly tyrosine) and proline residues and interacts with proline-containing ligands. This ability can be regulated by tyrosine phosphorylation. Proteins containing WW domain are dystrophin, Nedd4 or FE65 (Ilsley *et al.*, 2002).

The Armadillo repeat was firstly identified in the *Drosophila* segment polarity protein Armadillo. It comprises about 42 amino acids folded into three α-helices and its particular ligands bind into its positively-charged groove. The proteins with this repeat are β-catenin, plakophilin 1 and α-importin (Tewari *et al.*, 2010).

3.3.2. Regulation of Gα activation by accessory proteins

Regulators of G-protein signalling

The signal transduction via GPCRs and G-proteins is a rapid process *in vivo*. Nevertheless, purified Gα-proteins exhibit a very slow rate of GTP hydrolysis *in vitro*. This paradox was explained by the discovery of proteins accelerating GTP hydrolysis which are similar to GAPs of small G-proteins. To date, the family of regulators of G-protein signalling (RGS) proteins counts 21 members (RGS1-22 omitting RGS15) which are divided into four subfamilies according to their structural and functional features. The last identified member RGS22 protein falls outside these four subfamilies. They interact with many different partners including GPCRs, Gα proteins, effectors (adenylyl cyclase, GIRK channels, cGMP phosphodiesterase, phospholipase Cβ, Ca^{2+} channel), signalling proteins (small G-proteins, D-AKAP2 or components of the Wnt signalling pathways) or scaffold and adaptor proteins (GIPC, 14-3-3 proteins, spinophilin). They can be regulated by cations such as sodium, potassium and magnesium or phospholipids (Abramow-Newerly *et al.*, 2006; Siderovski and Willard, 2005).

Members of the A/RZ subfamily (RGS17, RGS19, RGS20) have about 20-30 kDa containing one RGS domain and a cysteine string region which might be palmitoylated. Moreover, RGS19 (GAIP) can bind to PDZ domain-containing proteins via its C-terminal PDZ domain. It was shown that it forms a protein complex with the D_2 dopamine receptor due to interaction with PDZ domain-containing scaffolding protein GIPC (RGS-GAIP interacting protein C terminus). GIPC can also interact with β_1-adrenergic receptor and decrease β_1-AR mediated ERK activation. It associates with clathrin-coated pits or other microdomains on the plasma membrane which implies its participation in vesicle trafficking (Abramow-Newerly *et al.*, 2006).

Members of the B/R4 subfamily (RGS1-5, RGS8, RGS13, RGS16, RGS18 and RGS21) are similar in structure to members of the A/RZ subfamily. They contain a simple RGS domain with short N- and C-termini. It was found that some members of this subfamily can bind to the IC3 of some GPCRs (Miyamoto-Matsubara *et al.*, 2010; Roy *et al.*, 2006) while others can be capable of recruitment to the plasma membrane without interactions with receptors or G-proteins, e.g. via PIP_3 (Ishii *et al.*, 2002). They are able to bind to many effectors and affect their activity. Some RGS inhibit $G_s\alpha$-stimulated AC activation and others inhibit the effects of $G_i\alpha$ protein on AC activity (Abramow-Newerly *et al.*, 2006). It was observed that 19 amino acids in N-terminus of RGS2 is necessary for its binding to the cytoplasmic catalytic C1 domain of adenylyl cyclase V and thus inhibits it (Salim *et al.*, 2003). RGS2, RGS5 and RGS8 were shown to contribute to deactivation of G protein-gated inwardly rectifying potassium channels (GIRK) (Herlitze *et al.*, 1999). The signalling pathway via phospholipase Cβ can be also inhibited by some RGS proteins, probably by binding to $G_q\alpha$ and preventing the interaction between $G_q\alpha$ and PLCβ (Abramow-Newerly *et al.*, 2006). It was observed

that some RGS proteins interact with Ca^{2+}/calmodulin in a Ca^{2+}-dependent manner without the effect on the GAP activity of RGS proteins (Popov *et al.*, 2000).

The C/R7 subfamily includes RGS6, RGS7, RGS9 and RGS11 which contain three functional domains. Except for a RGS domain, they have the Gγ-like (GGL) domain forming a stable complex with $G\beta_5$ or interacting with GPCRs and a DEP (Disheveled/EGL-10/Plextrin homology) domain binding to many different interacting partners such as R9AP (RGS9 associated protein) and R7BP (R7 binding protein). Both proteins are DEP domain-associated syntaxin-like proteins interacting with G-proteins (Abramow-Newerly *et al.*, 2006).

The D/R12 subfamily has two large multidomain members (RGS12 and RGS14) with an RGS domain and a Gα protein-binding domain GoLoco and one member RGS10 resembling to members of the B/R4 subfamily. Some splice variants of RGS12 contain PDZ domain as well. RGS12 and RGS14 proteins were shown to bind to some GPCRs. They probably interact with two Gα proteins simultaneously because they contain two Gα protein-binding domains. Nevertheless, the GoLoco domain preferentially interacts with an inactive Gα protein and inhibits the dissociation of GDP. The members of this subfamily can affect the activity of PLCβ and the voltage-dependent calcium channels (Abramow-Newerly *et al.*, 2006).

The RGS proteins are able to couple with some scaffold and adaptor proteins. One of them is spinophilin, which was proven to interact with the third IC3 of some isoforms of α-adrenergic receptors and the N-terminal domain of RGS2 leading to the inhibition of Ca^{2+} signalling (Wang *et al.*, 2005). It can also bind to protein phosphatase-1, F-actin, doublecortin or nucleotide exchange factor Tiam-1 through its PDZ domain (Abramow-Newerly *et al.*, 2006).

Another scaffold proteins interacting with RGS proteins are isoforms of 14-3-3 protein (α, β, γ, δ, ε, η, τ, ζ, σ). It seems that they bind to two binding sites (an RGS domain and the N-terminus) of RGS3, RGS7 and RGS8 and thus impede the ability of RGS proteins to inhibit the activity of Gα proteins. The affinity of these proteins is affected by phosphorylation/dephosphorylation of the serine residues in binding sites. While phosphorylation increases the affinity and contributes to inhibition of RGS proteins, dephosphorylation appears to increase the level of active RGS proteins (Benzing *et al.*, 2002; Benzing *et al.*, 2000).

Activators of G-protein signalling

Activators of G-protein signalling (AGS) are able to affect the G-protein activity independently of receptors. The group of AGS is comprised of ten members (AGS1-10), which can be divided into three distinct classes according to their functional features.

The class I of AGS proteins has only one member AGS1, which is closely related (about 60%) to the small G-protein Rhes/TEM2/RASD2. It contains a Ras-like motif and the N- and C-terminal extensions. It binds $G_{i2}\alpha$ and $G_{i3}\alpha$ proteins and functions as a guanine exchange factor (GEF)

promoting GTP binding (Cismowski, 2006). It also interacts with the Ras binding domain of PI3-kinase, inhibits protein kinase Cδ and GPCR-mediated regulation of GIRK channels and Erk1/2 (Blumer *et al.*, 2007).

The class II includes AGS3-6 proteins acting as guanine nucleotide dissociation inhibitors (GDI). They contain 1-4 GRP motifs consisting of 20-25 amino acids and interacting with $G_i\alpha$, $G_o\alpha$ and $G_t\alpha$ proteins. The binding site of the GRP motif in Gα protein is a switch II/α3-helix binding pocket, which is accessible in the heterotrimeric G-proteins (Blumer *et al.*, 2007). It is believed that this interaction inhibits GDP release and induces conformational changes in the switch II region leading to G-protein dissociation. This can dampen agonist-induced signal transduction or impede the re-association of the G-protein heterotrimer and promote activity of Gβγ-regulated effectors (Cismowski, 2006). Next protein region important for protein-protein interactions (e.g. with the small G-protein Rac) is the TPR (tetratricopeptide) motif, which is repeated in their N-terminus (Cismowski, 2006).

Members of the class III (AGS2, AGS7 and AGS8) directly bind to Gβγ dimers and some receptors as well. Thus, they can dissociate G-protein heterotrimers or capture Gβγ dimers after G-protein activation (Cismowski, 2006). AGS9 and AGS10 exhibit similar properties as AGS2, AGS7 and AGS8 and are included in the class III (Blumer *et al.*, 2007).

Ric8 proteins

There are two isoforms of Ric8 (resistance to inhibitors of cholinesterase, synembryn) proteins, Ric-8A and Ric-8B, which function as receptor-independent GEF proteins. They are coded by the *ric-8* gene and differ in their abilities to bind to distinct Gα proteins (Tall *et al.*, 2003). Ric-8A exhibits the specificity to $G_{i/o}\alpha$ nd $G_q\alpha$ proteins while Ric-8B to $G_s\alpha$ and $G_q\alpha$. These proteins are composed of 10 armadillo domains folded into a right-twisted α-alpha super helix (Figueroa *et al.*, 2009).

RhoGEF proteins

RhoGEF proteins represent another family of Gα protein regulators. Besides an RGS domain, they also contain a catalytic Dbl homology (DH) domain, one or two PH domains and in some cases a PDZ domain or RGS homology (RH) domain. The Dbl domain is an extended helical domain (about 200 amino acids) with the GEF activity (Aittaleb *et al.*, 2010). RhoGEF proteins can be regulated by direct interaction with activated Gα proteins or through phosphorylation by PKA and PKC. Activated $G_{12/13}\alpha$ proteins are able to bind to p115RhoGEF, LARG and PDZ-RhoGEF via its RH domain composed of nine α-helices in a flat domain with two lobes (Tesmer, 2009). PDZ-RhoGEF and LARG contain a PDZ domain, which can interact with other receptors than GPCRs, and thus mediate a crosstalk between different signalling pathways.

Activated $G_{q/11}\alpha$ proteins were shown to interact with LARG or p63RhoGEF, which directly competes with the activation of phospholipase Cβ (Lutz *et al.*, 2005). The binding site for activated $G_{q/11}\alpha$ proteins is the C-terminal helix of the PH domain in p63RhoGEF and this interaction results in the p63RhoGEF activation. However, $G_{16}\alpha$ protein which also binds to this site fails to activate it, suggesting that this isoform of Gα protein can serve as an inhibitor of $G_{q/11}$ protein signalling through p63RhoGEF (Aittaleb *et al.*, 2010). It has been reported that $G_q\alpha$ protein interacts with the DH/PH tandem domains of LARG independently of the phospholipase Cβ activation. It seems that this interaction mediates activation of the small GTPase Rho and Rho-dependent transcriptional activity of serum response factor (Pfreimer *et al.*, 2012).

RhoGEF proteins activated by Gβγ dimers are P-Rex1, P-Rex2 and p114RhoGEF. P-Rex proteins are composed of the DH/PH tandem domains, two DEP domains, two PDZ domains and the C-terminal domain. They are regulated not only by Gβγ dimers but also by phosphatidylinositol (3,4,5)-triphosphate (PIP_3) (Welch *et al.*, 2002). p114RhoGEF is structurally less complicated compared to P-Rex proteins. It contains only the DH/PH tandem domain with higher basal GEF activity and the C-terminal proline-rich region with the inhibitory effect (Aittaleb *et al.*, 2010; Blomquist *et al.*, 2000).

G-protein coupled receptor kinases

Another family of RGS domain-containing proteins is the G-protein coupled receptor kinase (GRK) family. As mentioned above, they phosphorylate the serine or threonine residues in the third intracellular loop and the C-terminal region of activated GPCRs and initiate homologous desensitization. Except for receptors, they are able to phosphorylate many non-receptor substrates such as tubulin, synucleins, phosducin, ribosomal protein P2, the inhibitory γ subunit of the type 6 retina cGMP phosphodiesterase, the β subunit of the epithelial Na^+ channel, the ezrin-radixin-moesin (ERM) family protein ezrin, receptor regulated Smads (R-Smad), the calcium-binding protein DREAM, IκBα, p38 MAPK and others (Penela *et al.*, 2010; Ribas *et al.*, 2007).

Seven GRK isoforms (GRK1-7) are diversely expressed in various tissues and engaged in different cellular processes due to their ability to interact with a variety of proteins. Visual GRK1 and GRK7 are expressed in retina and are mostly anchored to the plasma membrane by their farnesylated C-termini (Ribas *et al.*, 2007). The palmitoylated GRK4 is highly expressed in the testes and in cerebellum and kidney. The other GRKs (GRK2, GRK3, GRK5 and GRK6) are ubiquitously expressed and they are primarily responsible for phosphorylation of GPCRs. GRK2 and GRK3 are cytosolic proteins while GRK5 and GRK6 are associated with the plasma membrane via the PIP_2 binding proteins and the palmitoyl residue, respectively (Ribas *et al.*, 2007). The interaction of Gβγ dimers with cytosolic GRKs mediated through the PH domains of kinases is required for their translocation to the plasma membrane (Bunemann and Hosey, 1999).

They contain a well-conserved catalytic domain, the N-terminal domain with the RH domain and the C-terminal domain of a variable size. Some GRKs also comprise the PH domain in their C-terminal domains. It was suggested by molecular modelling that intramolecular interactions between all three domains keep the enzyme in an inactive basal state and that disruption of this state by interactions with regulatory proteins promotes a conformational change causing GRK2 activation and translocation (Sarnago *et al.*, 2003).

GRKs can be phosphorylated at some positions in the N-terminal or C-terminal domains. The best explored modifications are those found in the molecule of GRK2. The MAP kinase ERK phosphorylates a serine residue Ser^{670} located in the C-terminal domain of GRK2 leading to the inhibition of the kinase activity (Elorza *et al.*, 2000). On the contrary, the phosphorylation of the Ser^{685} residue by PKA facilitates the kinase activation and the binding with Gβγ dimers (Ribas *et al.*, 2007). Three tyrosine residues Tyr^{13}, Tyr^{86} and Tyr^{92} near the calmodulin-binding site and within the RH domain of GRK2 are phosphorylated by c-Src and these modifications appear to enhance its interaction with $G_q\alpha$ protein and to promote its degradation by the proteasome pathway (Penela *et al.*, 2003). Phosphorylation by PKC takes place at residue Ser^{29} within the calmodulin-binding site of GRK2 and abolishes the inhibition of the kinase activity by binding of calmodulin (Krasel *et al.*, 2001). It is worth noting that the activity of GRKs can be regulated by Ca^{2+} and calcium-binding proteins. GRK1 is inhibited by the calcium-binding protein recoverin while GRK2, GRK5 and GRK6 appear to be inhibited by Ca^{2+}-calmodulin (Bunemann and Hosey, 1999). This inhibition occurs when calmodulin binds to the N- and C-terminal domains of the kinase (Ribas *et al.*, 2007).

GRK2 and GRK3 interact with members of the $G_q\alpha$ protein class via its RH domain but not with members of the other Gα protein classes. They carry out the GAP activity towards $G_q\alpha$ proteins and are able to inhibit $G_q\alpha$-mediated phospholipase C activity (Ribas *et al.*, 2007). The interaction between GRK2 and $G_q\alpha$ protein seems to be enhanced through tyrosine phosphorylation by c-Src (Mariggio *et al.*, 2006).

GRKs have the ability to interact with different scaffold and signalling proteins. Caveolin which is a major component of caveolae serves as a scaffold for receptors, G-proteins and MAP kinases. It was found out that GRK-mediated phosphorylation can be inhibited when GRK is bound to caveolin-1 and caveolin-3 via its caveolin binding motifs in the PH domain and the N-terminal domain (Carman *et al.*, 1999; Schutzer *et al.*, 2005). Moreover, the association of GRK with caveolin scaffold can facilitate its interactions with other signalling and regulatory protein and thus mediate the crosstalks between different signalling pathways.

Clathrin binds to the clathrin-box located in the C-terminal domain of GRKs. This interaction appears to play a role in agonist-promoted internalization of some receptors in dynamin-dependent mechanism as well as in modulation of the kinase activity (Shiina *et al.*, 2001).

The multifunctional proteins GIT1 and GIT2 (GRK-interacting proteins 1 and 2) are interacting partners of GRK2, GRK3, GRK5 and GRK6. They are involved in many cellular processes concerned with cytoskeletal dynamics, membrane trafficking, cell adhesion and signal scaffolding. They have GAP activity towards ARF1 and ARF6 (ADP-ribosylation factor) proteins (Claing *et al.*, 2001).

The Raf kinase inhibitor protein RKIP is interacting with GRK2 and inhibits the kinase activity after receptor stimulation, phosphorylation of RKIP at residue Ser^{153} by PKC and its dissociation from Raf-1 (Lorenz *et al.*, 2003).

GRKs regulate the PI3K/Akt signalling pathway by interactions with the phosphoinositide 3-kinase (PI3K) as well as the serine/threonine protein kinase Akt. The PIK domain of GRK2 was proved to associate with PI3Kγ and it mediates recruitment of the kinase to the plasma membrane after receptor activation while the agonist-dependent Akt interaction with the C-terminus of GRK2 leads to Akt inhibition (Ribas et al., 2007).

GRKs are also able to inhibit the MAPK signalling pathway. It was shown that GRK2 and MAP2K MEK1 are in the same multimolecular complex and thus the regulation of ERK activity is controlled by direct or indirect interactions of these two proteins (Jimenez-Sainz *et al.*, 2006).

3.3.3. Scaffold proteins

β-arrestin

The family of arrestin has four members: visual arrestins (arrestin-1 and arrestin-4) expressed mainly in retina and non-visual arrestins (β-arrestin 1 and β-arrestin 2, also known as arrestin-2 and arrestin-3) expressed ubiquitously (Ma and Pei, 2007). β-arrestin consists of the N-terminal, a C-terminal domains and a long C-terminal tail interacting with the N-terminal domain (Gurevich and Gurevich a, 2006). Arrestin binding to an activated receptor induces the re-arrangements of its domains and-separation of the C-terminal tail from the N-terminal domain (Gurevich and Gurevich a, 2006). Some isoforms of arrestin contain a hydrophobic amino acid-rich region at the C-terminus which serves as a nuclear export signal responsible for its extranuclear localization. On the contrary, the N-terminal domain which also regulates the arrestin localization can bind to various interacting partners such as GPCRs, c-Src or Mdm2 (Wang *et al.*, 2003).

Arrestins are cytoplasmic phosphoproteins known mainly for their binding to the phosphorylated active receptors during receptor internalization. Agonist-stimulated interaction of arrestin with the β_2-adrenergic receptor leads to arrestin dephosphorylation. The β-arrestin can be phosphorylated by different kinases. Its amino acid residue Ser^{412} is modified by ERK1/2 or GRK5 (Barthet *et al.*, 2009; Lin *et al.*, 1997) while the phosphorylation of the tyrosine residue Tyr^{54} is mediated by c-Src and inhibits the interaction with the μ-2 subunit of the AP complex (Marion *et al.*,

2007). Its dephosphorylation is carried out by the protein phosphatase PP2A, which forms a protein complex with β-arrestin-2 and Akt (Beaulieu *et al.*, 2005). The dephosphorylation of β-arrestin seems to be required for clathrin binding and receptor endocytosis and the receptor internalization leads to its re-phosphorylation (Lin *et al.*, 1997).

The interaction between β-arrestin and the E3 ubiquitin ligase Mdm2 was shown to occur after stimulation of the β_2-adrenergic receptor and induce the β-arrestin ubiquitynation which is necessary for the subsequent receptor internalization (Shenoy *et al.*, 2001). Next, the interaction of β-arrestin-2 and Mdm2 after activation of opioid or bradykinin receptors can affect the transcription and apoptosis mediated by p53 because Mdm2 distributed primarily in the nucleus is able to ubiquitinate and target p53 for degradation (Wang *et al.*, 2003). In addition to ubiquitination, β-arrestin-2 can be modified by SUMO (small ubiquitin like modifier) in the SUMO motif LKDE in the C-terminal region (Wyatt *et al.*, 2011).

Important modification of β-arrestin-2 is N-nitrosylation at residue Cys^{410} by endothelial NO synthase which β-arrestin-2 binds to. This modification promotes the interaction of β-arrestin-2 with clathrin and β-adaptin (Shenoy and Lefkowitz, 2011).

β-Arrestins represent important scaffold proteins which serve as endocytic adaptors recruited after GPCR activation by agonists. Apart from clathrin and adaptor AP-2, they interact with a trafficking regulator N-ethylmaleimide-sensitive fusion protein (NSF), the GTPases ADP-ribosylation factor 6 (ARF6) and RhoA and their regulators ARNO (ARF nucleotide-binding site opener) (Gurevich and Gurevich a, 2006). The ARF6 and ARNO as well as NSF proteins were shown to enhance the internalization of the β_2-adrenergic receptor after β-arrestin-1 binding (Marchese *et al.*, 2003).

β-Arrestins are able to interact with non-receptor tyrosine kinases such as c-Src, Hck, Fgr or Yes and with many members of the mitogen-activated kinase pathway such as extracellular signal-regulated kinases (ERK1, ERK2), apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase 3 (JNK3), MEK1 (MAP2K1), p38 MAP kinases or c-Raf-1 (DeFea, 2011; Gurevich and Gurevich a, 2006). It is worth pointing out that GPCRs can activate ERKs through two separate mechanisms. The first mechanism involves the G-protein-dependent pathway via PKA or PKC and appears to be rapid and transient. The activated ERK translocates from the cytoplasm to the nucleus where it phosphorylates many substrates, including transcriptional factors. The second mechanism is β-arrestin-dependent, slow and long-acting and occurs without translocation of activated ERK from the cytoplasm (Ma and Pei, 2007).

Some receptors can promote formation of distinct signalling complexes via interactions with β-arrestins. For example, the angiotensin II receptor form the ERK1/2 module involving Raf, MEK1/2 and ERK1/2 while the β_2-adrenergic receptor constitutes a less stable complex comprising c-Src and ERK1/2 (DeFea, 2011). MEK1, ERK and Raf make contact with β-arrestin via its N- and C-terminal

regions and the SH3 domain of c-Src associates with N-terminal proline residues (Luttrell *et al.*, 1999). Both terminal regions of β-arrestin also associate with another complex, the Jnk3 module, which involves ASK1, MKK4 and Jnk (Song *et al.*, 2009).

β-Arrestins can regulate the IκB/NF-κB pathway through two mechanisms. They directly bind to an inhibitor protein IκBα, which forms an inactive dimer with a pleiotropic transcription factor NF-κB. When IκBα is phosphorylated after stimulation by an extracellular signal, IκBα is degraded and NF-κB is translocated to the nucleus where initiates the transcription. The interaction between β-arrestin and IκBα elicited by activation of the β_2-adrenergic receptor prevents phosphorylation and degradation of IκBα and thus reduces the NF-κB-mediated transcription (Gao *et al.*, 2004). The second mechanism is based on modulation of signalling by the Toll-like-interleukin-1 receptor (TLR-IL1R) and its adaptor tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). After activation of this receptor, TRAF6 interacts with β-arrestin which prevents the autoubiquitination of TRAF6 and activation of NF-κB (Wang *et al.*, 2006).

Another important function of β-arrestin consists of transferring signals from GPCRs into the nucleus. The nuclear localization of β-arrestins is provided by the N-terminal domain. β-Arrestin is able to regulate transcription via Mdm2 protein as mentioned previously or by the association with transcription cofactors p300 and CREB leading to increased acetylation of histone H4, reorganization of chromatin and subsequently increased gene expression. In this way, the activation of opioid receptors induces the transcription of the *p27* gene encoding the cyclin-dependent kinase inhibitor p27 and the *FOS* gene encoding the transcription factor FOS (Kang *et al.*, 2005).

It seems that β-arrestins can also regulate the PI3K/Akt signalling pathway. This pathway can be activated or inhibited depending on the activation of a particular receptor. The activation of IGF-1 receptor or PAR-1 receptor induces PI3K as well as Akt activation through interaction with β-arrestin while the activation of dopamine D2 receptor or PAR-2 receptor causes the opposite effect (DeFea, 2011).

Next interacting partner of β-arrestin is cAMP phosphodiesterase PDE4D5, which is recruited to the plasma membrane via interaction with β-arrestin after the β_2-adrenergic receptor activation. This association inhibits PKA activity triggered by increased concentration of cAMP and switches signalling from G_s to G_i, which results in subsequent MAPK activation (Lynch *et al.*, 2005).

β-Arrestin also serves as a scaffold protein for actin assembly proteins and can facilitate chemotaxis. The proteins participating in control of actin reorganization and simultaneously interacting with β-arrestin are cofilin, chronophin, LIM domain kinase (LIMK), filamin, RhoA and ral-GDS (DeFea, 2011).

Na^+/H^+ exchanger regulatory factor (NHERF)

Some GPCRs such as β_2-adrenergic receptor modulate the activity of the Na^+/H^+ exchanger 3 (NHE3). Its increased activity after receptor activation is at least partly given by the agonist-dependent interaction of the receptor C-terminal region with the PDZ domain of the NHE regulatory factor (NHERF) as the interaction of this regulatory protein with NHE inhibits the ionic exchange in a PKA-dependent manner (Weinman *et al.*, 1998). Next, it was shown that NHERF is able to bind to two isoforms of phospholipase Cβ (PLCβ1 and PLCβ2) in a protein complex with a transient receptor potential channel 4 (TRP4). This transmembrane protein is a Ca^{2+} channel which can act as an effector of GPCRs. NHERF interacts with PLCβ and TRP4 through its PDZ domain but associates also with the cytoskeletal protein ezrin through the FERM domain and thus indirectly binds to the active cytoskeletal filaments (Mahon and Segre, 2004; Suh *et al.*, 2001). Although the functions of all these interactions have not yet been completely elucidated, it seems that NHERF could connect the PLC signalling pathway with TRP4 channels.

Caveolin and flotillin

Caveolins and flotillins are scaffold proteins enriched in membrane microdomains such as caveolae and lipid rafts and they have been shown to interact with the $G_q\alpha$ protein (Oh and Schnitzer, 2001). It was observed that caveolin-1 prefer to bind to the activated $G_q\alpha$ protein and prevent the interaction of $G_q\alpha$ with Gβγ dimer. This would prolong the activated state with rising concentration of intracellular Ca^{2+} (Sengupta *et al.*, 2008).

Flotillin possesses a conserved SPFH (stomatin/prohibitin/flotillin/Hf1K/C) domain at its N-terminus which is necessary for the interaction with $G_q\alpha$ protein. This interaction may represent a significant crosstalk as it is connected with the signal transfer from $G_q\alpha$ to p38 MAPK through Src family tyrosine kinase in lipid rafts (Mizuno and Itoh, 2009).

A-kinase anchoring proteins (AKAPs)

The scaffold proteins which were shown to interact with β_2-adrenergic receptors, protein kinase A and other signalling proteins are known as A-kinase anchoring proteins (AKAPs). They are involved in receptor phosphorylation, regulation of receptor desensitization as well as downstream mitogenic signalling. The other interacting proteins of AKAPs are protein kinase C, phosphatases, adenylyl cyclases, phosphodiesterases or ERK kinases (Tilakaratne and Sexton, 2005).

3.3.4. Effectors of G-proteins

Both Gα protein and Gβγ dimer transfer signals from GPCRs to different effectors thereby regulating their activity. The effectors are various enzymes regulating the concentration of second messengers and permeability of ion channels.

Adenylyl cyclase

The primary effector of members of the G_s and G_i classes is adenylyl cyclase (AC), which has nine isoforms in vertebrates. Twelve transmembrane α-helices of AC form two symmetric domains, which are connected by two cytoplasmic domains necessary for its catalytic activity. It contains two catalytic domains C1 and C2. All AC isoforms can be activated by $G_s\alpha$ proteins and inhibited by $G_{i/o}$ proteins and Gβγ dimers and some of them can also be activated by Gβγ dimers under certain conditions. The switch II of $G_s\alpha$ proteins bind to both catalytic domains of AC although most of binding surface interacts with the C2 domain. This interaction induces rotation of the C1 domain around the C2 domain and causes a conformational change of the active site important for the enzyme catalytic function. On the other hand, $G_i\alpha$ proteins bind to the C1 domain and stabilize the two helices of the C1 domain. A crucial region for binding of Gβγ dimers includes amino acid residues 956 to 982 in the middle of the C2 domain (Sunahara and Taussig, 2002).

The activated AC catalyzes the conversion of adenosine triphosphate (ATP) to the second messenger cyclic 3',5'-adenosine monophosphate (cAMP), which subsequently activates cAMP-dependent protein kinase (PKA). PKA phosphorylate serine and threonine residues of many intracellular and plasma membrane-bound proteins which are involved in cell growth, division, differentiation and secretion. The activity of some AC isoforms is regulated also by Ca^{2+}/calmodulin, RGS proteins and/or through phosphorylation by PKA, PKC or CaM kinase II (Sunahara and Taussig, 2002).

Phospholipase Cβ (PLCβ)

Phosphatidylinositol specific phospholipases C are Ca^{2+}-dependent enzymes catalyzing the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2) to inositol-1,4,5-triphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). The thirteen known isoforms of PLCs can be divided into six families (β, γ, δ, ε, ζ and η), which differ in tissue localization and specific activity (Drin and Scarlata, 2007). The PLC β family includes four isoforms differing in their response to $G_{q/11}\alpha$ proteins, their major activators. $G_{q/11}\alpha$ proteins activate PLC β1 and PLC β3 isoforms more efficiently than PLC β2 isoform (Smrcka and Sternweis, 1993). Moreover, PLC β2 and PLC β3 can be activated by Gβγ dimers (Camps *et al.*, 1992; Park *et al.*, 1993).

PLC β consists of an N-terminal PH domain, four EF hands, a catalytic X/Y domain, a C2 domain and C-terminal extension of 400 amino acid residues. The PH domain functions as an interacting site for phosphoinositides and thus the phospholipase is targeted to the plasma membrane. This domain is a major region for interaction with the blades 1, 2, 5 and 7 of Gβγ dimer. Each EF hand is composed of four helix-loop-helix motifs folded into two lobes and the whole EF domain is connected to the PH domain by a short linker. The catalytic X/Y domain is composed of two regions X and Y, which are linked by a sequence exhibiting high flexibility (Drin and Scarlata, 2007). It has been reported that the removal of this linker led to a higher basal activity of PLC β2 suggesting that the linker has an inhibitory effect on this enzyme (Zhang and Neer, 2001). The C2 domain is a tight sandwich consisting of β sheets, which serve as scaffolds for the loops with specific functions. This domain specifically interacts with $G_q\alpha$ protein but not with $G_i\alpha$ protein or Gβγ dimer. Moreover, the interaction of this domain and the non-activated $G_q\alpha$ protein is much weaker than with the activated $G_q\alpha$ protein (Drin and Scarlata, 2007). The C-terminal extension is a characteristic feature of PLC β and determines the binding site for $G_q\alpha$ protein. It consists of three long α-helices folded into a coiled-coil structure and containing phosphorylation sites with regulatory roles (Singer *et al.*, 2002). The PLC β interacting site on $G_q\alpha$ protein includes amino acid residues in the α3 helix and β4 sheet which are adjacent to the switch regions (Venkatakrishnan and Exton, 1996).

cGMP phosphodiesterase

The family of cyclic nucleotide phosphodiesterases (PDE) includes 11 subfamilies with different isoforms of this enzyme encoded by 21 genes in human genome (Conti and Beavo, 2007). These phosphodiesterases are classified according to their features and selectivity to cyclic GMP (cGMP) or cAMP which they are able to hydrolyze by breaking the phosphodiester bonds in these second messengers (Omori and Kotera, 2007).

cGMP phosphodiesterase plays an important role in the signalling pathway via rhodopsin and transducin ($G_t\alpha$ protein) in rodes and cones of vertebrates. Photo-excited rhodopsin induces the GDP/GTP exchange of transducing, which subsequently activates the cGMP phosphodiesterase. This enzyme hydrolyzes cGMP, which leads to closure of cGMP-gated channels and hyperpolarization of the plasma membrane (Granovsky and Artemyev, 2001).

This enzyme comprises two catalytic subunits (α and β) and two regulatory subunits (γ) forming the heterotetramer. The function of the γ subunit is regulation of the phosphodiesterase activity. After transducin activation, the C-terminus of the γ subunits interacts with its α3 helix and switch regions which results in conformational changes making the catalytic subunits accessible for their substrate (Grant *et al.*, 2006). Additionally, it has been reported that this interaction increases the hydrolysis of GTP and deactivation of transducin due to the ability of the γ subunit to increase the

affinity of RGS9 for transducin. The GTP hydrolysis decreases the affinity of the γ subunit for transducin and its re-association with the catalytic subunits (Skiba *et al.*, 1999).

Phosphoinositide 3-kinases

PI3-kinases (PI3-kinases, PI3-K) are enzymes catalysing phosphorylation of the 3'-OH position of the inositol ring of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI-4)P) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and producing PI-(3)P, PI-$(3,4)P_2$ and PI-$(3,4,5)P_3$ (Cantrell, 2001). Multiple isoforms of PI3-K are divided into three classes (I-III) but only members of class I were shown to couple to extracellular stimuli and signalling pathways via G-proteins (Vanhaesebroeck *et al.*, 1997). Members of class I are composed of a catalytic subunit p110 and a regulatory adaptor subunit. The catalytic subunit p110 has four isoforms (α, β, γ and δ) encoded by separate genes and three isoforms of the regulatory subunit (p85α, p85β and p55γ) are encoded by three genes. Besides the adaptor feature, the regulatory subunit exhibits the ability to inhibit the activity of the p110 catalytic subunit (Cantrell, 2001).

Some Gα proteins as well as Gβγ dimers were proved to be activators or inhibitors of the PI3K signalling pathway. G_tα and G_{i1}α proteins can activate p110γ PI3K (Yeung and Wong, 2010) whereas G_qα protein interacts with an adaptor binding domain (ABD) of p110α PI3K and thus inhibits the Akt activation mediated by this enzyme (Ballou *et al.*, 2003). Furthermore, the bound G_qα protein blocks Ras protein from binding to p110α PI3K and ensures the additional mechanism to suppress the PI3K activation (Ballou *et al.*, 2006). Besides activation of p110α PI3K, G_{16}α protein is even able to mediate this inhibition through p110β PI3K as well (Yeung and Wong, 2010). It is worth noting that Gβγ dimers bind directly to p110β PI3K and p110γ PI3K and activate them (Ballou *et al.*, 2006). Altogether, individual subunits of G-proteins exert distinct effects on the PI3K signalling pathway and can modulate it in dependence upon incoming signals to the final cellular response.

Btk (Bruton's tyrosine kinase) family kinases

This family of kinases includes Btk/Atk, Itk/Emt/Tsk, Bmx/Etk and Tec, which contain an N-terminal PH domain, a Tec homology (TH) domain, an SH3 domain, an SH2 domain and a C-terminal catalytic kinase domain. By virtue of these numerous binding domains they can bind to many different upstream and downstream interacting partners. The PH-TH domain of Btk can associate with G_qα protein, G_{12}α protein and Gβγ dimer, which results in increase of the kinase activity and subsequent regulation of RhoA activity (Mao *et al.*, 1998).

Ion channels

The α and βγ subunits of heterotrimeric G-proteins are able to gate and modulate Na^+, K^+, Ca^{2+} and Cl^- selective ion channels through direct interaction or indirectly via second messengers (Morris

and Malbon, 1999). The signalling pathways initiated by M2 muscarinic, somatostatin or α_2-adrenergic receptors activate G-protein-gated inwardly rectifying K^+ channels through $G_i\alpha$ proteins and Gβγ dimers (Landry *et al.*, 2006). Ca^{2+} channels can be activated or inhibited by both Gα proteins and Gβγ dimers. $G_o\alpha$ protein inhibits these channels after activation of opioid or catecholamine receptors while $G_s\alpha$ protein can activate them (Morris and Malbon, 1999).

3.3.5. G-protein independent GPCR signalling

GPCRs are able to convey signals in a G-protein-independent manner. As mentioned previously, some of these pathways are regulated by scaffold proteins (β-arrestin, NHERF). However, others are mediated due to the interaction of receptors with tyrosine kinases such as Src and JAK. The JAK/STAT (Janus protein kinase/signal transducers and activators of transcription) signalling pathway is mediated from the cytokine receptor to STAT proteins. Cytokine receptors dimerize after cytokine binding to the extracellular part of the receptor. This causes tyrosine phosphorylation and subsequent activation of JAK. The cytokine receptor is phosphorylated by JAK and associate with STAT. The phosphorylated STATs dissociate from the receptor, form dimers which translocate into the nucleus where they activate transcription of target genes (Tilakaratne and Sexton, 2005). Nevertheless, the JAK/STAT signalling pathway can be activated by some GPCRs including the angiotensin AT_1 receptor, the platelet-activating factor (PAF) receptor or the chemokine receptors CCR2, CCR5 and CXCR4. It was shown that the AT_1 receptor is able to activate this pathway by direct interaction with JAK2. However, this interaction may depend on some other conditions. For example, the N-terminal SH2 domain of protein tyrosine phosphatase SHP-2 is required for response to angiotensin II (Godeny *et al.*, 2007).

The tyrosine kinase c-Src is known to be activated by GPCRs when β-arrestin is recruited to the receptor during the desensitization process. It can be also activated by Gβγ dimers detached from $G_i\alpha$ protein (Daaka *et al.*, 1997). However, it seems that members of the Src family tyrosine kinases are able to transfer signals from GPCRs directly through the interaction with the C-terminal region of these receptors. In the case of β_2-adrenergic receptor, the concentration of agonist determines how the signal is conveyed from the receptor. Low agonist concentrations cause activation of G-proteins while high concentrations lead to coupling of Src to the β_2-adrenergic receptor. Another member of this family, Fyn, associates directly with the C-terminal region of the serotonin 5-HT_6 receptor and subsequently activates the ERK1/2 signalling pathway (Yun *et al.*, 2007). These studies suggest that GPCRs can transfer signals via Src in a G-protein- and β-arrestin-independent manner.

3.3.6. Transactivation of GPCRs and RTKs

Both GPCRs and receptor tyrosine kinases (RTKs) are able to transactivate each other. RTKs such as the epidermal growth factor (EGF) receptor, the platelet-derived growth factor (PDGF) receptor, fibroblast growth factor (FGF) receptor can be transactivated by some GPCRs (e.g., thrombin, angiotensin, endothelin, acetylcholine and lysophosphatidic acid receptors) in a ligand-dependent manner. Another possibility of such a transactivation consists in direct association of both receptors or in phosphorylation of RTK by tyrosine kinases downstream of GPCR signalling. Both mechanisms occur in a ligand-independent manner (Delcourt *et al.*, 2007).

Similar mechanisms are applied in the transactivation of GPCRs by RTKs. It has been reported that the insulin-like growth factor 1 (IGF-1) can interact with $G_i\alpha$ protein as well as Gβγ dimers to activate the ERK pathway while the EGF receptor stimulate cAMP production by activation of $G_s\alpha$ protein (Luttrell *et al.*, 1995; Poppleton *et al.*, 1996).

3.3.7. Regulation of transcription factors by heterotrimeric G-proteins

Modulation of various transcription factors and cofactors is one of important roles of the GPCR signalling and two major regulatory inputs for transcription control are the MAPK signalling pathway activated via distinct Gα proteins and free Gβγ dimers. Other signalling intermediates participating in transcription control include PKA, PKC, PLC β, small GTPase and RhoGEFs, CaMKII, PDE or PI3K/Akt (Ho *et al.*, 2009).

The transcription factors NF-κB and STAT can be activated by triggering the signalling pathways through all Gα proteins while the activation of other factors is more specific. For example, c-FOS and nuclear factor of activated T-cells (NFAT) are modulated by $G_s\alpha$, $G_{q/11}\alpha$ and $G_{12}\alpha$ proteins and c-Jun by $G_s\alpha$ and $G_{12}\alpha$ proteins. A well-known example of transcription control by GPCRs is the activation of the nuclear transcription factor CREB (cAMP-responsive element (CRE) binding protein) by the $G_s\alpha$/AC/PKA signalling pathway. After its activation, CREB binds to p300/CREB-binding protein (CBP) and regulates the transcription (Ho *et al.*, 2009).

3.3.8. The TRH-R/$G_{q/11}\alpha$ signalling pathway

The TRH receptor is coupled to $G_{q/11}$ proteins, which in turn stimulate the activity of PLCβ cleaving $PI(4,5)P_2$ into $PI(1,4,5)P_3$ and DAG. The increased concentration of $PI(1,4,5)P_3$ causes the release of Ca^{2+} from intracellular stores and the elevation of intracellular Ca^{2+} activates Ca^{2+}/calmodulin-dependent protein kinase. DAG activates protein kinase C which is then translocated from the soluble to the particulate subcellular fraction.

Nevertheless, TRH can stimulate the MAPK signalling pathway mediating gene transcription. Treatment with the MAP2K inhibitor PD98059 blocked the TRH-induced activation of MAPK and decreased TRH-mediated gene transcription. The PKC inhibitor GF109203X and an L-type Ca^{2+} channel blocker nimodipine reduced TRH-induced MAPK activation and gene transcription suggesting that the MAPK activation by TRH is PKC- and Ca^{2+}-dependent (Wang and Maurer, 1999). In some cases, receptors require the recruitment of β-arrestin to activate the MAPK signalling pathway. However, the TRH receptor does not seem to interact with β-arrestin (Smith *et al.*, 2001). The TRH receptor was also found to activate MAPK in PKC-independent manner via Gβγ dimer (Palomero *et al.*, 1998).

The TRH receptor has been shown to transactivate the epidermal growth factor (EGF) receptor in GH_3 cells and related tyrosine kinase receptor HER2 in the phosphorylation-dependent manner leading to the recruitment of the adaptor proteins Grb2 and Shc. This phosphorylation was shown to be necessary for full TRH-induced activation of the MAPK signalling pathway and it was reduced by the PKC inhibitor GF109203X (Wang *et al.*, 2000).

4. Methods

4.1. Materials

All materials for tissue culture were supplied by Sigma–Aldrich (Milwaukee, WI), Gibco-BRL (Gaithersburg MD), NUNC (Rochester, NY) and PAA (Pasching, Austria). Complete Protease Inhibitor cocktail was from Roche (Basel, Switzerland). Immobiline Dry-Strips, Pharmalyte buffer, secondary anti-rabbit antibody labeled with horseradish peroxidase and CytoScint cocktail were purchased from GE Healthcare (Piscataway, NJ) and BioScint cocktail (National Diagnostics, Hessle Hull, UK). SYPRO Ruby stain was from Molecular Probes (Eugene, OR), trypsin from Promega (Madison, WI), acrylamide and bis-acrylamide from Serva (Heidelberg, Germany), nitrocellulose membrane from Schleicher-Schuell (Erdmannhausen, Germany), polyvinyldifluoride membrane from Bio-Rad (Hercules, CA), Whatman GF/C filters from Whatman Ltd. (Oxford, UK) and SuperSignal Substrate (Pierce Biotechnology, Rockford, IL). Dynabeads Protein G and Lipofectamine^{TM} RNAiMAX was from Invitrogen (Carlsbad, CA), BS^3 from Thermo Fisher Scientific (Waltham, MA) and [^{35}S]GTPγ was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All other chemicals and materials were from Sigma–Aldrich and were of the best quality available. Preparation and characterization of rabbit polyclonal serum against $G_{q/11}\alpha$ and $G_{i1,2}\alpha$ protein was described previously (Ihnatovych *et al.*, 2002; Matousek *et al.*, 2004). All other primary antibodies, blocking peptides, secondary anti-goat or anti-mouse antibodies labeled with horseradish peroxidase and all siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA).

4.2. Cell culture

HEK293 cells (clone E2 stably expressing the long isoform of the rat TRH receptor (TRH-R1) and clone E2M11 stably expressing both the long isoform of TRH-R1 and the mouse $G_{11}\alpha$ protein) prepared in Dr. G. Milligan´ laboratory (University of Glasgow, U.K.) were obtained by courtesy of Dr. P. Svoboda (Institute of Physiology, Academy of Science of the Czech Republic). HEK293 cells were grown in DMEM supplemented with 10% heat-inactivated newborn calf serum, geneticin (0.8 mg/ml) and hygromycin B (0.2 mg/ml) at 37 °C in 5% CO_2 humified atmosphere (Svoboda *et al.*, 1996).

4.3. Preparation of the postnuclear supernatant and the plasma membrane-enriched fraction and cytosol

Nearly confluent cell cultures grown in 80 cm^2 tissue culture flasks (15 flasks per sample) were treated with or without 10 μM TRH for 10 min, 30 min, 1 h, 2 h, 4 h, 8 h or 16 h and subsequently harvested. After collection by centrifugation at 1,800 rpm for 10 min (Hettich Universal

30RF centrifuge), cells were diluted in homogenization buffer STEM (250 mM sucrose, 50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$; pH 7.5) for sample preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and 2D electrophoresis or in homogenization buffer H (750 mM aminocaproic acid, 50 mM Bis-Tris, 0.5 mM EDTA; pH 7.0) for sample preparation for clear native electrophoresis (CN-PAGE). In both cases, Complete Protease Inhibitor Cocktail was supplemented to the samples, which were subsequently homogenized in a Teflon-glass homogenizer at 1,500 rpm for 5 min on ice. The homogenate was centrifuged for 3 min at 1,500 rpm at 4 °C in a Hettich centrifuge and samples of the postnuclear supernatant (PNS) were used for fractionation on Percoll density gradient or frozen in liquid nitrogen and stored at -80 °C. The resulting PNS was applied on the top of 30% or 25% (w/v) Percoll^R in TMEN buffer (20 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl; pH 7.4) and centrifuged in a Beckman Ti70 rotor at 65,000 x g for 30 min at 4 °C or in a Beckman Ti50.2 rotor at 50,000 x g for 15 min at 4 °C. Two layers of protein-rich opalescent material were separated from a cytosolic fraction on the top of the gradient. The cytosol was subsequently prepared from the top gradient layer by high speed centrifugation (300,000 x g, 1 h). The upper fraction (with lower buoyant density) enriched with plasma membranes was diluted in TMEN buffer and centrifuged in a Beckman Ti 50.2 rotor at 150,000 x g for 1 h (at 4 °C). Protein pellets were resuspended in a small volume of TMEN buffer. Samples were then frozen in liquid nitrogen and stored at -80 °C.

4.4. 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 TM buffer (50mM Tris–HCl, 5mM MgCl2, pH 7.4) 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.* (DeBlasi *et al.*, 1989). The binding reaction was terminated by rapid filtration through Whatman GF/B filters. Filters were subsequently washed 3 times with 3ml of ice-cold TM buffer and 3 times with 0.9% NaCl. Radioactivity retained on the filters was determined by liquid scintillation counting using CytoScint cocktail.

4.5. Agonist-stimulated [^{35}S]GTPγ binding assay

Agonist-stimulated [^{35}S]GTPγ binding was measured according to Bourova *et al.*(Bourova *et al.*, 2003). Aliquots of PNS or PM (10 mg protein per assay) were incubated with (total) or without (basal) TRH in a final volume of 100 ml of reaction mixture containing 20 mM HEPES, pH 7.4, 3 mM MgCl2, 100 mM NaCl, 2 mM 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. In routine analysis, TRH-stimulated $[^{35}S]GTP\gamma S$ binding was determined by a one-point assay using 5 nM $[^{35}S]GTP\gamma S$ ± 10 mM TRH. When analyzing dose-response curves, TRH concentration was gradually increased from 10^{-11}–10^{-4} M. Non-specific binding was determined by parallel assays containing 10 mM unlabeled GTP and it was subtracted from total bound radioactivity. The EC50 values were calculated from the dose-response curves by the GraphPad Prism 3.02 software (San Diego, CA).

4.6. SDS-PAGE 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. 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 TBS-T (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20; pH 8.0) 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 and exposed to Kodak MXB film.

4.7. Sample preparation for isoelectric focusing and 2D electrophoresis

Samples of PNS or PM were disposed of salts and other interfering compounds by trichloroacetic acid (TCA) precipitation followed by ethanol washing. A sample containing 100 mg of proteins for quantitative analysis (Sypro Ruby staining) or 1 mg of proteins for mass spectrometry analysis (Coomassie staining) was diluted with H_2O to the final volume of 90 ml and precipitated with 30 ml 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 ml 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 solubilised with 250 ml 2-DE 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 unsolubilised material by centrifugation (16,000 g, 1 min), the sample was pipetted into a groove of the Immobiline DryStrip Reswelling Tray (GE Healthcare).

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 solubilised 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: 150V for 5 h, 500 V for 1 h, and 3,500V 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% dithiotreaitol (DTT) for 15 min in order to reduce the disulfide bridges and other oxidized groups present in protein molecules. The strips were subsequently alkylated in the same equilibration buffer E containing 2.5% iodoacetamide (IAA) for 15 min. The equilibrated strips were then transferred onto 10% SDS–polyacrylamide slab gels (14 cm x 16 cm x 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.

4.8. Staining of polyacrylamide gels

The proteins in polyacrylamide gels were visualized with SYPRO Ruby according to the manufacturer's instructions. 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.

4.9. Image acquisition and analysis

After staining, the 2-DE 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 analysed 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 analysing 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). The levels of proteins increased or decreased at least two fold were taken into account as altered. Master gels for 2D proteome analyses were obtained by averaging the data of 16 replicas of each experimental group.

4.10. In-gel digestion, mass spectrometry and data processing

Individual spots cut from 2D gel were placed in microtubes and covered with 100 ml of 50 mM ammonium bicarbonate (ABC) buffer in 50 % acetonitrile (ACN) with 50mM 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 ml of 50 mM ABC/50% ACN with 50 mM IAA and sonicated for 5 min. After 25 min, the supernatant was discarded and exchanged for 100 ml 50 mM ABC/50% ACN with 50 mM DTT and sonicated for 5 min to remove excess IAA. Supernatant was discarded and samples were sonicated for 5 min in 100 ml of ultra-pure water for HPLC. The water was then discarded and samples sonicated for another 5 min in 100 ml 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 ml 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 ml drop was transferred onto MALDI target and let to dry. Dried droplets were covered with 0.5 ml 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 nonredundant NCBI database of protein sequences (6573034 sequences; 2244863856 residues). Database search criteria were 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.

4.11. CN-PAGE

Samples (50 μg protein) of the postnuclear supernatant, the plasma membrane-enriched fraction or the cytosol were solubilised in sample buffer (75 mM Bis-Tris, 750 mM ε-aminocaproic acid, 0.5% Coomassie G-250) containing varying concentrations of different detergents (digitonin, lauryl maltoside, Brij 56, Triton X-100, CHAPS, and SDS) for 30 min on ice or at 37 °C in some cases. The insoluble material was removed by centrifugation at 10,000 rpm for 10 min (at 4 °C) and subsequent separation of protein complexes was performed on 6-15%, 10-15% or 12-15% linear gradient polyacrylamide slab gels cooled to 2 °C using the Mighty Small SE 260 apparatus (GE Healthcare). A discontinuous buffer system using different anode (50 mM Bis-Tris, pH 7.0) and cathode (50 mM tricine and 15 mM Bis-Tris, pH 7.0) buffers was employed in the current CN-PAGE. Electrophoresis was carried out at a constant voltage 50 V for 30 min and then at a constant current 15 mA for 3 h till the Coomassie G-250 dye reached the end of the gel. After electrophoresis, proteins were transferred onto PVDF membrane at a constant voltage 100 V for 2 h. The blocking of PVDF membranes and the incubation with specific antibodies were performed as described above in SDS-PAGE. Each experiment was carried out at least in triplicate and one representative blot was chosen for illustration.

4.12. CN/SDS-PAGE

After the first dimension CN-PAGE, the gel strips were cut and incubated with 1% dithiotreitol (DTT) in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 0.1 mM EDTA, 2% SDS, 30% glycerol, 0.01% bromphenol blue) for 15 min and subsequently with 2.5% iodoacetamide (IAA) in equilibration buffer for 15 min. The strips were placed onto denaturating gels (4% stacking and 10% separating gel). The electrophoresis was performed in Laemmli running buffer (25 mM Tris, 192 mM glycin, 1% (w/v) SDS) at a constant voltage 200 V at room temperature. The separated proteins were transferred onto nitrocellulose membrane and probed with specific primary antibodies. Western blots for each detergent and antibody were replicated at least three times, one representative blot was chosen. The density of some selected spots was quantified by ImageQuant^{TM} TL software (GE Healthcare, Chalfont St. Giles, UK).

4.13. Autoradiography

Two hundreds μg of postnuclear supernatant from control HEK293-E2M11 cells were incubated with Mix buffer (final concentration 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl) and with or without 10 μM TRH and 10 nM [^{35}S]GTPγS (specific activity 1000 Ci/mmol) for 30 min at 20 °C in a total volume of 50 μl. The reaction was terminated by cooling of samples at 0 °C for 10 min. The samples were solubilised 1% lauryl maltoside for 30 min at 0°C and resolved by CN-PAGE

at 6-15 % non-denaturating linear polyacrylamide gels. Gels were fixed twice in fixation solution (50 % methanol, 10 % acetic acid) for 10 min, dried for 100 min at 60°C and put into cassette with foil for rise of intensity. The exposition on Kodak MXB film was performed for 5 days at -80 °C.

4.14. siRNA inhibition of specific proteins

E2M11 cells were plated in 6-well plates at 30% confluence 24 h before transfection. Eighty picomoles of each siRNA against specific protein or 160 picomoles of control siRNA were mixed with 5 µl of Lipofectamine™ RNAiMAX in 500 µl of DMEM for 30 min at room temperature to produce liposome-enclosed nucleotides, which were then added to each well containing 2 ml of serum-free medium. After 24-h incubation, cells were grown in serum-supplemented DMEM for next 48 h.

4.15. Immunoprecipitation

E2M11 cells (one 80 cm^2 cell culture flasks per sample) were lysed in 5 ml of NP buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 20 mM NaF, 2 mM Na_3VO_5, 10% glycerol, 1 % Nonidet P-40 (NP-40); pH 8.0) supplemented with Complete Protease Inhibitor Cocktail for 1 h on ice. Cell debris were removed by centrifugation at 10,000 rpm and 4 °C for 10 min. Fifty µl of Dynabeads Protein G per sample was incubated with 10 µl of primary antibody against $G_{q/11}\alpha$ protein or TRH-R for 1 h at room temperature using the tube rotator (10 rpm). Beads were cross-linked to primary antibodies using 5 mM BS^3 in 250 µl conjugation buffer (20 mM sodium phosphate, 150 mM NaCl; pH 7.0) for 30 min at room temperature. The cross-linking reaction was quenched by adding 12.5 µl of quenching buffer (1 M Tris-HCl; pH 7.5) and rotating on the tube rotator. After washing with 200 µl of PBS-T buffer (137 mM NaCl, 2.7 mM KCl, 2.8 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.02% Tween 20; pH 7.4), 500 µl of lysate was incubated with antibody-coated beads for 1 h using the tube rotator. After brief washing with 200 µl of PBS, the bound proteins were eluted with 20 µl of 50 mM glycine, pH 2.8 for 5 min using the tube rotator. The final eluate was mixed with appropriate amount of 4-times concentrated Laemmli sample buffer (0.25 M Tris-HCl, 8% SDS, 40% glycerol, 0.04% bromphenol blue, 0.4 M dithiotreitol; pH 6.8), boiled at 90 °C for 3 min and the proteins were separated by SDS-PAGE using standard 10% polyacrylamide gels. After blotting onto nitrocellulose membrane and probing with specific antibodies, the $G_{q/11}\alpha$ and Gβ proteins were visualized by ECL. No bands were detected when incubating Dynabeads and antibodies without cell lysates or Dynabeads and cell lysates without antibodies (negative controls).

5. Results

5.1. A proteomic study on the TRH effects

5.1.1. Characterization of fractions obtained from Percoll^R density gradient

For observation of protein alterations in cells after hormone exposure, the postnuclear supernatant from HEK293-E2M11 cell line was fractionated on Percoll^R density gradient. The cytosolic fraction on the top of the gradient and two membrane fractions were isolated (Fig. 5.1A). To find out which fraction contains the higher portion of the plasma membranes, the samples of PNS and both membrane fractions were separated by SDS-PAGE, transferred on nitrocellulose membrane and incubated with antibody against Na^+,K^+-ATPase, which was used as a prototypical marker of the plasma membrane. As shown at Fig. 5.1B, the immunoblot signal was significantly higher in the upper layer (Fraction 1) compared to the sample of PNS, while it was slightly decreased in the lower layer (Fraction 2) than in PNS. These findings suggest that the upper layer is highly enriched with plasma membranes and is suitable for observation of membrane-bound proteins.

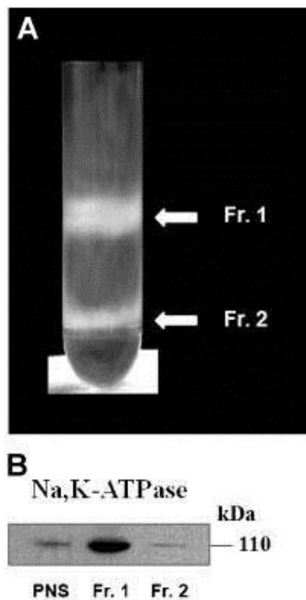


Fig. 5.1

Separation of postnuclear supernatant on the Percoll^R density gradient. The postnuclear supernatant from HEK293-E2M11 cell line was centrifuged on the 30% Percoll^R density gradient in a Beckman Ti70 rotor at 65,000 x g for 30 min at 4 °C (A). A cytosolic fraction from the top of gradient was centrifuged at 300,000 x g for 1 h. Two distinct membrane layers were collected and centrifuged at 150,000 x g for 1 h. The enrichment of fractions with plasma membranes was proved by separation of

proteins from PNS and particular fractions by SDS-PAGE, Western blotting and incubation with specific antibody against Na^+,K^+-ATPase (B).

In order to assess fractionation efficiency, it was of interest to evaluate the levels of Na^+,K^+-ATPase as a marker of the plasma membrane and lactate dehydrogenase B (LDH) as a marker of the cytosol in the PM-enriched fraction and the cytosolic fraction. Using SDS-PAGE and immunolotting, it was found that the level of Na^+,K^+-ATPase was markedly increased in the PM-enriched fraction compared to PNS but no immunoblot signal was observed in the cytosol (Fig. 5.2). On the other hand, the immunoblot signal of LDH was slightly increased in the cytosol compared to the sample of PNS and no signal was in the cytosol (Fig. 5.2). Hence, the fractionation on the Percoll^R density gradient was found to be suitable for separation of membrane-bound proteins from soluble proteins and this approach has been applied in next experiments.

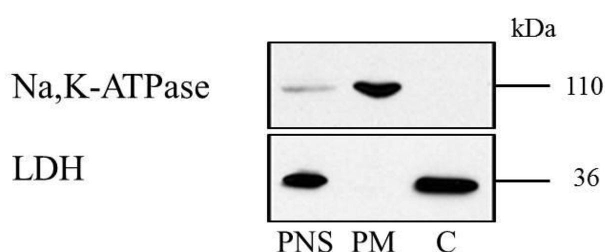


Fig. 5.2

Assessment of separation of the PM-enriched fraction and cytosol. The samples of the PNS, PM-enriched fraction and cytosol were separated by SDS-PAGE. After Western blotting, nitrocellulose membranes were incubated with antibodies against Na,K-ATPase and lactate dehydrogenase.

5.1.2. Characterization of TRH receptor and $G_{q/11}\alpha$ protein in the postnuclear supernatant and PM-enriched fraction

The TRH-R distribution in samples of the PNS and PM-enriched fraction were measured by analysis of [^{3}H]TRH/TRH displacement curves according to the method of DeBlasi *et al.* (DeBlasi *et al.*, 1989). The affinity of [^{3}H]TRH binding to TRH binding sites was similar in both fractions (K_D = 21.4 ± 4.5 nM in the PNS, K_D = 28.9 ± 6.2 nM in the PM-enriched fraction) whereas the number of TRH binding sites was significantly increased in the PM-enriched fraction compared to the PNS (B_{max} = 2.2 ± 0.8 pmol/mg protein in the PNS, B_{max} = 11.3 ± 1.5 pmol/mg protein in the PM-enriched fraction) (Fig. 5.3A).

The functional activity of G-proteins in the PNS and PM-enriched fraction was measured by [^{35}S]GTPγS binding assay. The assessment of basal and TRH stimulated [^{35}S]GTPγS bindings in both samples demonstrated that the sample of the PM-enriched fraction exerted almost 10 times higher

basal as well as TRH (1.10^{-5} M)-stimulated [^{35}S]GTPγS binding than the sample of the PNS (Fig. 5.3B).

The higher concentrations of TRH (10^{-4}–10^{-6} M) doubled the value of [^{35}S]GTPγS binding in control samples of both the PNS and PM-enriched fraction. However, the rise of [^{35}S]GTPγS binding was more noticeable and significant in the PM-enriched fraction than in the PNS. Next, [^{35}S]GTPγS binding was also assessed in samples from TRH-treated cells (16 h). [^{35}S]GTPγS binding was diminished in TRH-treated cells compared to control samples and this diminution was higher in the PM-enriched fraction than in the PNS (Fig. 5.3C).

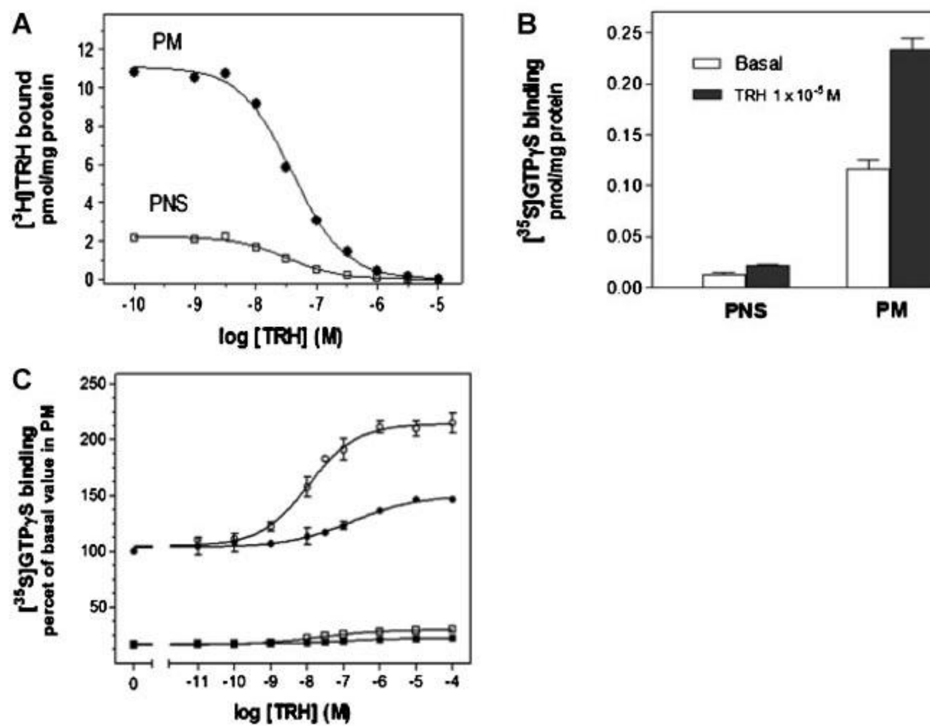


Fig. 5.3

[^{3}H]TRH and [^{3}H]GTPγS binding assays in the PNS and PM-enriched fraction. The TRH distribution was compared in samples of the PNS and PM-enriched fraction using [^{3}H]TRH/TRH competition binding experiments which were performed in triplicates and calculated according to DeBlasi *et al.* (DeBlasi *et al.*, 1989) (A). [^{35}S]GTPγS binding assay was carried out as a one-point assay with 5 nM [^{35}S]GTPγS and 2 mM GDP in samples of PNS and the PM-enriched fraction from control cells. Open and solid bars represent basal and TRH-stimulated [^{35}S]GTPγS binding (B). Samples of PNS (squares) and the PM-enriched fraction (circles) isolated from control cells (open symboles) or TRH stimulated cells (16 h, 10^{-4}–10^{-11} M; closed symboles) were used for assessment of [^{35}S]GTPγS binding in the presence of 2 mM GDP. The [^{35}S]GTPγS binding curves are expressed as a percentage of maximum binding in the absence of TRH (100%) (C).

5.1.3. Down-regulation and solubilisation of $G_{q/11}\alpha$ proteins

In order to determine down-regulation and solubilisation of $G_{q/11}\alpha$ protein after TRH treatment, the samples of the PNS, plasma membrane-enriched fraction and cytosol from control and TRH-treated (1.10^{-5} M; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were resolved by SDS-PAGE, transeferred on nitrocellulose membranes and incubated with specific antibodies against $G_{q/11}\alpha$ protein. The prolonged TRH treatment (4–16 h) resulted in down-regulation of $G_{q/11}\alpha$ protein (Fig. 5.4A) as well as its translocation from membranes to cytosol (Fig. 5.4B).

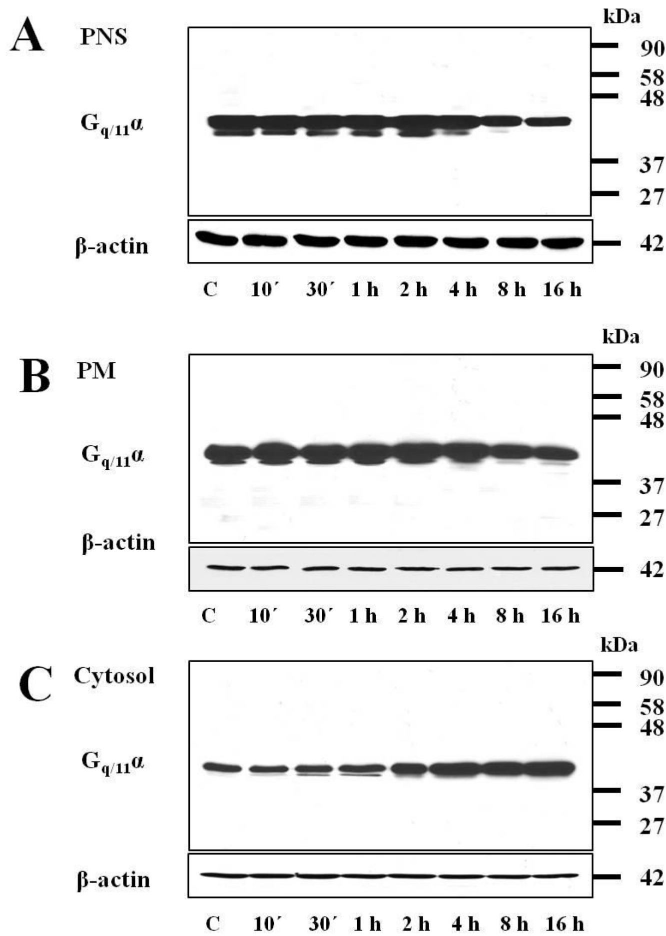


Fig. 5.4

Determination of down-regulation and solubilisation of $G_{q/11}\alpha$ protein after TRH treatment. The samples of the postnuclear supernatant (A) or PM-enriched fraction and cytosol (B) from control and TRH-treated (10 μM; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were resolved by SDS-PAGE. After electrotransfer on nitrocellulose membrane, $G_{q/11}\alpha$ proteins were detected by specific antibodies against $G_{q/11}\alpha$ proteins.

5.1.4. Comparison of protein composition of the postnuclear supernatant and PM-enriched fraction

In order to determine the extent of similarity of samples of the PNS and PM-enriched fraction, proteins of these two samples were separated by 2D electrophoresis. The proteins were resolved by isoelectric focusing on linear IPG strips (pH 4-7) in the first dimension and subsequently by SDS-PAGE on 10% polyacrylamide gels in the second dimension. The gels were stained with the fluorescence dye Sypro Ruby. Sixteen pairs of gels were evaluated using PDQuest software. Two hundred spots were found to be in both samples while other 229 spots were present only in the PNS and other 166 spots only in the PM-enriched fraction (Fig. 5.5). It can be supposed that the spots found only in samples of the PNS represent soluble proteins which were isolated in the cytosolic fraction. These results suggest that fractionation on the Percoll^R density gradient is useful for detection of proteins which are less abundant in cells.

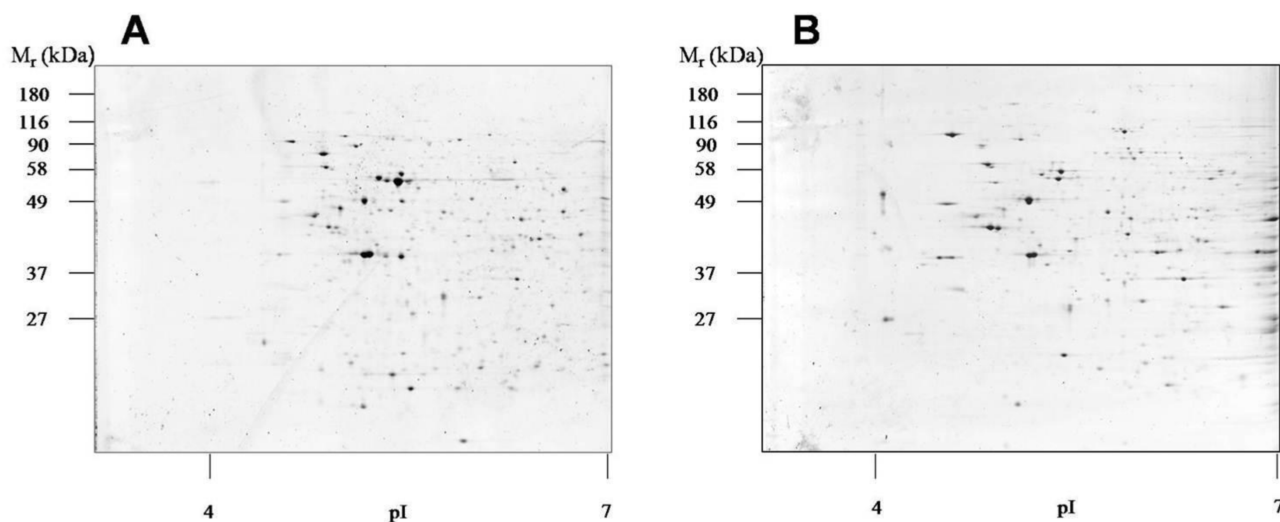


Fig. 5.5

Comparison of major protein composition of 2D representative maps of the PNS (A) and PM-enriched fraction (B) whose samples were separated on linear IPG strips (pH 4-7) in the first dimension and by SDS-PAGE on 10% polyacrylamide gels in the second dimension. The proteins in gels were stained with the fluorescence dye Sypro Ruby.

5.1.5. Effect of long-term TRH treatment on protein levels in the PM-enriched fraction

Proteins in the PM-enriched fraction of control and TRH-treated (1.10^{-5} M, 16 h) cells were resolved by 2D electrophoresis and the stained spots analysed by PDQuest software. In sample of TRH-treated cells, 4 proteins were found to disappear, the levels of 18 proteins decreased and the levels of 39 proteins increased. Forty-two proteins out of 61 observed altered proteins were identified by mass spectrometry. The final master gel with depicted identified proteins is shown in Fig. 5.6 and the numbers assigned to proteins in master gel correspond to the numbers of proteins in Table 1. It is

noteworthy that only four altered proteins were detected by analysis of the PNS of control and TRH-treated (1.10^{-5} M, 16 h) cells (data not shown). Analysis of the lower fraction from the Percoll[R] density gradient revealed that protein composition of this fraction is very similar to protein composition of the PM-enriched fraction. Hence, the lower fraction is likely to contain cell fragments with higher buoyant density derived from compartments such as mitochondria, Golgi apparatus or endoplasmic reticulum but no fragments of the plasma membranes, whereas the PM-enriched fraction apparently contains cell fragments from all membrane compartments.

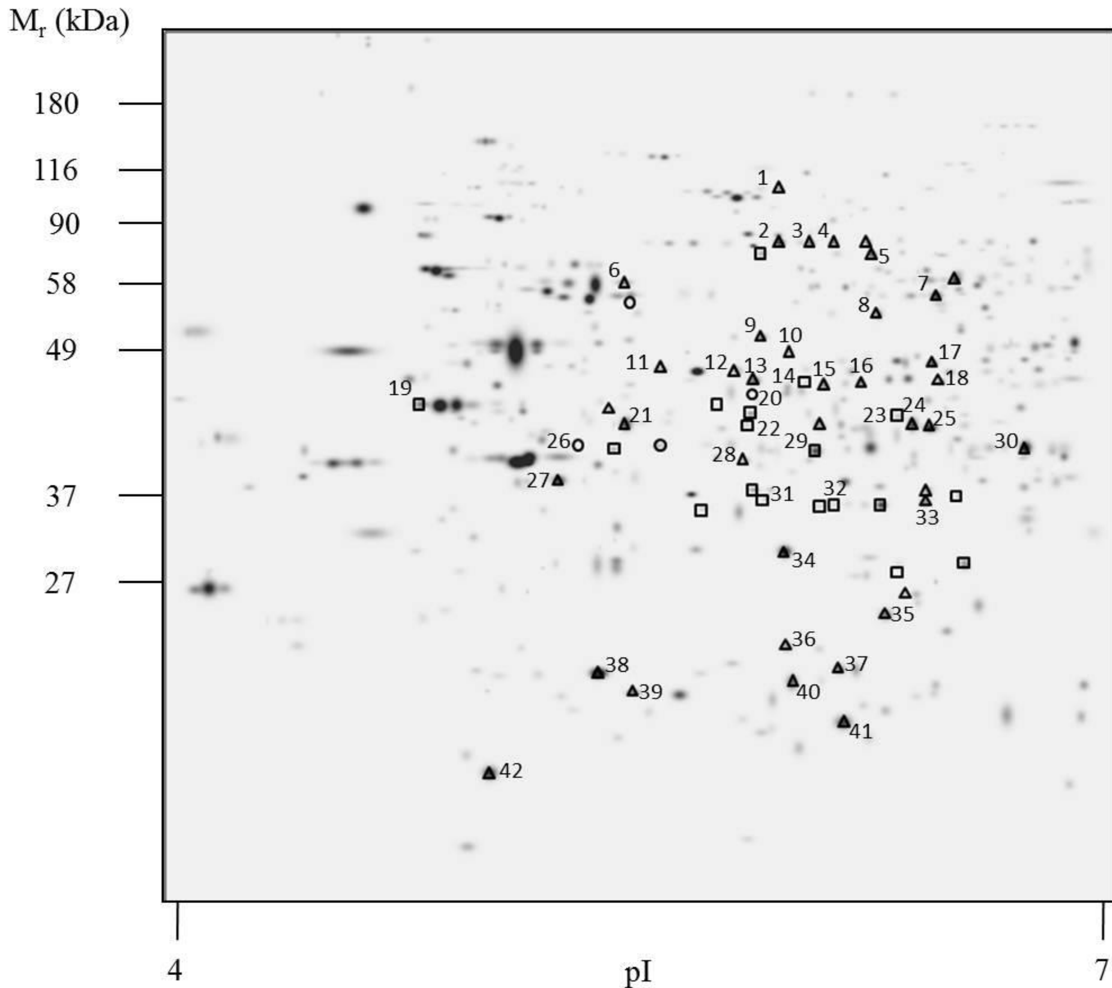


Fig. 5.6

Effect of long-term TRH treatment on protein levels in the PM-enriched fraction. Master gel of major proteins in the PM-enriched fraction of HEK293-E2M11 was derived from 16 gel replicas. The qualitative differences in protein expression found in 2D maps of samples from control and TRH-treated cells are marked by circles, the quantitative alterations representing down-regulation or up-regulation of proteins after TRH treatment are marked by squares or triangles, respectively.

Table 1

List of proteins identified by mass spectrometry in the PM-enriched fraction of HEK293-E2M11 which were altered after long-term (16 h) treatment with TRH. The spot numbers correspond to protein numbers in Master gel shown in Fig. 5.5. The increased or decreased expression levels of proteins are denoted by upward (↑) or downward (↓) arrows, respectively. The numbers in parentheses reflect the fold change is proteins expression caused by TRH.

Spot	Accession no.	Protein name	Change (fold)
1	gi|2274968	Glucosidase II	↑ (2.0)
2	gi|516764	Motor protein	↑ (2.2)
3	gi|48145703	Inner membrane mitochondrial protein (mitofilin)	↑ (3.0)
4	gi|62414289	Vimentin	↑ (2.7)
5	gi|46249758	Ezrin	↑ (2.0)
6	gi|292059	MTHSP75	↑ (2.7)
7	gi|156416003	Succinate dehydrogenase complex, subunit A, flavoprotein precursor	↑ (2.5)
8	gi|4506753	RuvB-like 1	↑ (4.6)
9	gi|36796	T-complex polypeptide 1	↑ (2.4)
10	gi|403456	26S protease (S4) regulatory subunit	↑ (2.6)
11	gi|860986	Protein disulfide isomerase	↑ (2.2)
12	gi|303618	Protein disulfide isomerase A3	↑ (2.0)
13	gi|6137677	Chain A, human mitochondrial aldehyde dehydrogenase complexed with NAD^+ and Mn^{2+}	↑ (3.4)
14	gi|1710279	Dihydrolipoamide acetyl transferase	↓ (5.2)
15	gi|18605506	PMPCA protein	↑ (2.2)
16	gi|5453603	Chaperonin containing TCP1, subunit 2	↑ (2.0)
17	gi|7657381	PRP19/PSO4 pre-mRNA processing factor 19 homolog	↑ (2.3)
18	gi|5771523	3-Phosphoglycerate dehydrogenase	↑ (2.2)
19	gi|32189394	ATP synthase, H^+ transporting, mitochondrial F1 complex, beta subunit precursor	↓ (2.5)
20	gi|14124984	Chaperonin containing TCP1, subunit 3	↓ (4.5)
21	gi|46593007	Ubiquinol-cytochrome c reductase core protein I	↑ (2.0)
22	gi|5031753	Heterogeneous nuclear ribonucleoprotein H1	↓ (3.8)
23	gi|31543831	Tubulin, gamma 1	↓ (5.0)
24	gi|10835067	Autoantigen La	↑ (3.7)
25	gi|2697005	Cell cycle protein p38-2G4 homolog	↑ (2.7)
26	gi|30311	Cytokeratin 18	↓ (7.1)
27	gi|7305503	Stomatin (EPB72)-like 2	↑ (2.1)
28	gi|386854	Type II keratin subunit protein	↑ (2.5)
29	gi|39644794	EEF1G protein	↓ (2.2)
30	gi|1706611	Elongation factor Tu, mitochondrial precursor	↑ (2.4)
31	gi|181969	Elongation factor 2	↓ (2.5)
32	gi|11935049	Keratin 1	↓ (5.0)
33	gi|9910244	Mitochondrial ribosomal protein S22	↑ (2.0)
34	gi|12654583	Ribosomal protein, large, P0	↑ (3.0)
35	gi|16924265	Enoyl coenzyme A hydratase 1, peroxisomal	↑ (2.0)
36	gi|110349780	ATP synthase mitochondrial F1 complex assembly factor 1 isoform 1 precursor	↑ (2.0)
37	gi|5803013	Endoplasmic reticulum protein 29 isoform 1 precursor	↑ (2.8)
38	gi|4505773	Prohibitin	↑ (2.2)
39	gi|4758788	NADH dehydrogenase (ubiquinone) Fe-S protein 3	↑ (2.4)
40	gi|12707570	Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor	↑ (2.5)
41	gi|32483377	Peroxiredoxin III isoform b	↑ (2.7)
42	gi|51479152	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit d, isoform b	↑ (2.0)

Many of the herein identified proteins fall into structural or functional protein families whose members are often detected by 2D electrophoresis. These families include keratins, annexins, peroxiredoxines, actins, enolases, protein disulphide isomerases (PDIs), tubulins, vimentins, proteasome subunits, heterogeneous ribonucleoprotein particle subunits or elongation factors (Petrak *et al.*, 2008). Frequent identification of these proteins in different cell types and under different experimental conditions is likely given by technical limitations (limited sensitivity) of 2D electrophoresis. Changes found in the expression of these proteins after cell stimulation can apparently correspond to the common cellular stress responses.

The localization of the identified proteins differs from the plasma membrane to the nucleus suggesting that the PM-enriched fraction contains cell fragments with lower buoyant density from all membrane compartments and the cytoskeleton. Sixteen altered proteins were from the mitochondria, 11 proteins from cytoskeleton and 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 (Drastichova *et al.*, 2010).

5.1.6. Effect of long-term TRH treatment on the levels of selected proteins and apoptotic markers

Some proteins identified by 2DE/MS represent proteins with anti-apoptotic and protective effects and their levels after TRH treatment were increased. To verify these findings, the samples of the postnuclear supernatant from control and TRH-treated (1.10^{-5} M, 16 h) cells were resolved by SDS-PAGE and peroxiredoxin III, prohibitin and MTHSP75 were detected by immunoblotting with specific antibodies (Fig. 5.7A). The immunoblot signals of all three proteins were increased several times in TRH-treated samples compared to the corresponding controls. These observations support the results obtained by 2DE/MS and suggest that TRH can exert cytoprotective effects.

Next, the potential anti-apoptotic effect of TRH was assessed by evaluation of the caspase-3 activity and the Bcl-2/Bax ratio. Samples of the postnuclear supernatant from control and TRH-treated (1.10^{-5} M, 16 h) cells as well as control and TRH-treated (1.10^{-5} M, 12 h) cells subsequently incubated with camptothecin (1.10^{-6} M, 4 h), a drug causing DNA damage and subsequent apoptosis, were resolved by SDS-PAGE, transferred on nitrocellulose membranes and incubated with specific antibodies against caspase-3, PARP (poly(ADP-ribose)polymerase), Bcl-2 and Bax (Fig. 5.7B). Whereas the levels of pro-apoptotic caspase-3 in both TRH-treated samples were decreased, the levels of PARP, an enzyme engaged in repair of DNA, were roughly the same in these and corresponding control samples. These results suggest that TRH treatment caused down-regulation of caspase-3 because the uncleaved form of PARP, which is cleaved by the active form of caspase-3, was not changed in both TRH-treated cell samples.

The effect of TRH was also determined by evaluation of the Bcl-2/Bax ratio, which was assessed as a ratio of the intensities of Bcl-2 and Bax immunoblot signals in particular samples. The Bcl-2/Bax ratio in control sample was 1. This ratio in TRH-treated sample, camptothecin-treated sample and TRH/camptothecin sample were 3.9, 0.6 and 1.6, respectively.

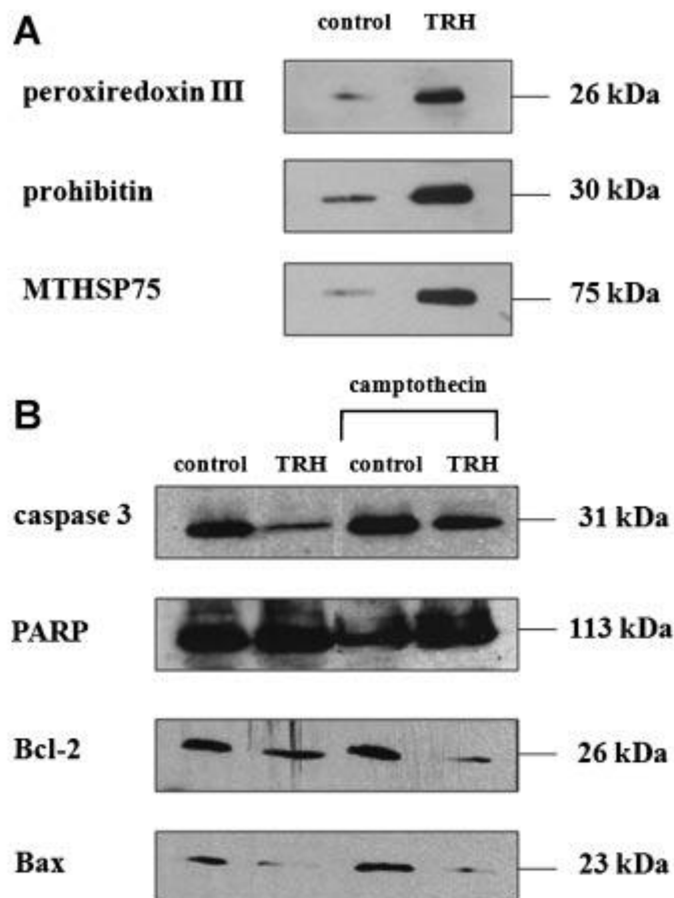


Fig. 5.7

Effect of long-term TRH treatment on the levels of selected apoptotic markers. The levels of the anti-apoptotic and protective proteins identified by 2DE/MS were verified by SDS-PAGE and immunoblotting with specific antibodies. The immunoblot signals of peroxiredoxin III, prohibitin and MTHSP75 in control and TRH-treated (1.10^{-5} M, 16 h) corroborated their increased levels after TRH treatment (A). The levels of caspase-3, PARP, Bcl-2 and Bax were observed in control and TRH-treated cells (1.10^{-5} M, 16 h) and the control and TRH-treated (1.10^{-5} M, 12 h) subsequently incubated with campthotecin (1.10^{-6} M, 4 h). The levels of these apoptotic markers were evaluated by SDS-PAGE and immunoblotting (B).

5.2. Exploring molecular complexes of TRH-R1 and $G_{q/11}$ proteins

5.2.1. Determination of GPCRs and G-proteins by SDS-PAGE

Because native electrophoresis with subsequent immunoblotting requires highly specific antibodies, we checked the quality of selected antibodies against $G_{q/11}\alpha$ proteins, $G_{i1,2}\alpha$ protein, Gβ protein, TRH-R1, α_{1B}-adrenergic receptor and α_{2A}-adrenergic receptor by SDS-PAGE and the specific blocking peptides provided that they were available (Fig. 5.8). Two bands were detected using antibodies against $G_{q/11}\alpha$ protein. The major band (43 kDa) corresponds to $G_{11}\alpha$ while the minor one (40 kDa) to $G_q\alpha$. Only one band was observed by immunoblotting with the $G_{11}\alpha$ antibody which proved that the band with lower mobility in the gel is $G_{11}\alpha$. These observations are in line with previous reports testifying the mobility of $G_{11}\alpha$ and $G_q\alpha$ proteins in polyacrylamide gels (Blank *et al.*, 1991; Mullaney *et al.*, 1993).

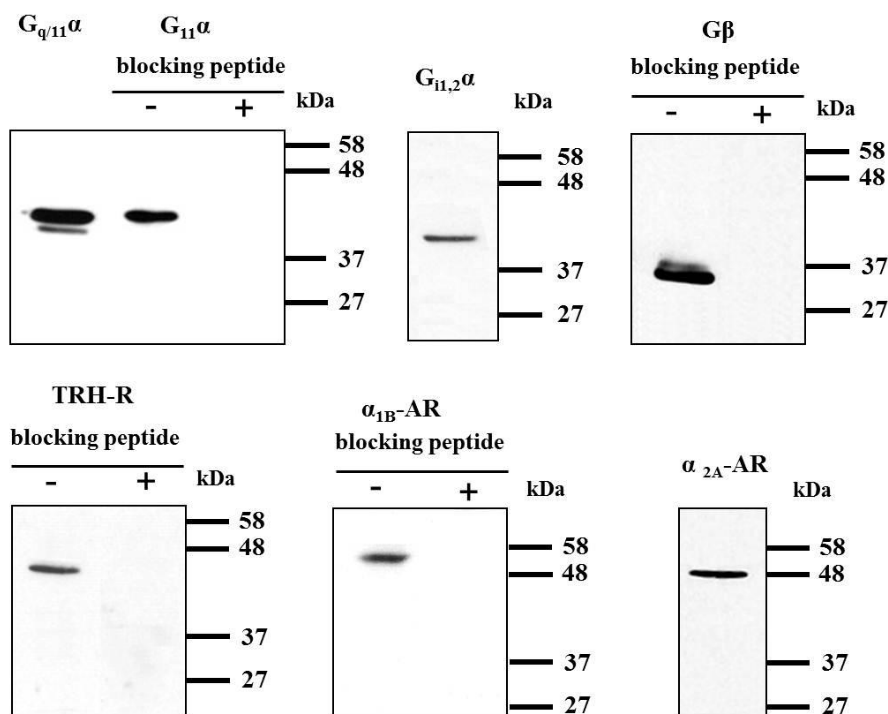


Fig. 5.8

Determination of selected GPCRs and G-protein subunits by SDS-PAGE and immunoblotting. Fifty μg of the postnuclear supernatant from control HEK293-E2M11 cells were resolved on 10% polyacrylamide gels by SDS-PAGE. After transfer on nitrocellulose membrane, proteins were detected by specific antibodies. If relevant blocking peptide was available, the specificity of antibody was verified by incubation with a synthetic peptide (0.5 μg/ml) corresponding to the protein epitope recognized by appropriate antibody. Two distinct bands were detected by incubation with the $G_{q/11}\alpha$ protein antibody. The upper band corresponds to $G_{11}\alpha$ and the lower one to $G_q\alpha$.

5.2.2. Expression of TRH-R1 and $G_{11}\alpha$ protein

The alteration of TRH-R1 expression induced by exposure of cells to 10 μM TRH at different time intervals was evaluated in samples of the PNS by SDS-PAGE and immunoblotting (Fig. 5.9A). The decrease of TRH-R level observed after 1 h TRH treatment and its subsequent increase after prolonged hormone treatment (4-16 h) is in agreement with the previously reported study of Cook and co-workers (Cook and Hinkle, 2004).

To compare the expression of TRH-R1 and $G_{q/11}\alpha$ protein in HEK293-E2M11 cells to their endogenous expression in tissue, 50 μg of the postnuclear supernatants from rat brain cortex and HEK293-E2M11 cells were separated by SDS-PAGE and immunoblotted (Fig. 5.9B). The expression level of TRH-R1 was roughly the same in both samples while the level of $G_{q/11}\alpha$ protein was slightly higher in sample of HEK293-E2M11 cells compared to brain cortex. The overexpression of proteins in transfected systems can significantly affect the formation of protein complexes which are absent in cells with endogenous protein expression. Nevertheless, neither of these proteins was excessively overexpressed in HEK293-E2M11 cells and overexpression artefacts could be excluded.

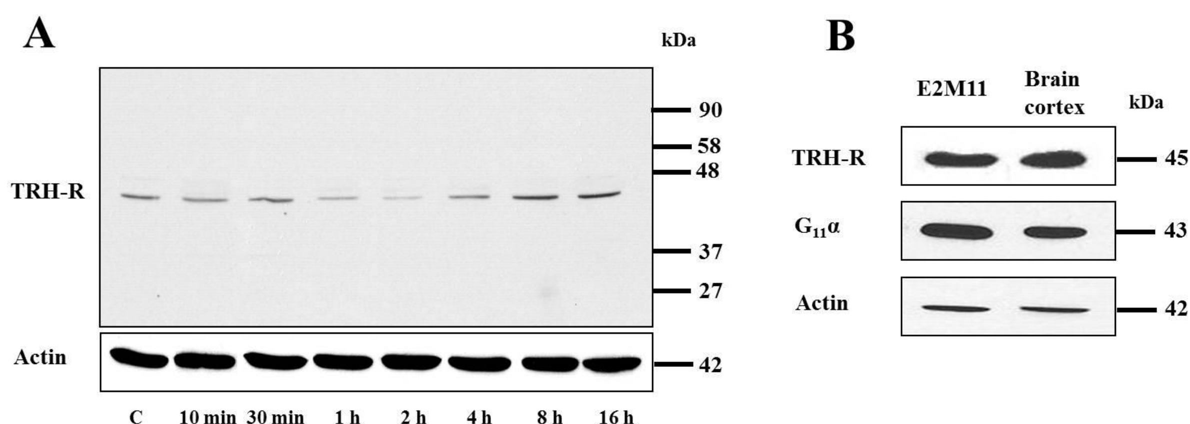


Fig. 5.9
Determination of the expression of TRH-R1 and $G_{11}\alpha$ protein. The effect of 10 μM TRH on the expression of TRH-R1 was assessed in the PNS by SDS-PAGE and immunoblotting at different time intervals (A). The expression of TRH-R1 and $G_{11}\alpha$ protein was compared in samples of postnuclear supernatants from HEK293-E2M11 cell line and rat brain cortex (B). In both experiments, β-actin was used as a loading control.

5.2.3. Identification of TRH-R and $G_{q/11}$ protein complexes by CN-PAGE after detergent solubilisation

Samples of the postnuclear supernatant from control HEK293-E2M11 cell line were solubilised with detergents digitonin, lauryl maltoside (LM), Brij 56, Triton X-100, CHAPS and SDS

at different concentration for 30 min. The solubilised protein complexes were separated on 6-15% linear non-denaturating polyacrylamide gels by CN-PAGE. After immunoblotting and incubation with TRH-R1 antibodies, two distinct protein complexes involving TRH-R1 were detected in the areas of 140 kDa and 80 kDa (Fig. 5.10A). Immunoblot density of the complex with lower mobility, which was detectable with all applied detergents, was markedly higher than the immunoblot density of the complex found about 80 kDa. The complexes of $G_{q/11}\alpha$ and Gβ proteins which were detectable only with detergent lauryl maltoside were found in the 140 kDa region (Fig. 5.10B,C). The increased concentration of LM caused the separation of two $G_{q/11}\alpha$ protein complexes in the 140 kDa region. The identified signals of TRH-R1, $G_{q/11}\alpha$ and Gβ proteins in the area of 140 kDa suggest that these proteins could form a presumed TRH-R1-$G_{q/11}$ protein complex in control HEK293-E2M11 cells.

As shown at Fig. 5.10, the signal intensity of protein complexes was significantly affected by the character of applied detergent as well as their concentration, which can be connected with different efficiency of individual detergents. Detergents may also influence the mobility of complexes in the gel. The shift in mobility of amphiphilic molecules induced by application of detergents and Coomassie G-250 was described previously (Helenius and Simons, 1977; Wittig *et al.*, 2007). Based on these preliminary experiments, the optimal concentration of detergents used for solubilisation was 1% for LM, Brij 56, Triton X-100, CHAPS and digitonin and 0.05% for SDS.

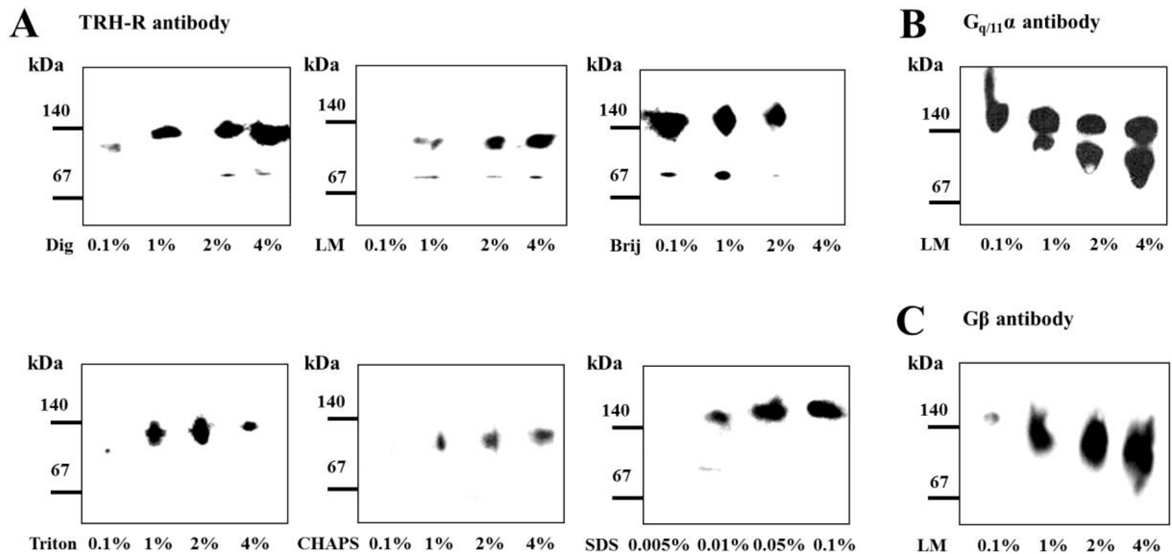


Fig. 5.10

Detection of protein complexes of TRH-R or $G_{q/11}$ protein. Samples of the postnuclear supernatant from HEK293-E2M11 cell line were solubilised with detergents digitonin (Dig), lauryl maltoside (LM), Brij, Triton X-100, CHAPS and SDS in sample buffer at different concentrations. The solubilised protein complexes were resolved on 6-15% linear gradient polyacrylamide gels under non-denaturating conditions and immunoblotted with specific antibodies. Two distinct complexes of TRH-

R were detected in the areas of 140 kDa and 80 kDa (A). Protein complexes of $G_{q/11}\alpha$ and Gβ proteins were detected only in the area of 140 kDa (B, C).

5.2.4. Effect of temperature on the stability of TRH-R1 and $G_{q/11}\alpha$ protein complexes

TRH receptor is able to form dimers and substantial portion of them is glycosylated at their N-terminus (Zhu *et al.*, 2002). The dimers of glycosylated TRH receptors were observed at molecular size of 150 kDa and were stable at increased temperature (37 °C). Deglycosylation of these dimers by PNGase F resulted in decrease of their molecular size (Zhu *et al.*, 2002). Therefore, it was necessary to find out whether the TRH-R1 complex detected in the region about 140 kDa could be a dimer of glycosylated TRH receptor.

Protein deglycosylation by PNGase F requires incubation of samples at increased temperature causing protein denaturation and dissociation of protein complexes. Hence, the deglycosylation experiments were not suitable for our study. Nevertheless, it was of interest to check the stability of the observed complexes at increased temperature. The sample solubilisation performed at 37 °C for 30 min resulted in disappearance of TRH-R1 and $G_{q/11}\alpha$ signals in the region of 140 kDa and appearance of TRH-R1 and $G_{q/11}\alpha$ signals at molecular size about 80 kDa and 45 kDa, respectively (Fig. 5.11). Thus, it can be concluded that the TRH-R1 complex found about 140 kDa does not correspond to the glycosylated TRH-R1 dimer because the stability of this complex would not be influenced by solubilisation at increased temperature.

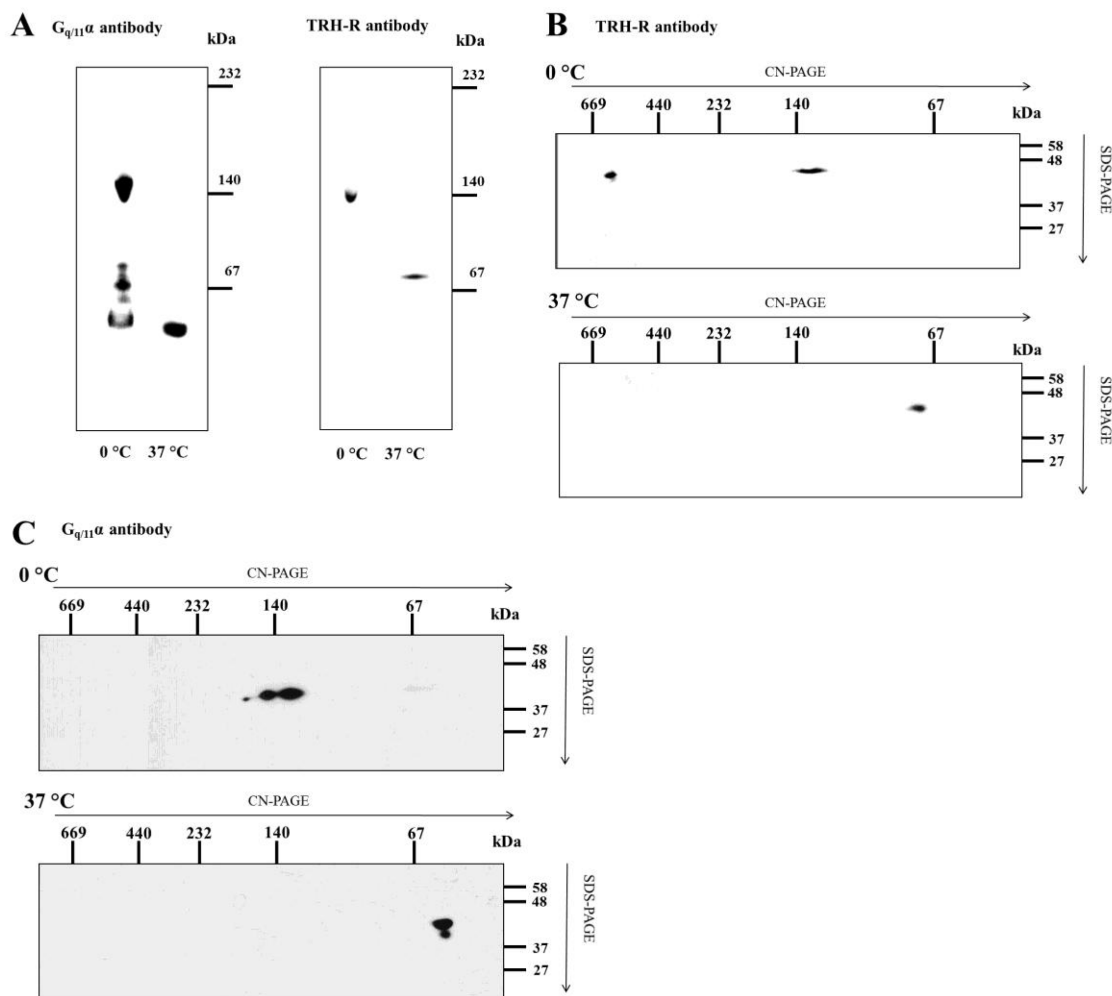


Fig. 5.11

Effect of temperature on the stability of protein complexes in the 140 kDa region. Samples of the postnuclear supernatant from control cells were solubilised with 1% LM in sample buffer at 0 °C or 37 °C for 30 min. The solubilised protein complexes were resolved on 6-15% linear gels by CN-PAGE and immunoblotted with specific TRH-R1 or $G_{q/11}\alpha$ antibodies (A). The same samples were used for detection of these complexes by CN/SDS-PAGE (B, C).

5.2.5. Identification of the TRH-R interacting G-protein subunits by co-immunoprecipitation

In order to verify the interaction between the TRH-R and $G_{q/11}$ protein in control cells, co-immunoprecipitation was used. Lysate of HEK293-E2M11 cells or NP-40 buffer as a negative control was incubated with Dynabeads Protein G and specific receptor or G-protein antibodies. Afterwords, the eluted proteins from Dynabeads were separated by SDS-PAGE and identified by immunoblotting with antibodies against $G_{q/11}\alpha$ or Gβ. Successful detection of $G_{q/11}\alpha$ after immunoprecipitation with TRH-R antibody along with concomitant identification of the Gβ subunit after immunoprecipitation

with TRH-R or $G_{q/11}\alpha$ antibodies confirmed the presumed interaction among TRH-R, $G_{q/11}\alpha$ a $G\beta$ proteins in control cells (Fig. 5.12).

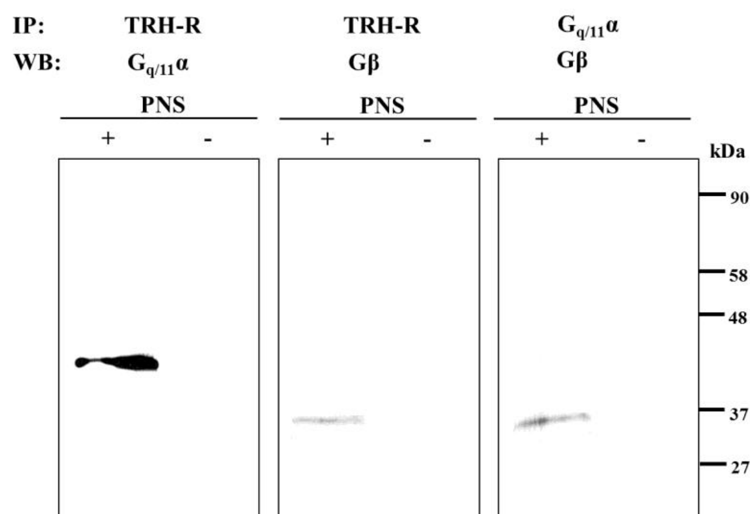


Fig. 5.12

Assessment of the interaction between TRH-R and $G_{q/11}\alpha$ or $G\beta$, and between $G_{q/11}\alpha$ and $G\beta$ in control HEK293-E2M11 cells by co-immunoprecipitation. TRH-R or $G_{q/11}\alpha$ antibodies were bound to Dynabeads Protein G and after 1-h incubation with cell samples, the eluted proteins were resolved on 10% polyacrylamide gels using SDS-PAGE, which was followed by immunoblotting with $G_{q/11}\alpha$ or $G\beta$ antibodies. NP-40 buffer without cell lysate was used as a negative control to ascertain the specificity of the signals detected on immunoblots.

5.2.6. Effect of decreased expression of $G_{q/11}$ protein

The TRH-R1 and $G_{q/11}\alpha$ complexes found in the region of 140 kDa can represent a single complex or be two distinct complexes comprising different interacting partners. To verify whether these proteins are involved in one complex or not experiments with decreased expression of $G_{q/11}\alpha$ or $G\beta$ proteins were performed. For this purpose it was useful to assess and compare the $G_{q/11}\alpha$ levels in HEK293-E2 and HEK293-E2M11 cell lines, because HEK293-E2 cells exogenously expressing the long isoform of TRH-R1 served as a maternal cell line for preparation of HEK293-E2M11 cells (Svoboda *et al.*, 1996).

Samples of the postnuclear supernatant from HEK293-E2 and HEK293-E2M11 cell lines were resolved by SDS-PAGE and the expression of TRH-R and $G_{q/11}\alpha$ protein was evaluated by immunoblotting (Fig. 5.13A). Whereas the level of TRH-R was roughly the same in both samples, the level of $G_{q/11}\alpha$ protein was several times higher in HEK293-E2M11 than in HEK293-E2 cells. Thus, HEK293-E2 cells were suitable for determination whether decreased level of $G_{q/11}\alpha$ protein may affect the formation of the presumed TRH-R1-$G_{q/11}$ protein complex detected in HEK293-E2M11 cells.

Samples of the postnuclear supernatant of both cell lines were resolved by CN/SDS-PAGE. The immunoblot signal of $G_{q/11}\alpha$ in the complex detected in the 140 kDa region was significantly lower in HEK293-E2 than in HEK293-E2M11 cells and similar decrease was observed for TRH-R (Fig. 5.13B). These results suggest that the band with higher gel mobility below the 140 kDa marker corresponds to a single complex containing both TRH-R and $G_{q/11}\alpha$. If this complex was composed of two distinct types of TRH-R and $G_{q/11}\alpha$ complexes, the immunoblot signal of TRH-R must have been similar in samples from both cell lines because the amount of other potential interacting receptor partners did not differ.

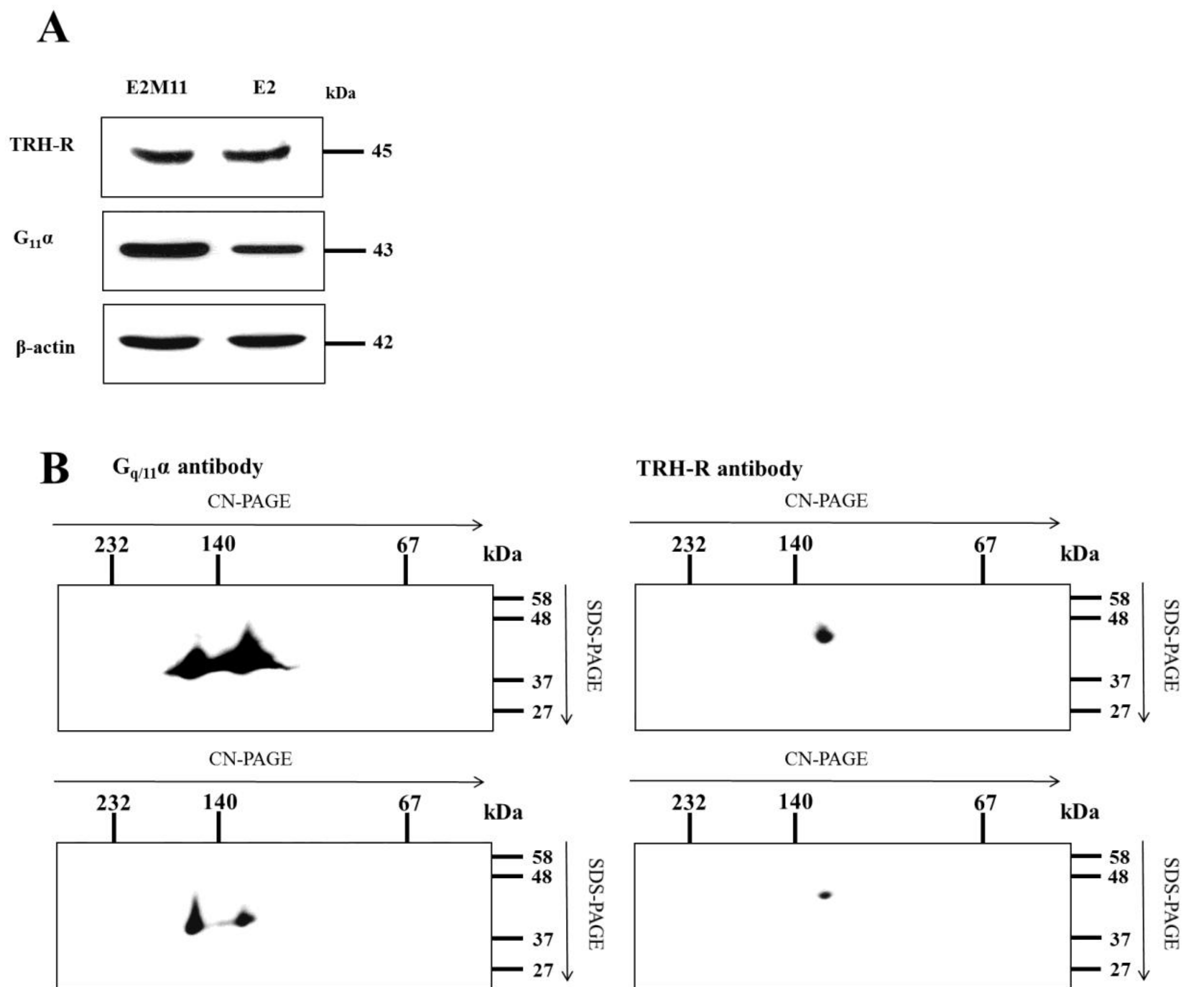


Fig. 5.13

The presence and expression levels of protein complexes of TRH-R1 and $G_{q/11}\alpha$ protein in HEK293-E2M11 and HEK293-E2 cell lines. Fifty µg of PNS from both cell lines were separated on 10% polyacrylamide gels by SDS-PAGE (A) or on 12-15% linear gels by CN-PAGE and subsequently on 10% polyacrylamide gels by SDS-PAGE (B). Proteins were transferred to nitrocellulose membranes which were then incubated with specific antibodies against TRH-R1 or $G_{q/11}\alpha$ proteins.

In order to decrease the expression level of Gβ protein, HEK293-E2M11 cells were transfected with siRNA against $G\beta_1$ and $G\beta_2$ proteins. The successful transfection and subsequent decline in their expression levels were proved by SDS-PAGE and immunoblotting (Fig. 5.14A). The levels of $G\beta_1$ and $G\beta_2$ proteins were several times lower compared to HEK293-E2M11 cells transfected with control siRNA but the expression of $G_{q/11}\alpha$ protein and TRH-R1 was not changed. However, the reduced expression of $G\beta_1$ and $G\beta_2$ proteins noticeably affected the formation of a presumed TRH-R1-$G_{q/11}$ protein complex because the immunoblot signals of $G_{q/11}\alpha$ protein and TRH-R1 after separation by CN/SDS-PAGE were much weaker, similarly as in the case of Gβ protein (Fig. 5.14B).

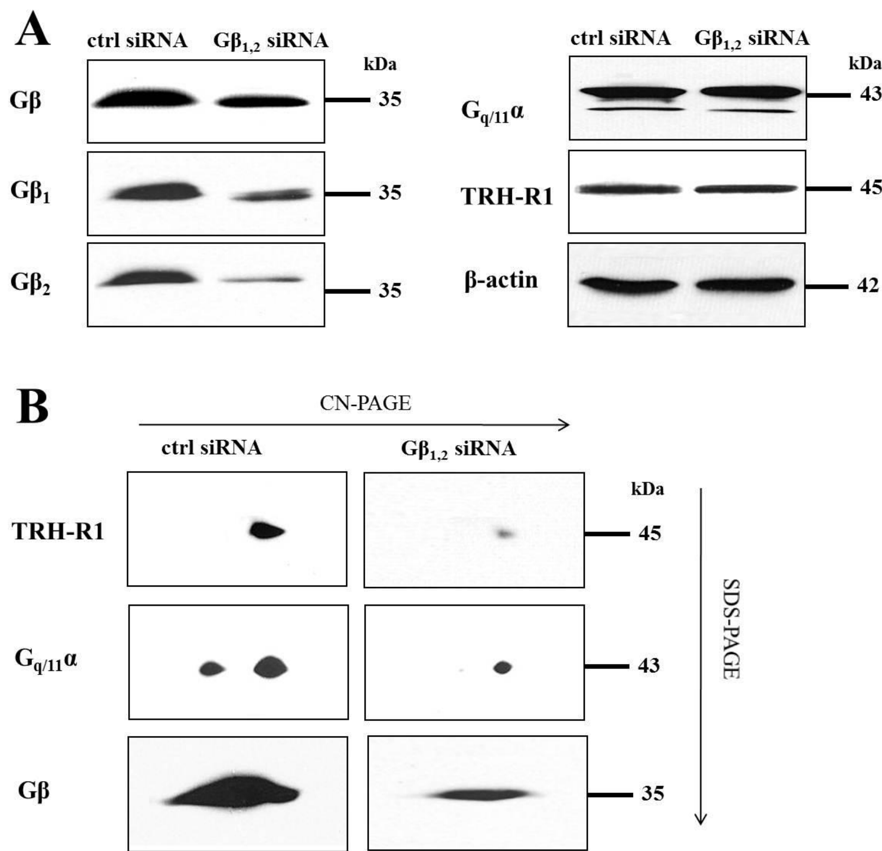


Fig. 5.14

Effect of reduced expression of Gβ on the level of TRH-R1 and $G_{q/11}\alpha$ protein and the stability of the presumed TRH-R1–$G_{q/11}$ protein complex. HEK293-E2M11 cells were transfected with control siRNA (ctrl siRNA; 160 pmol/sample) or siRNA against $G\beta_1$ or $G\beta_2$ proteins (80 pmol of each siRNA/sample) in 5 µl Lipofectamine^{TM} RNAiMAX. The samples were resolved by SDS-PAGE and immunoblotting to evaluate the levels of TRH-R1, $G_{q/11}\alpha$ and Gβ proteins (A). β-Actin was used as a loading control. Next, the samples from control and RNAi-transfected cells were resolved by CN/SDS-PAGE and the stability of the presumed TRH-R1–$G_{q/11}$ protein complex was assessed by immunoblotting (B).

5.2.7. Effect of TRH on the stability of the presumed TRH-R1–$G_{q/11}$ protein complex

One can anticipate that TRH might possibly influence the stability of the presumed TRH-R1–$G_{q/11}$ protein complex. To find out whether this was the case, the postnuclear supernatant was prepared from control and TRH-treated (1.10^{-5} M; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells and these samples were solubilised with different detergents. The solubilised protein complexes were resolved on 12-15% linear polyacrylamide gels by CN-PAGE and transferred on PVDF membranes which were then incubated with antibodies against TRH-R1, $G_{q/11}\alpha$ and Gβ proteins (Fig. 5.15).

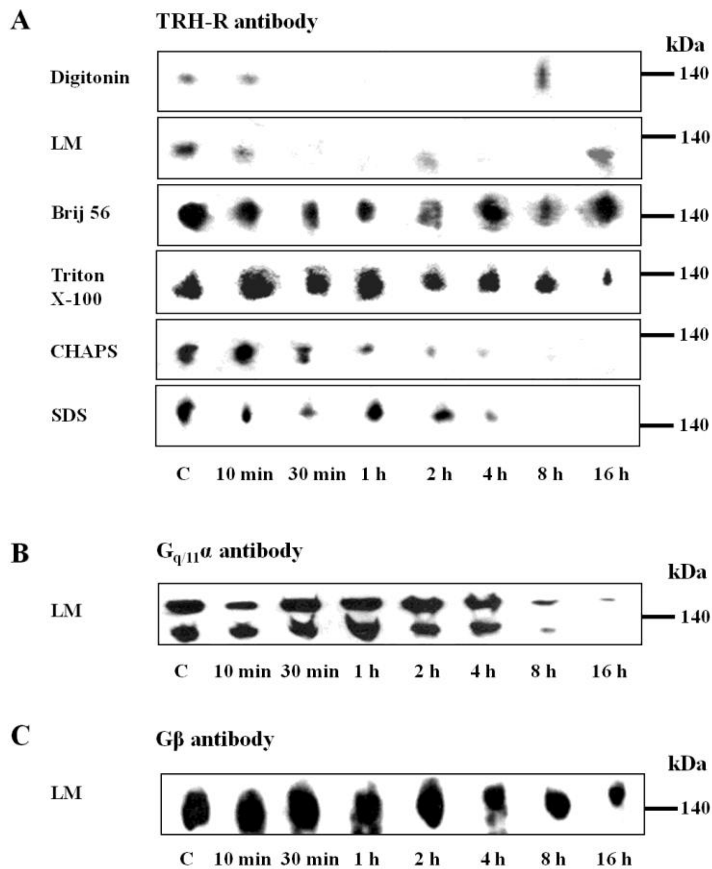


Fig. 5.15

Effect of TRH on the stability of the presumed TRH-R1–$G_{q/11}$ protein complex. The protein complexes in the postnuclear supernatant from control and TRH-treated (10 µM; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% digitonin, 1% lauryl maltoside, 1% Brij 56, 1% Triton X-100, 1% CHAPS or 0.05% SDS in sample buffer and separated by CN-PAGE. By immunoblotting, the signal of TRH-R1 in the area of 140 kDa was detected after solubilisation with all applied detergents (A) while the signals of $G_{q/11}\alpha$ and Gβ proteins in the same region were identified only after solubilisation with lauryl maltoside in sample buffer (B, C).

By detection of this complex using TRH-R1 antibody, it was observed that short-term TRH treatment (10–30 min) resulted in gradual dissociation of the presumed TRH-R1-$G_{q/11}$ protein complex while prolonged TRH treatment (4–16 h) led to partial re-association of this complex (Fig. 5.15A), probably due to increased expression of TRH-R1 induced by prolonged TRH treatment (Fig. 5.9A). Detection of heterotrimeric $G_{q/11}$ protein in the area of 140 kDa revealed that the pattern of immunoblot signals of this complex in samples from control and TRH-treated cells differs from the pattern of TRH-R1 immunoblot signals because the significant dissociation of this complex occurred after prolonged TRH treatment (4–16 h) (Fig. 5.15 B,C). This discrepancy can be explained by the existence of several pre-associated complexes of different $G_{q/11}$ protein-coupled receptors with heterotrimeric $G_{q/11}$ protein and down-regulation of $G_{q/11}\alpha$ after prolonged hormone treatment.

Molecular protein complexes can form higher-order structures whose parts can be buried in central areas of the complexes. Thus, specific epitopes for binding of antibodies can be inaccessible which would lead to incorrect estimation of quantitative changes dependent on hormone treatment. Therefore, it was advantageous to perform CN-PAGE followed by denaturing SDS-PAGE in the second dimension (CN/SDS-PAGE). The solubilised protein complexes of the postnuclear supernatant from control and TRH-treated (1.10^{-5} M; 30 min, 2 h, 4 h, 8 h and 16 h) cells were then analysed by CN/SDS-PAGE and immunoblotting. The detergents applied for detection of TRH-R1 in the presumed TRH-R1-$G_{q/11}$ protein complex were 1% Brij, 1% digitonin, 1% lauryl maltoside and 1% Triton X-100 (Fig. 5.16A and Fig. 5.17A). $G_{q/11}\alpha$ or $G\beta$ proteins in the presumed TRH-R1-$G_{q/11}$ protein complex were detected not only by application of 1% lauryl maltoside as in case of CN-PAGE but also by application of 1% digitonin or 1% Triton X-100 (Fig. 5.16A and Fig. 5.17C). The patterns of immunoblot signals of all observed proteins in control and TRH-treated cells were roughly the same as the patterns obtained by CN-PAGE suggesting that the requisite epitopes for binding of used antibodies were accessible.

The putative cell compartment in which the presumed TRH-R1-$G_{q/11}$ protein complex could be present is supposed to be the plasma membrane. Therefore, it was obvious that this complex should be detected in the PM-enriched fraction isolated by centrifugation on Percoll[R] density gradient. Samples of this fraction from control and TRH-treated (30 min, 2 h, 4 h, 8 h and 16 h) cells were solubilised with 1% digitonin and the solubilised protein complexes were resolved by CN/SDS-PAGE. The components of the presumed TRH-R1-$G_{q/11}$ protein complex were then detected by immunoblotting (Fig. 5.16B and 5.17B,D). The results of analyses of spot densities correspond well to the results which were observed in samples of the postnuclear supernatant.

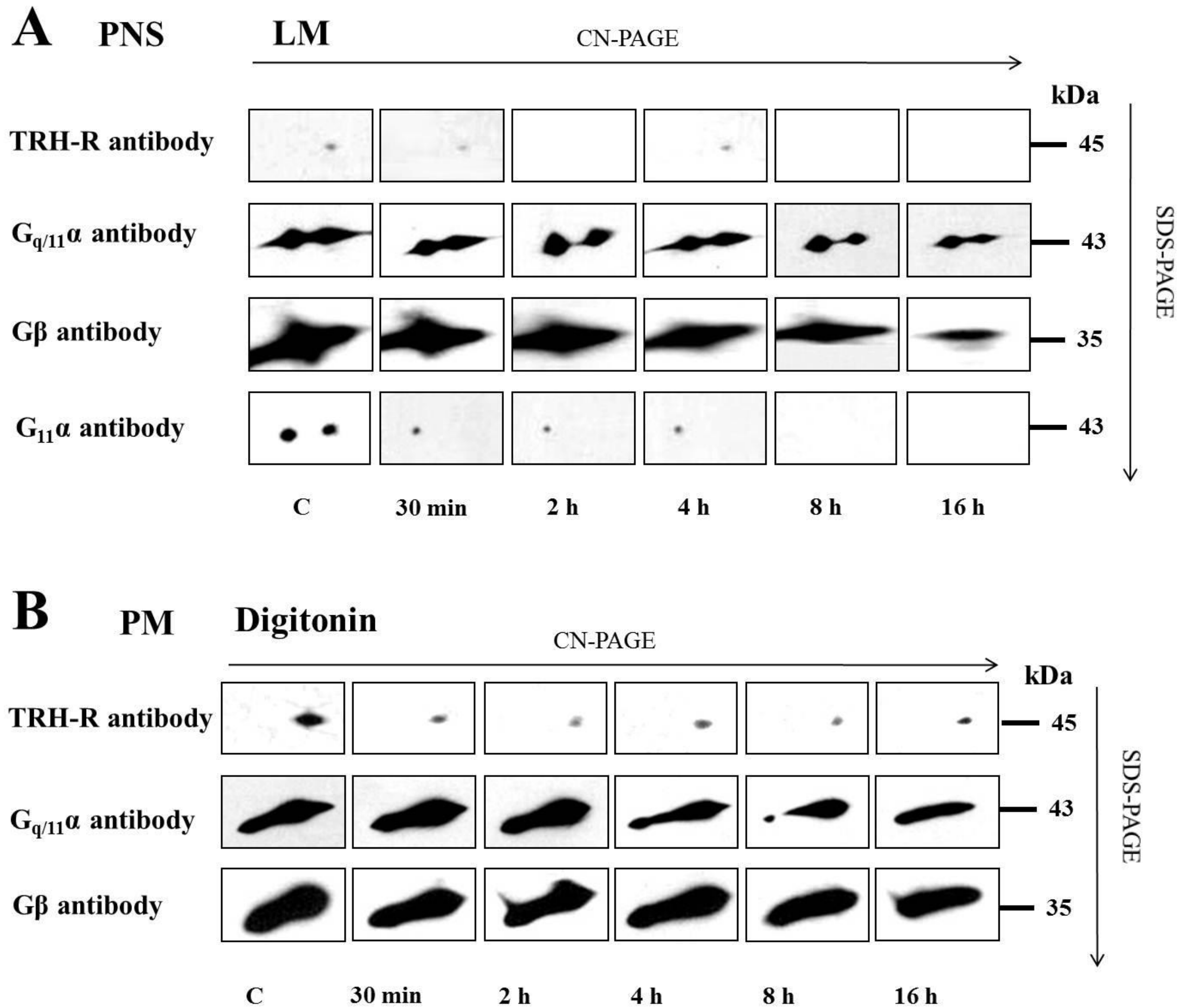


Fig. 5.16

Effect of TRH on the stability of the TRH-R1-$G_{q/11}$ protein complex separated by CN/SDS-PAGE. Protein complexes in the postnuclear supernatant from control and TRH-treated (1.10^{-5} M; 30 min, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% lauryl maltoside in sample buffer (A). After separation by CN-PAGE in the first dimension, the gel strips were cut and incubated in the equilibration buffer with 1% DTT and subsequently in the equilibration buffer with 1% IAA. The separated protein complexes dissociated into individual proteins during SDS-PAGE in the second dimension. Specific antibodies against TRH-R1, $G_{q/11}\alpha$, $G_{11}\alpha$ and Gβ proteins were used for detection of the components forming the presumed TRH-R1-$G_{q/11}$ protein complex. The same set of experiments was performed on samples of the PM-enriched fraction solubilised with 1% digitonin in sample buffer (B).

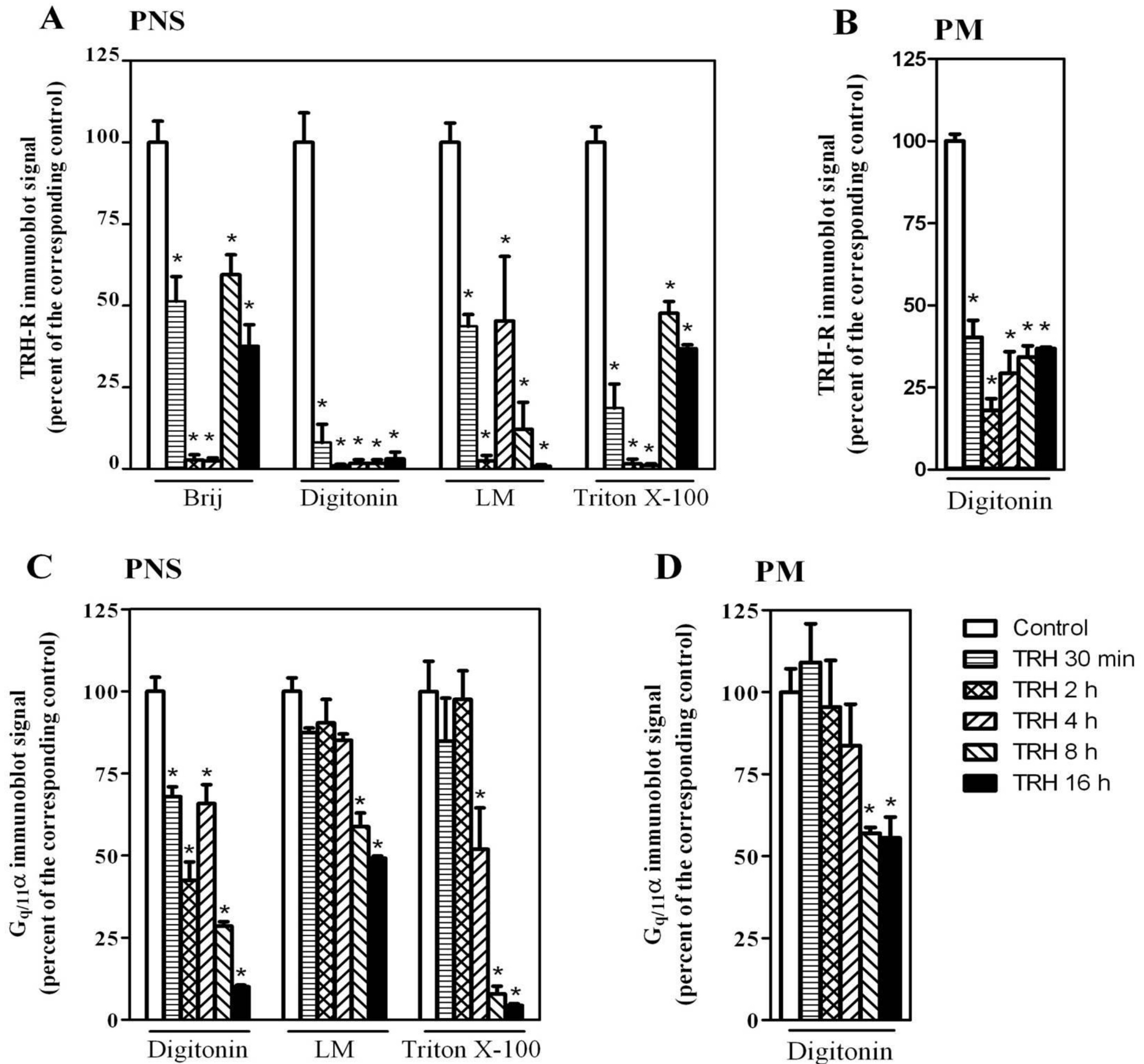


Fig. 5.17

Densitometric analysis of the TRH-R1 or $G_{q/11}\alpha$ immunoblot signals. The spots of TRH-R1 (A, B) or $G_{q/11}\alpha$ (C, D) protein complexes, which were separated by CN/SDS-PAGE after solubilisation of the postnuclear supernatant (A, C) or the PM-enriched fraction (B, D) from control and TRH-treated (1.10^{-5} M; 30 min, 2 h, 4 h, 8 h and 16 h) cells with different detergents and subsequently detected in the area of 140 kDa by immunoblotting, were analysed by ImageQuant^{TM} TL Software (Amersham Biosciences). The analyzed spot densities were expressed as percentage of signal intensity (control sample corresponds to 100%). Data represent the mean±S.E.M. of three separate experiments. *$p<0.05$ compared to the corresponding control in each group.

5.2.8. Effect of TRH on the stability of the presumed TRH-R1 dimer

Another low-molecular-weight protein complex of TRH-R1 was observed around 80 kDa. This complex was not affected by incubation of samples at increased temperature (37 °C) during

solubilisation (Fig. 5.10) suggesting that it could be TRH-R1 dimer. This supposition is supported by the finding of Zhu and co-workers who observed that the stability of TRH-R1 homodimer was not affected by increased temperature (Zhu *et al.*, 2002). It might be expected that if this 80 kDa complex was composed of TRH-R1 monomer and its possible interacting partners, it would dissociate at increased temperature as in case of the presumed TRH-R1-$G_{q/11}$ protein complex. Nevertheless, this complex was disintegrated only under denaturating conditions (Fig. 5.11B).

It was of interest to find out if TRH may affect the stability of this presumed TRH-R1 dimer. Samples of the postnuclear supernatant from control and TRH-treated (10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) cells were solubilised with different detergents and resolved by CN-PAGE. The presumed TRH-R1 dimer was then detected by immunoblotting (Fig. 5.18A). The increased level of this complex was observed after short-term TRH treatment (10 min – 2 h) whereas prolonged TRH treatment (8–16 h) led to decrease in the level of TRH-R1 dimer suggesting that it could be a consequence of partial re-association of the TRH-R1-$G_{q/11}$ protein complex observed in the same time interval. The increased level of TRH-R1 dimer after 2- and 4-h TRH treatment was also observed by CN/SDS-PAGE (Fig. 5.18B).

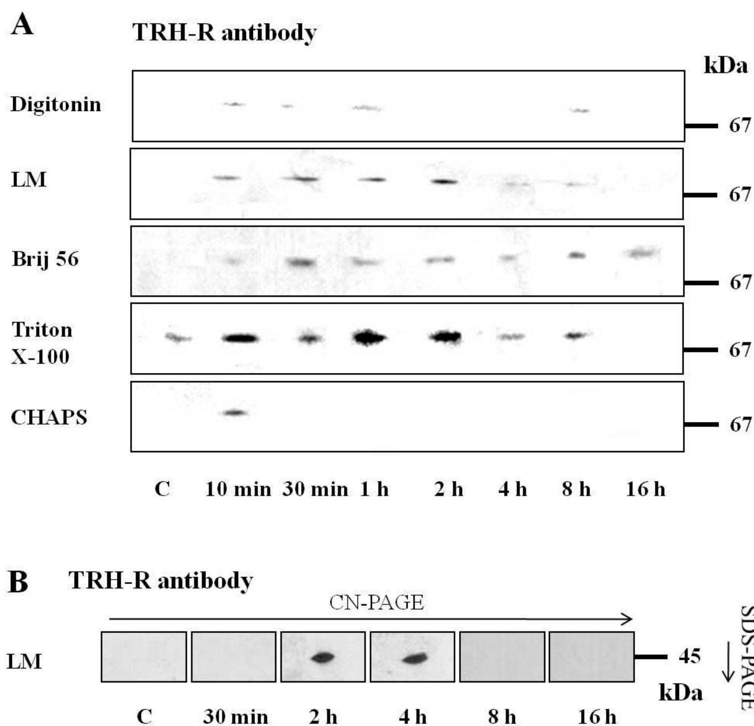


Fig. 5.18

Effect of TRH on the stability of the presumed TRH-R1 dimer. Samples of the postnuclear supernatant from control and TRH-treated (10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% digitonin, 1% lauryl maltoside, 1% Brij 56, 1% Triton X-100 or 1% CHAPS in

sample buffer. The solubilised protein complexes were resolved by CN-PAGE (A) or CN/SDS-PAGE (B) and TRH-R1 complex in the area of 80 kDa was detected by immunoblotting.

5.2.9. Identification of protein complexes of α-adrenergic receptors by CN-PAGE

As described above, changes in the intensity of TRH-R signals evoked by TRH differed from those observed for both $G_{q/11}\alpha$ and Gβ proteins (Fig. 5.15 and Fig. 5.16). These results could be explained by the possible existence of $G_{q/11}$ protein complexes containing different GPCRs which have similar molecular weight and properties. In order to verify this assumption, specific antibodies against α_{1B}-adrenergic receptors (ARs) were used in the next set of experiments. These receptors were detected in samples of the PNS from control and TRH-treated cells solubilised with 1% Brij 56 and resolved by CN-PAGE (Fig. 5.19A). A remarkable portion of immunochemical signal appeared in complexes found around 140 kDa, mainly in the complex with a somewhat lower electrophoretic mobility. Intriguingly, the amount of these receptors dynamically changed in the course of hormone treatment. The α_{1B}-AR antibody recognized also a single band migrating at 50 kDa, which corresponds to receptor monomers (Fig. 5.19A). Similar results were obtained after solubilisation of samples with 1% digitonin or 1% Triton X-100 (data not shown).

In order to find out whether the complexes of $G_{q/11}$ proteins and their cognate receptors found in the region of 140 kDa are not only artificial structures formed during the solubilisation step, the presumed complexes of α_{2A}-AR were also investigated. Although this receptor is known to interact with G_i proteins, it was found not to be precoupled with $G_{i1}\alpha$ to a significant extent (Hein *et al.*, 2005; Qin *et al.*, 2008). In our experiments, samples of the PNS from HEK293-E2M11 cells were solubilised with different detergents (1% Brij 56, 1% digitonin or 1% Triton X-100) and resolved on 10%-15% linear gradient polyacrylamide gels. After electrotransfer to PVDF membrane, specific antibodies against $G_{i1,2}\alpha$ or α_{2A}-AR were used to identify these proteins on immunoblots. In this way, five bands containing $G_{i1,2}\alpha$ and two bands containing α_{2A}-AR were visualized (Fig. 5.19B). Both these proteins were found in a complex of 140 kDa but the signal corresponding to α_{2A}-AR was relatively very low. A substantial amount of α_{2A}-AR migrating at 80 kDa may represent the putative receptor homo- and heterodimers. A significant portion of $G_{i1,2}\alpha$ was resolved as a single 40 kDa band corresponding to protein monomer. It might be expected that if Gα proteins and GPCRs form the artificial complexes during detergent solubilisation, the amounts of such detected complexes should be comparable for different types of Gα proteins and GPCRs because distinct Gα are structurally and functionally similar, which is valid for G-protein coupled receptors as well. However, this was not the case, especially for α_{2A}-AR. To elaborate this issue further, we also checked whether another G_i-coupled receptor, muscarinic M2 receptor, might form molecular complexes with these G-proteins. Interestingly, only the 50 kDa monomeric form of this receptor was detected on immunoblots after solubilisation of samples with the same detergents as above (data not shown). These observations support the notion

that the 140 kDa complexes containing $G_{q/11}$ and their cognate GPCRs do not represent only artificial structures formed during solubilisation.

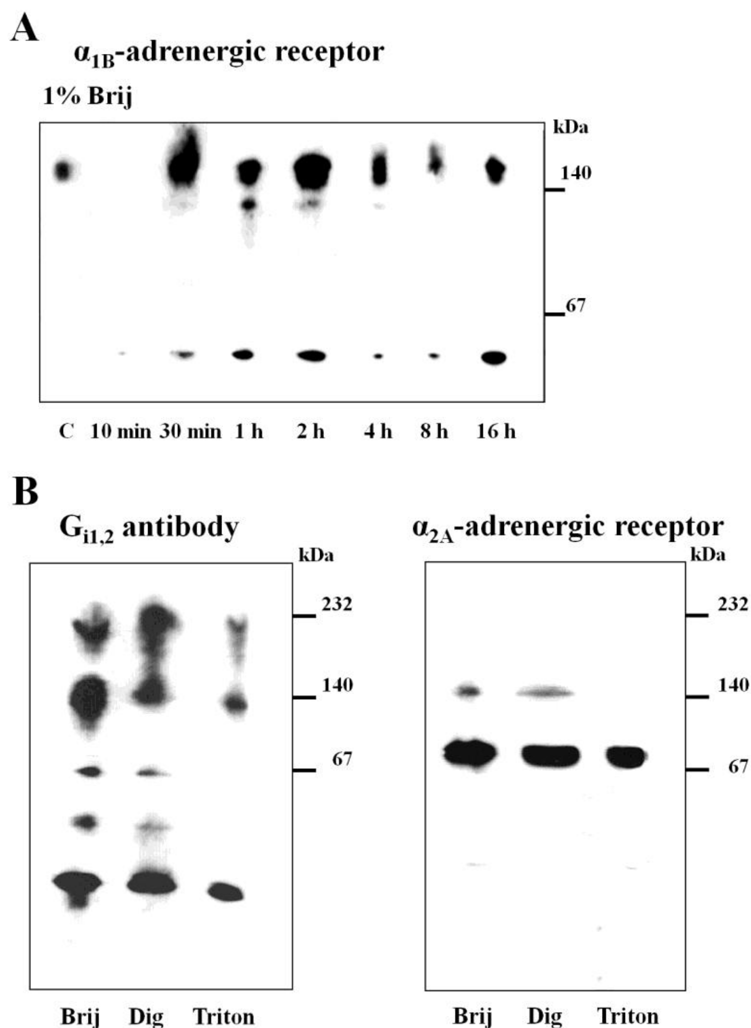


Fig. 5.19

Detection of protein complexes of α-ARs and $G_{i1,2}\alpha$ protein. The postnuclear supernatants prepared from control and TRH-treated (10 min, 30 min, 1h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% Brij 56, 1% digitonin or 1% Triton X-100 in sample buffer and protein complexes were separated on 10-15% linear gradient polyacrylamide gels under native conditions. After electrotransfer of proteins to PVDF membrane, the blots were probed with α_{1B}-AR antibodies (A), $G_{i1,2}\alpha$ and α_{2A}-AR antibodies (B).

5.2.10. Detection of $G_{q/11}$ protein complexes after solubilisation with Brij 56

In order to detect other types of $G_{q/11}$ protein complexes, other solubilisation conditions were applied. Samples of the postnuclear supernatant from control HEK293-E2M11 cells were solubilised with 1% Brij 56 at different concentrations (0.1%, 1%, 2% or 4%). The solubilised protein

complexes were separated by CN-PAGE and transferred on PVDF membranes, which were then probed with $G_{q/11}\alpha$ or Gβ antibodies. Using both these antibodies, one strong immunoblot signal was detected in the area of 300 kDa (Fig. 5.20A). This complex is likely to involve heterotrimeric $G_{q/11}$ protein and it was not detectable by solubilisation with other detergents.

To determine whether the complex found around 300 kDa is membrane-bound or cytosolic, samples of the PM-enriched or cytosolic fraction were solubilised under the same solubilisation conditions as in case of the postnuclear supernatant and resolved by CN-PAGE. The $G_{q/11}$ protein complex in the region of 300 kDa was observed only in the PM-enriched fraction (Fig. 5.20B). In all next experiments exploring this complex, solubilisation with 1% Brij 56 was carried out.

Since no immunoblot signals of some selected GPCRs (TRH receptor, α_{1B}-adrenergic receptor, muscarinic M1 receptor or bradykinin B2 receptor) were observed in the area corresponding in the size to the above detected $G_{q/11}$ protein complex (data not shown), it can be supposed that this high-molecular-weight complex does not contain receptors.

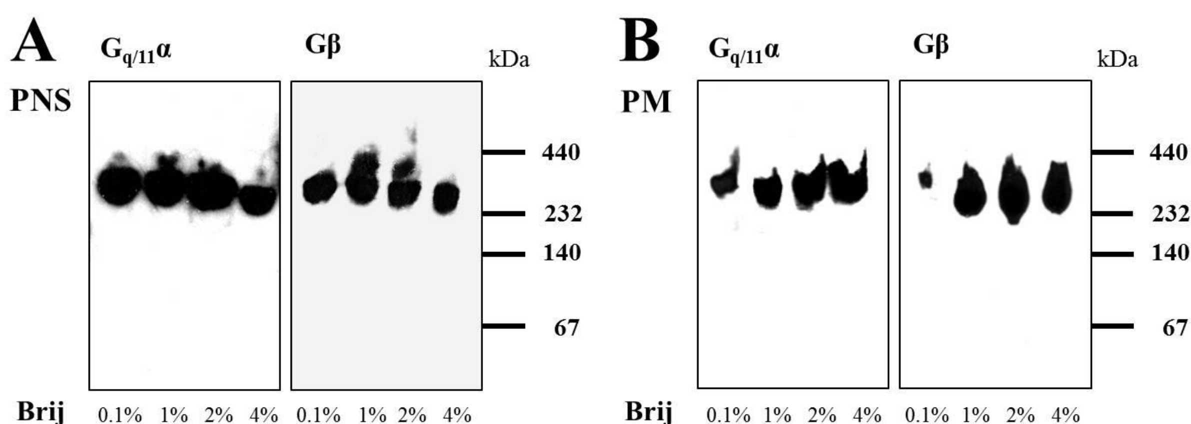


Fig. 5.20

Detection of a $G_{q/11}$ protein complex around 300 kDa. Samples of the postnuclear supernatant (A) or the PM-enriched fraction (B) were solubilised with different concentrations of Brij 56 in sample buffer. The solubilised protein complexes were resolved on 5-16% linear non-denaturating gels by CN-PAGE. After electrotransfer of proteins to PVDF membrane, the blots were probed with $G_{q/11}\alpha$ and Gβ antibodies (B).

5.2.11. TRH-induced dissociation of high-molecular-weight $G_{q/11}\alpha$ protein complex

Samples of the postnuclear supernatant (Fig. 21) as well as the PM-enriched fraction (Fig. 5.22) from control and TRH-treated (10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) cells were used to study the effect of TRH on the stability of the high-molecular-weight complex found in the region of 300 kDa. Protein complexes after sample solubilisation were separated on 6-15% linear non-

denaturating gels by CN-PAGE (Fig. 5.21A and Fig. 5.22A) or by CN/SDS-PAGE (Fig. 5.21B and Fig. 5.22B). Detection of this $G_{q/11}$ protein complex by immunoblotting with $G_{q/11}\alpha$ or Gβ antibodies revealed that prolonged TRH treatment (4–16 h) resulted in dissociation of this complex. A similar pattern of dissociation was also observed in case of the 140 kDa TRH-R1–$G_{q/11}$ protein complex.

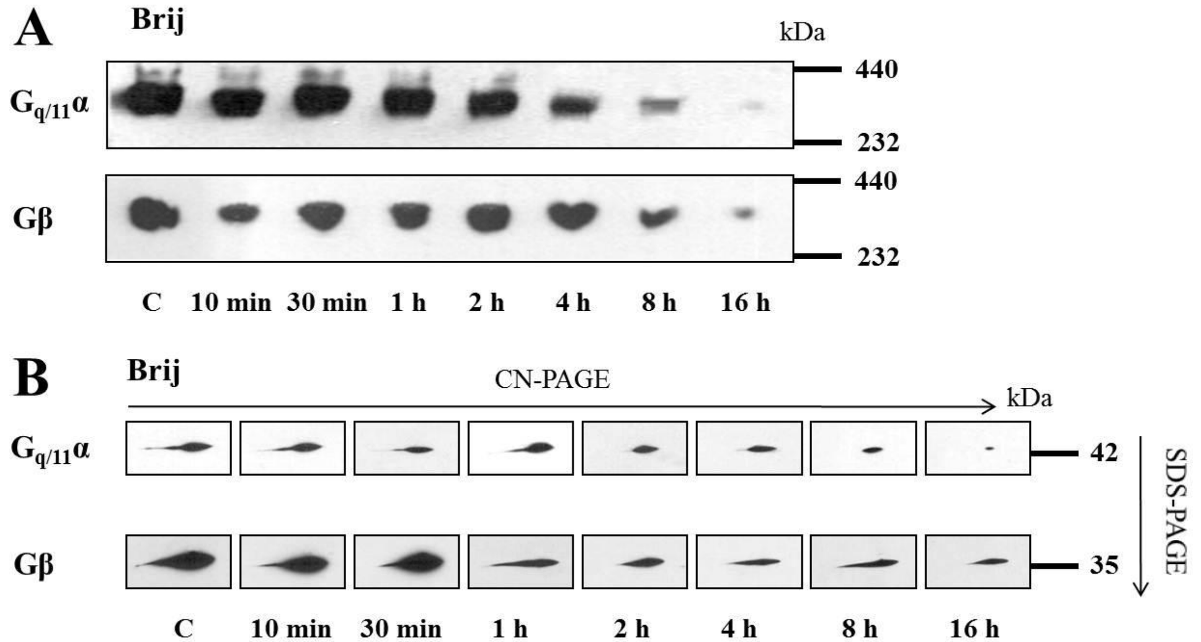


Fig. 5.21

Effect of TRH on dissociation of the $G_{q/11}$ protein complex detected around 300 kDa in the postnuclear supernatant. Protein complexes in the postnuclear supernatant from control and TRH-treated (1.10^{-5} M; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% Brij 56 in sample buffer, resolved by CN-PAGE (A) or by CN/SDS-PAGE (B) and detected by immunoblotting with $G_{q/11}\alpha$ antibodies.

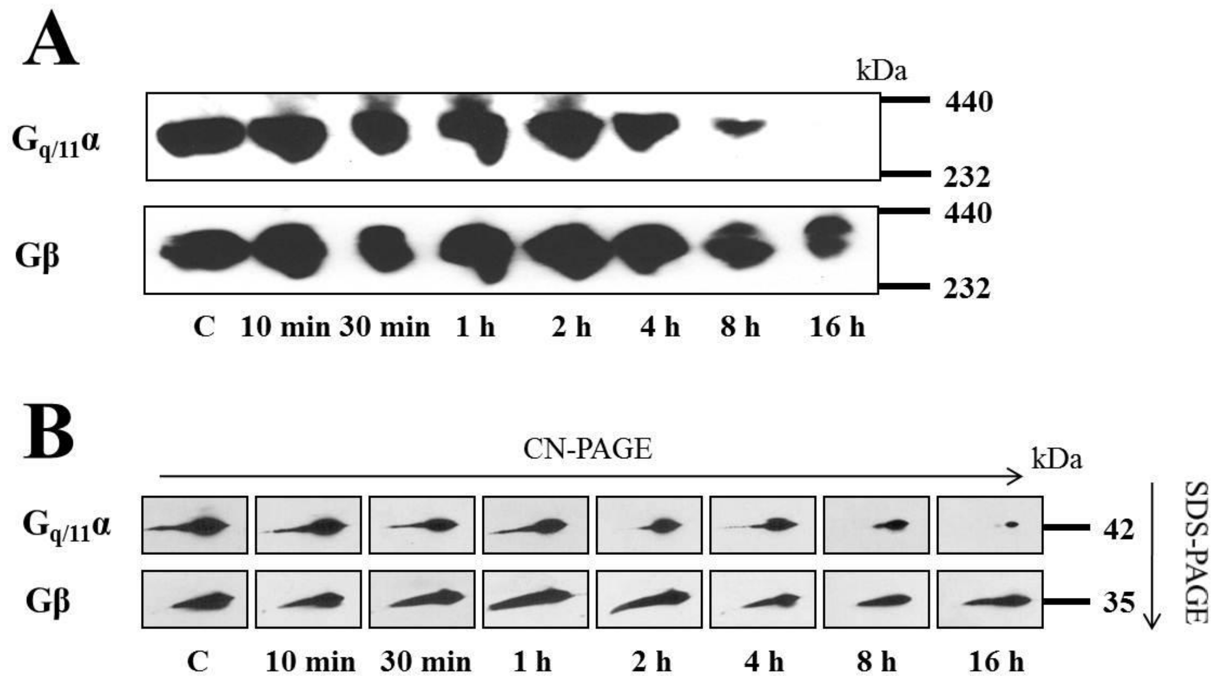


Fig. 5.22

Effect of TRH on dissociation of $G_{q/11}$ protein complex detected around 300 kDa in the PM-enriched fraction. Protein complexes in the PM-enriched fraction from control and TRH-treated (1.10^{-5} M; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% Brij 56 in sample buffer, resolved by CN-PAGE (A) or by CN/SDS-PAGE (B) and detected by immunoblotting with $G_{q/11}α$ antibodies.

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

When the experiments observing TRH effect on the stability of the TRH-R1-$G_{q/11}$ protein complex and the $G_{q/11}$ protein complex were performed, yet another $G_{q/11}α$ protein complex was detected mainly in samples of cells after prolonged TRH treatment. Samples of the postnuclear supernatant from control and TRH-treated (10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with different detergents dissolved in sample buffer and resolved by CN-PAGE. After immunoblotting with $G_{q/11}α$ antibody, two distinct low-molecular-weight $G_{q/11}α$ protein complexes were identified in the region of 70 kDa and their levels were rising dependently on time of TRH treatment (Fig. 5.23).

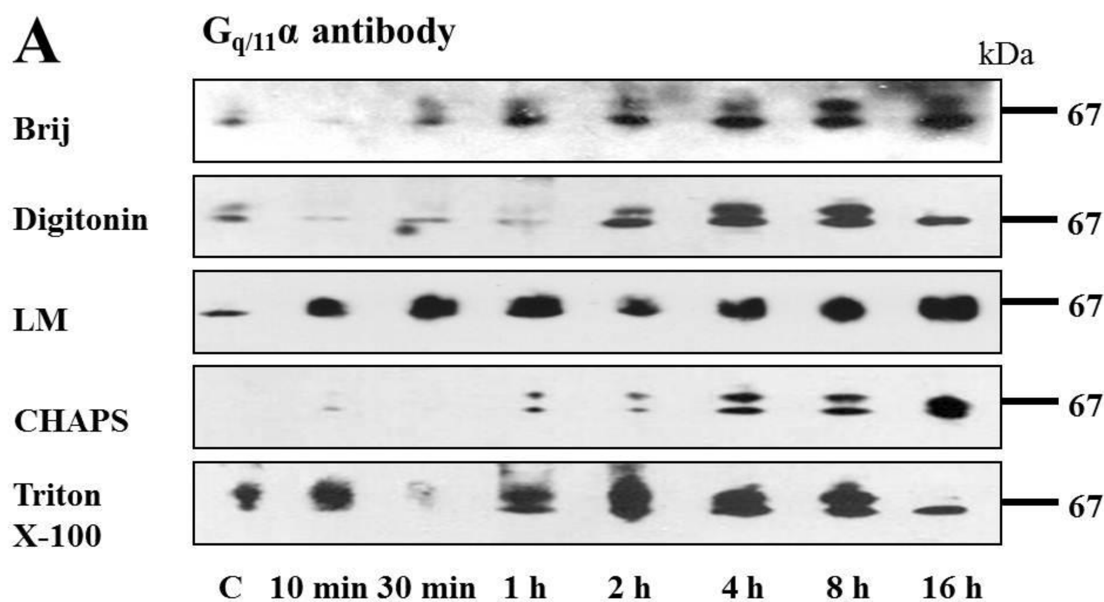


Fig. 5.23

Effect of TRH on the formation of the $G_{q/11}\alpha$ protein complex detected around 70 kDa in the postnuclear supernatant. Protein complexes of postnuclear supernatant from control and TRH-treated (1.10^{-5} M; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% Brij 56, 1% digitonin, 1% lauryl maltoside, 1% CHAPS and 1% Triton X-100 in sample buffer, resolved by CN-PAGE and detected by immunoblotting with $G_{q/11}\alpha$ antibodies.

To determine the localization of the low-molecular-weight $G_{q/11}\alpha$ protein complexes in isolated fractions, samples of the PM-enriched and cytosolic fractions of control and TRH-treated (10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were analysed in the same way as in case of the PNS. These complexes were found in the cytosolic fraction and even three distinct complexes were detected under some conditions (Fig. 5.24A). By solubilisation with Brij 56, a monomer of $G_{q/11}\alpha$ protein was identified roughly around 45 kDa. The levels of the low-molecular-weight $G_{q/11}\alpha$ protein complexes were increasing with prolonged time of TRH treatment.

In order to confirm the rise in levels of these $G_{q/11}\alpha$ protein complexes, samples of the cytosolic fraction were solubilised and resolved by CN/SDS-PAGE (Fig. 5.24B). Two intensive spots with a size of 43 kDa corresponding to $G_{11}\alpha$ protein and two fainter spots with a size of 40 kDa corresponding to $G_q\alpha$ protein were identified as components of these $G_{q/11}\alpha$ protein complexes. Although short-term TRH treatment (30 min – 2 h) induced formation of these complexes, significant rise in their levels was observed only after prolonged TRH treatment (4–16 h). It seems that both these complexes are comprised of $G_{11}\alpha$ as well as $G_q\alpha$ and a slight difference in their size is not substantiated by the presence of only one type of $G_{q/11}\alpha$ protein in a particular complex.

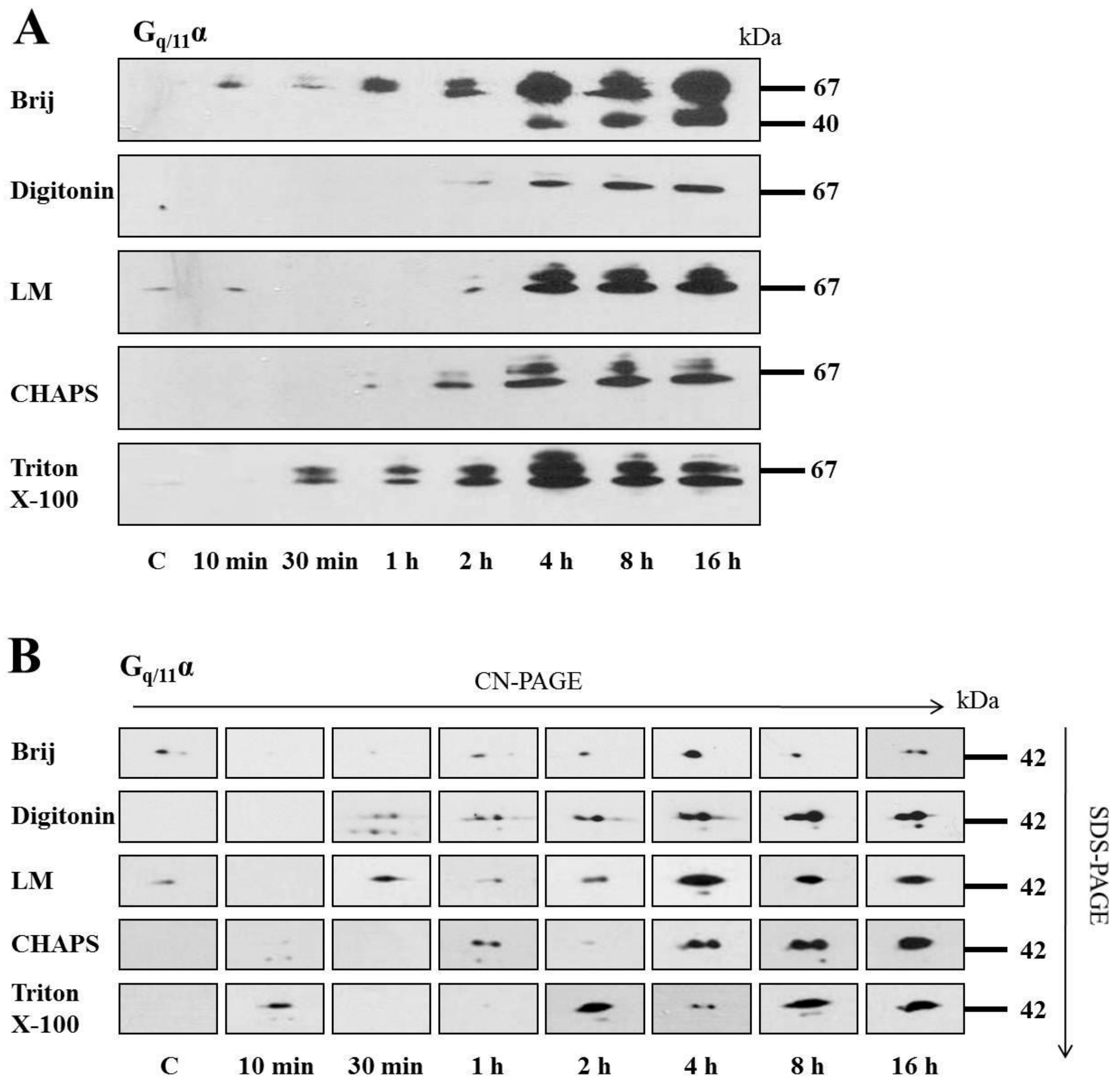


Fig. 5.24

Effect of TRH on the formation of the $G_{q/11}\alpha$ protein complex detected around 70 kDa in cytosol. Protein complexes in the cytosolic fraction from control and TRH-treated (1.10^{-5} M; 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) HEK293-E2M11 cells were solubilised with 1% Brij 56, 1% digitonin, 1% lauryl maltoside, 1% CHAPS and 1% Triton X-100 in sample buffer, resolved by CN-PAGE (A) or CN/SDS-PAGE (B) and detected by immunoblotting with $G_{q/11}\alpha$ antibodies.

It might be speculated that these complexes could be trimeric $G_{q/11}\alpha$ proteins varying in βγ heterodimers. In order to determine whether these complexes could include β subunit of trimeric G-proteins, specific primary antibody against Gβ protein was used for immunodetection. Although this experiment was repeatedly conducted after solubilisation with different detergents by CN-PAGE as

well as CN/SDS-PAGE, no immunoblot signal of Gβ was observed in the region of 70 kDa (data not shown).

The most marked increase in the levels of $G_{q/11}\alpha$ protein complexes were observed after prolonged TRH treatment (4–16 h), which can be connected with dissociation of the TRH-R1-$G_{q/11}$ and $G_{q/11}$ protein complexes during the same time intervals.

5.2.13. Effect of TRH concentration on dissociation/association of $G_{q/11}\alpha$ complexes

In the previous set of experiments, TRH was applied at a final concentration of 10 μM in order to detect the hormone effect. Nevertheless, hormone concentration is one of the most key factors determining which processes will actually take place in the cell. The TRH concentration (1.10^{-5} M) used in these experiments is very high and it can hardly be considered physiological. It was necessary to check whether the lower hormone concentrations would lead to the same dissociation/association patterns of the observed protein complexes. The postnuclear supernatants of control and TRH-treated (16 h; 10^{-5}–10^{-9} M) HEK293-E2M11 cells were solubilised by LM or Brij 56, the solubilised protein complexes were separated by CN-PAGE and $G_{q/11}\alpha$ proteins detected using specific antibody (Fig. 5.25).

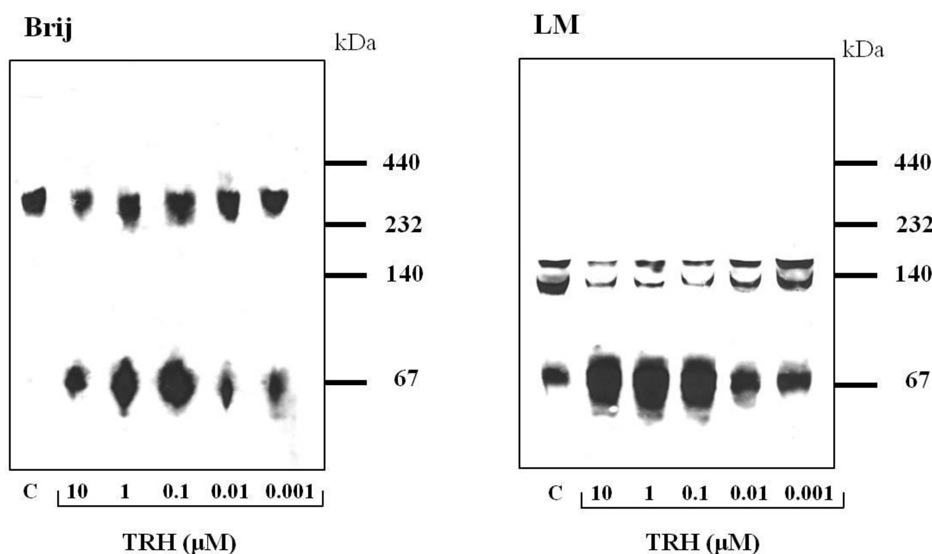


Fig. 5.25

Effect of TRH concentration on dissociation/association of $G_{q/11}\alpha$ complexes. Samples of the postnuclear supernatant from control and TRH-treated (16 h; 10-0.001 μM) HEK293-E2M11 cells were solubilised with 1% Brij 56 or 1% lauryl maltoside in sample buffer and protein complexes resolved on 6-15% linear non-denaturating gels by CN-PAGE. Protein complexes were detected by immunoblotting with $G_{q/11}\alpha$ antibodies.

More pronounced alterations in dissociation of membrane-bound complexes and association of cytosolic complexes were observed primarily in samples of cells which were treated with TRH in a concentration range of 10^{-5}–10^{-7} M. The lower concentrations of TRH (10^{-8} –10^{-9} M), which are more physiological, induced similar changes but to a lesser extent. Therefore, the observed dissociation and association of protein complexes can be regarded as a general process induced by a wide range of TRH concentrations, which can also occur under physiological conditions. Nevertheless, the level of $G_{q/11}$ complex in the 300 kDa region was not markedly affected by treatment with lower concentrations of TRH compared to control sample and thus the formation of low-molecular-weight cytosolic complexes in the 70 kDa region is more likely to be connected with dissociation of the complex found in the 140 kDa region.

5.2.14. TRH-induced formation of a high-molecular-weight protein complex of TRH-R

The putative TRH-R complex in the 500 kDa region was detected in experiments observing the effect of detergent concentration on solubilisation of TRH-R complexes (Fig. 26A). When using digitonin, one TRH-R complex in the 500 kDa region was found while solubilisation with CHAPS or Triton X-100 enabled to separate this complex into two distinct bands. The optimal concentration for solubilisation of this complex was assessed to be 1%.

Our experiments analysing the effect of TRH on the stability of the TRH-R1-$G_{q/11}$ complex revealed that the level of the TRH-R1 complex in the 500 kDa region increased after short-term TRH treatment (10–30 min) suggesting that the activated receptor can be relocated from the TRH-R1-$G_{q/11}$ complex into this complex. This relocation could enable interaction of the receptor with different signalling molecules and allow trigger signalling pathways conveying the signal into the cell interior.

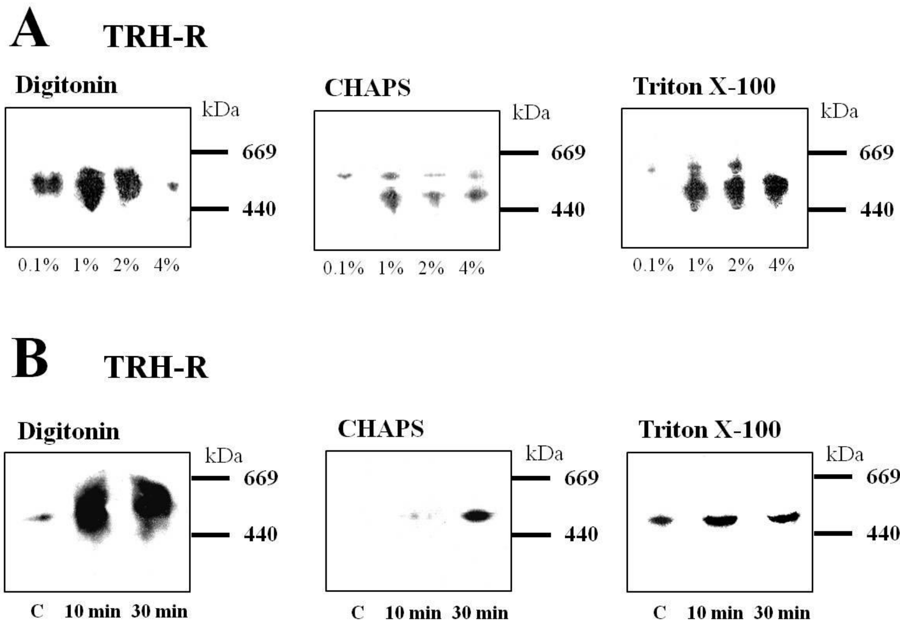


Fig. 5.26

Detection of a high-molecular-weight TRH-R protein complex in the 500 kDa region and its stability after TRH treatment. Samples of the postnuclear supernatant from HEK293-E2M11 cell line were solubilised with different concentrations of digitonin (Dig), CHAPS and Triton X-100 in sample buffer. The solubilised protein complexes were resolved on 6-10% linear gradient polyacrylamide gels under non-denaturating conditions and immunoblotted with specific antibodies against TRH-R1. The TRH-R1 complex was detected in the area of 500 kDa (A) and the amount of this complex rose after short-term (10–30 min) treatment with 10 μM TRH (B).

5.2.15. TRH-induced formation of a high-molecular-weight protein complex of $G_{q/11}\alpha$ protein

Another $G_{q/11}\alpha$ protein complex, which was noticed during the primary experiments exploring the effect of detergent concentration on solubilisation of complexes, was found in the 700 kDa region (Fig. 5.27A). This complex was solubilised by digitonin, Brij 56 or lauryl maltoside in sample buffer and the optimal concentration for solubilisation of this complex was assessed to be 1%. The level of this complex was found to increase after short-term (10–30 min) treatment similarly as in case of TRH-R1 complex in the 500 kDa region (Fig. 5.27B).

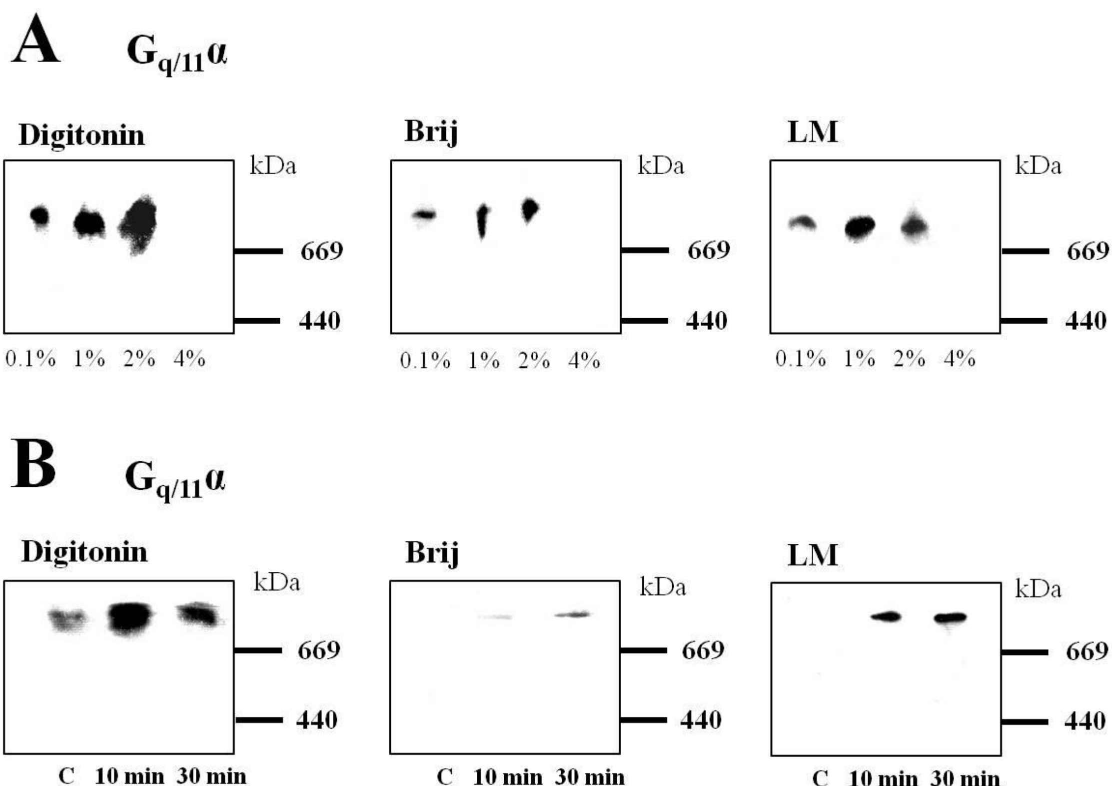


Fig. 5.27

Detection of a high-molecular-weight $G_{q/11}\alpha$ protein complex in the 700 kDa region and its stability after TRH treatment. Samples of the postnuclear supernatant from HEK293-E2M11 cell line were solubilised with different concentrations of digitonin (Dig), Brij and lauryl maltoside (LM) in sample buffer. The solubilised protein complexes were resolved on 6-10% linear gradient polyacrylamide gels under non-denaturating conditions and immunoblotted with specific antibodies against $G_{q/11}\alpha$. The $G_{q/11}\alpha$ complex was detected in the area of 700 kDa (A) and the level of this complex increased after short-term (10–30 min) treatment with 10 μM TRH (B).

These results suggest that the activated $G_{q/11}\alpha$ protein might be relocated from the TRH-R1-$G_{q/11}$ complex after TRH stimulation. Therefore, it was of interest to assess which $G_{q/11}\alpha$ complexes could involve $G_{q/11}\alpha$ protein in an activated state. In order to solve this issue, the [^{35}S]GTPγS binding assay followed by CN-PAGE and autoradiography was performed. Samples of the postnuclear supernatant from control HEK293-E2M11 cells were incubated with or without 10 μM TRH and 10 nM [^{35}S]GTPγS, solubilised with 1% lauryl maltoside and resolved by CN-PAGE.

As shown in Fig. 5.28, the basal activity of $G_{q/11}\alpha$ protein was detected in the 40 kDa and 700 kDa regions apparently corresponding to free $G_{q/11}\alpha$ subunit and the high-molecular-weight 700 kDa $G_{q/11}\alpha$ complex, respectively. Incubation of the postnuclear supernatant with 10 μM TRH in the reaction mixture for [^{35}S]GTPγS assay resulted in increase of signals in the 40 kDa, 140 kDa and 700

kDa regions (Fig. 27) indicating the increased $G_{q/11}\alpha$ activity. It can be concluded that the activated $G_{q/11}\alpha$ subunit is involved in the 700 kDa $G_{q/11}\alpha$ complex in its basal state as well as after its activation by TRH receptor.

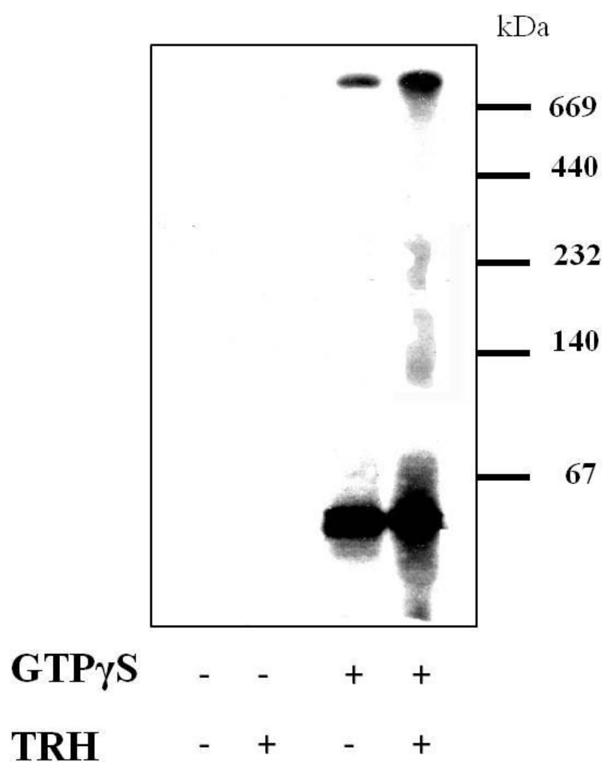


Fig. 5.28

Two hundreds μg of the postnuclear supernatant from control HEK293-E2M11 cells were incubated with or without 10 μM TRH and 10 nM [^{35}S]GTPγS in Mix buffer for 30 min at 20 °C. The samples solubilised with 1% lauryl maltoside were resolved by CN-PAGE and the gels exposed on Kodak MXB films for 5 days at -80 °C.

5.2.16. Identification of Gβ protein as a component of high-molecular-weight protein complexes

Gβ protein takes part in signal transduction and is able to trigger distinct signalling pathways compared to those triggered by $G_{q/11}\alpha$. As mentioned above, Gβ was recognised as a component of protein complexes found in the regions of 140 kDa and 300 kDa. By contrast, it seems that $G_{q/11}\alpha$ protein complex in the 70 kDa region does not contain Gβ subunit. It was of interest to check whether Gβ protein is part of the TRH-R complex in the 500 kDa region and $G_{q/11}\alpha$ complex in the 700 kDa.

Samples of the postnuclear supernatant from control HEK293-E2M11 cells were treated with different concentrations of digitonin in sample buffer and the solubilised protein complexes were then resolved on 6-10% non-denaturating linear gels by CN-PAGE and electrotransferred on PVDF membrane. Using Gβ antibodies, the immunoblot signals of Gβ were detected in the region of 700 kDa

when using different concentrations of digitonin and yet another one was found in the region of 500 kDa, but only after solubilisation with 1% digitonin (Fig. 5.29A).

In order to determine the stability of the above Gβ complexes, samples of control and TRH-treated (10 and 30 min) cells were solubilised with 1% digitonin in sample buffer and protein complexes were resolved by CN-PAGE and detected by immunoblotting with specific Gβ antibodies. The levels of both observed complexes were markedly increased after 30-min TRH treatment suggesting that these Gβ complexes are apparently formed after TRH-R activation (Fig. 5.29B). These data suggest that Gβ protein could be a component of high-molecular-weight TRH-R and $G_{q/11}\alpha$ complexes observed in the 500 kDa and 700 kDa regions, respectively.

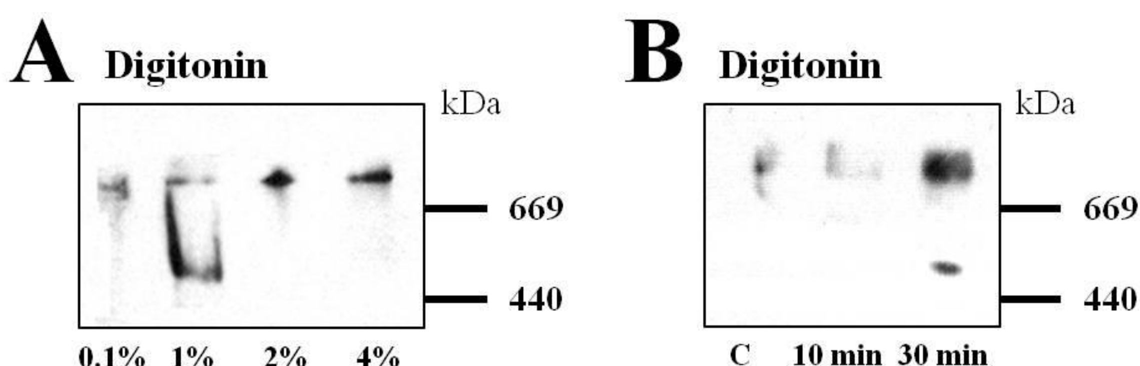


Fig. 5.29

Detection of Gβ in high-molecular-weight protein complexes. Samples of the postnuclear supernatant from HEK293-E2M11 cell line were solubilised with different concentrations of digitonin in sample buffer. The solubilised protein complexes were resolved on 6-10% linear gradient polyacrylamide gels under non-denaturating conditions and immunoblotted with specific antibodies against Gβ. The Gβ complexes were detected in the areas of 700 kDa and 500 kDa (A) and the levels of these complexes were found to increase after short-term (30 min) treatment with 10 μM TRH (B).

5.2.17. Identification of potential interacting partners of TRH receptor and $G_{q/11}\alpha$

In order to find the potential interacting partners which could be components of the identified TRH receptor and $G_{q/11}\alpha$ protein complexes we selected several proteins taking part in the TRH-R signalling pathway. These selected proteins included scaffolding proteins clathrin, NHERF1, caveolin-1 and flotillin-2 and proteins engaged in signal transduction PLCβ, GRK2, RGS2-5 and β-arrestin. Specificity of the antibodies against PLCβ and GRK2 is shown in Fig. 5.30A.

To detect if these selected proteins would be components of the identified protein complexes, samples of the postnuclear supernatant from control and TRH-treated cells were solubilised with different detergents and solubilised protein complexes were separated on 6-15% linear non-

denaturating linear gels by CN-PAGE. After electrotransfer on PVDF membrane, membranes were incubated with specific antibodies against selected proteins.

Most of the selected proteins (clathrin, NHERF1, caveolin-1, flotillin-2, RGS2-5 and β-arrestin) were not detected in the gel regions in which TRH-R or $G_{q/11}\alpha$ complexes were identified (data not shown). Only PLCβ and GRK2 were detected in the area of 500 kDa and 700 kDa (Fig. 5.30B) suggesting that these proteins could be components of TRH-R and $G_{q/11}\alpha$ complexes in these regions.

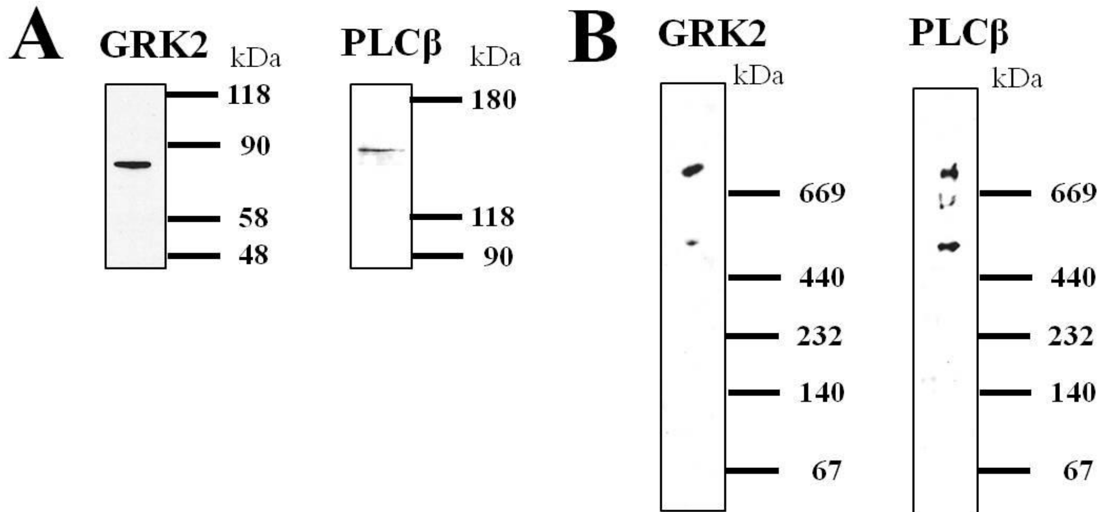


Fig. 5.30

Determination of potential interacting partners of TRH-R and $G_{q/11}\alpha$ protein in protein complexes. Specificity of antibodies against GRK2 and PLCβ was verified by SDS-PAGE (A). Sample of the postnuclear supernatant from control HEK293-E2M11 cells was solubilised with 1% digitonin in sample buffer and solubilised protein complexes were resolved on 6-10% non-denaturating polyacrylamide gels by CN-PAGE. After electrotransfer on PVDF membrane, the complexes of GRK2 and PLCβ were detected using specific primary antibodies against these proteins (B).

6. Discussion

6.1. A proteomic study of alterations induced by prolonged TRH exposure

Signal transduction across the plasma membrane into the cell interior is a crucial process which allows communication between cells. The transfer of information can be mediated by binding of signalling molecules such as hormones and neurotransmitters to receptors located at the plasma membrane which forms the surface of the cell and separates the extracellular environment from the cell interior. The length of hormone treatment determines how the cell will respond to the received signal and which cell processes will be executed. Prolonged hormone treatment can lead to cell damage. However, there is a feedback protective process called desensitization, which may take place in cells in order to prevent cell damage caused by excessive stimulation. Desensitization is a complex process resulting in decrease of the number of signalling molecules ensuring the specificity of transmembrane signal transduction (Marchese *et al.*, 2008).

Our study of focused on protein alterations after prolonged TRH treatment was based on experiments using HEK293 cell line exogenously expressing the rat long isoform of TRH receptor (TRH-R1) and mouse $G_{11}\alpha$ protein (E2M11 clone). The effects of the prolonged TRH treatment in this cell line were observed previously by immunofluorescence microscopy, [^{3}TRH] binding assay and 2D-electrophoresis (Drmota *et al.*, 1998; Matousek *et al.*, 2005; Matousek *et al.*, 2004). It was observed by immunofluorescence microscopy that $G_{q/11}\alpha$ proteins partly translocate from the plasma membranes to the cytosol after prolonged TRH treatment (10 μM) of HEK293-E2M11 cells. This translocation was detectable after 2 h treatment with TRH and it increased after 4 h and 16 h hormone treatment. The TRH concentration which were cells treated with is another key factor determining whether the translocation occurs. The level of $G_{11}\alpha$ protein was markedly diminished in the plasma membrane after long-term (16 h) treatment with TRH in a concentration range of 10^{-5}–10^{-8} M while it increased in the cytosol under the same conditions (Drmota *et al.*, 1998).

The translocation of $G_{q/11}\alpha$ proteins from the plasma membrane to the cytosol was earlier described using 2D electrophoresis and subsequent immunoblot (Matousek *et al.*, 2004). The membrane fractions obtained by different membrane preparations (crude membranes, detergent-insensitive domains, alkaline treated-domains and low-density membrane fractions) from control and TRH-treated (16 h; 1.10^{-5} M) HEK293-E2M11 cells were resolved by 2D electrophoresis and $G_{q/11}\alpha$ proteins were detected by immunoblotting with specific antibody. Prolonged TRH treatment resulted in reduction of $G_{q/11}\alpha$ immunoblot signals (Matousek *et al.*, 2004). On the other hand, separation of the cytosolic fraction from control and TRH-treated (1.10^{-5} M; 10 min, 30 min, 1 h, 2 h, 4 h and 16 h) HEK293-E2M11 cells by 2D-electrophoresis followed by immunoblotting with $G_{q/11}\alpha$ antibody revealed a gradual rise in the intensity of $G_{q/11}\alpha$ immunoblot signals (Durchankova *et al.*, 2008).

Altogether, these results confirmed that TRH treatment causes the translocation of $G_{q/11}\alpha$ proteins from the membrane-bound to soluble form and this process occurs chiefly at longer time intervals (2–16 h).

The translocation of TRH receptor was found to occur after short-term TRH treatment. It was shown by [^{3}TRH] binding assay that the number of TRH receptors in the plasma membrane decreased already after 10 min treatment with 10 μM TRH, which continued further with prolonged hormone treatment. Following 2 h TRH exposure a significant loss (roughly 80%) of cell surface binding sites was observed (Drmota *et al.*, 1998). This loss in the amount of TRH-R1 in the plasma membrane is likely connected with down-regulation of the receptor, which was observed in the postnuclear supernatant from HEK293-E2M11 cells mainly after 1 h and 2 h treatment with 10 μM TRH. However, prolonged TRH treatment (8 h and 16 h) resulted in the up-regulation of TRH receptor, which was previously reported in the study of Cook and Hinkle (Cook and Hinkle, 2004), where a gradual up-regulation of TRH receptor was detected after 8 h, 24 h and 48 h intervals of TRH treatment.

In the present study we aimed to determine protein alterations in the membrane fraction induced by prolonged TRH treatment. Two-dimensional electrophoresis was previously used to investigate protein alterations in the fraction of membrane microdomains prepared by different isolation procedures from HEK293-E2M11 cells (Matousek *et al.*, 2005). Nevertheless, no alterations in the levels of detected proteins (roughly about 150-170 proteins) were observed after prolonged TRH treatment (1.10^{-5} M; 16 h). Therefore, here we decided to prepare the plasma membrane-enriched fraction from the postnuclear supernatant by centrifugation on Percoll^R density gradient to detect the presumed hormone-induced protein alterations. This method is often applied to isolate different cell compartments (Pertoft, 2000) (Fauvel *et al.*, 1986) (Bourova *et al.*, 2009). We isolated the PM-enriched fraction by Percoll^R gradient centrifugation of the PNS and the effectiveness of this isolation was verified by detection of increased immunoblot signal of Na^+,K^+-ATPase (a marker of plasma membrane) in this fraction compared to the PNS (Fig. 5.1). Using [^{3}H]TRH and [^{35}S]GTPγS binding assays, the number of TRH receptors and the basal as well as agonist stimulated $G_{q/11}$ protein activities in this fraction were shown to be markedly increased compared to the PNS (Fig. 5.3).

Next, samples of the PNS and PM-enriched fraction were resolved by 2D electrophoresis and thusly separated proteins were stained by the fluorescence dye Sypro Ruby. By comparing of master gels representing proteins of the PNS and PM-enriched fraction, roughly one third of all detected proteins were found in both preparations, another third was only in the PNS and the remaining one in the PM-enriched fraction (Fig. 5.5). This observation can be explained by separation of the soluble, cytosolic proteins from the membrane-bound proteins which fell from the top to lower layers of the density gradient. The proteins which were detected only in the PNS are mostly the cytosolic proteins. Contrarily, the isolation of membrane-bound proteins enabled to detect other low-abundant proteins

from membranes. Thus, the PM-enriched fraction was suitable for determination of hormone-induced alterations of minor proteins.

It is noteworthy that we firstly compared the protein composition of the postnuclear supernatant from control and TRH-treated (1.10^{-5} M, 16 h) HEK293-E2M11 cells resolved by 2D electrophoresis followed by staining with Sypro Ruby. This comparison revealed only two qualitative and two quantitative protein alterations. However, 61 protein alterations were found by comparison of protein composition of the PM-enriched fraction from the same cell samples (Fig. 5.6). The TRH exposure led to disappearance of 4 proteins and to decrease and increase in the levels of 18 and 39 proteins, respectively. Forty-two altered proteins were successfully identified by mass spectrometry. None of the identified proteins was strictly plasma membrane-bound, which corresponds well to the previous study showing no changes in protein composition of membrane microdomains (Matousek *et al.*, 2005). The majority of the identified proteins were membrane-bound proteins from mitochondria, endoplasmic reticulum, Golgi apparatus, nucleus or vesicles.

One of the identified proteins whose level was enhanced by TRH treatment was ezrin which is a member of the ezrin-radixin-moesin (ERM) family. These proteins ensure cross-link of cortical actin with the plasma membrane via its N-terminal four-point-one ERM (FERM) domain (Cant and Pitcher, 2005). This domain interacts with PIP_2 and plasma membrane-bound proteins while the C-terminal domain includes an actin-binding module (McClatchey and Fehon, 2009). It was reported earlier that this protein directly interacts with α_{1B}-adrenergic receptor through a polyarginine motif in the C-terminal region of the receptor and this interaction contributes to receptor recycling to the plasma membrane (Stanasila *et al.*, 2006). GPCRs were shown to bind to ERM-binding protein 50 (EBP50), which is also known as NHERF1. Association of receptors with EBP50 appears to be important in receptor trafficking (Cao *et al.*, 1999; Li *et al.*, 2002; Weinman *et al.*, 2006). Ezrin can be phosphorylated at Thr^{567} which was shown to be mediated by GRK2, PKC or Rho kinase. This posttranslational modification is required for maintaining ezrin in its active conformation and agonist-induced internalization of the β_2-adrenergic receptor in HEK293 cells (Cant and Pitcher, 2005). Therefore, it can be supposed that the increase in the level of ezrin induced by prolonged TRH treatment can be connected with recycling of TRH receptor to the plasma membrane and receptor trafficking as well as actin re-organization.

Analysis of altered proteins by mass spectrometry enabled identification of several mitochondrial proteins which take part in formation of cristae and thus are necessary for function of mitochondria. The identified proteins included prohibitin, peroxiredoxin III, MTHSP75 (GRP75, mortalin), mitofilin and stomatin like-2 protein.

Prohibitin is a protein forming large complexes at the inner mitochondrial membrane and its loss lead to dysfunction of mitochondria, increased generation of reactive oxygen species, abnormal cristae morphology and an increased sensitivity towards stimuli which provoke apoptosis (Osman *et*

al., 2009). This protein interacts with GTPase OPA1 and the membrane phospholipids cardiolipin and phosphatidylethanolamine which are required for mitochondrial fusion and cristae morphogenesis (Osman *et al.*, 2009). It has been reported that depletion of cardiolipin caused a release of cytochrome c from the mitochondrial inner membrane and accelerated apoptosis (Choi *et al.*, 2007). Moreover, oxidation of cardiolipin can lead to a release of cytochrome c from the mitochondrial inner membrane because cardiolipin oxidized by reactive oxygen species (ROS) was shown to have a lower affinity for cytochrome c than the unoxidized form (Montero *et al.*, 2010). Thus, prohibitin complexes serving as protein and lipid scaffolds can control mitochondrial structure and integrity. Another identified mitochondrial protein, stomatin like-2 protein (SLP-2), was found to directly interact with prohibitin and stabilize it (Osman *et al.*, 2009). It also binds to cardiolipin. Up-regulation of SLP-2 increases cardiolipin content in mitochondrial membrane and induces mitochondrial biogenesis (Christie *et al.*, 2011).

Peroxiredoxin III is a mitochondrial antioxidant protein which scavenges H_2O_2 and protects cardiolipin from lipid peroxidation (Wolf *et al.*, 2010). This protein was shown to protect neurons against cell death induced by oxidative stress and its over-expression attenuates oxidative stress in cardiac muscle cells (Tsutsui *et al.*, 2009). Depletion of peroxiredoxin III in HeLa cells led to an increase of intracellular levels of H_2O_2, a release of cytochrome c, caspase activation and sensitization of cells to induction of apoptosis by staurosporine or TNF-α (Chang *et al.*, 2004; John *et al.*, 2005). It has been reported that this antioxidant protein is able to protect pancreatic β cells from cell stress induced by accumulation of H_2O_2 or induction of caspase-9 and -3 by pro-inflammatory cytokines (Wolf *et al.*, 2010).

Mitofilin is a part of a large multimeric protein complex which is anchored in the inner mitochondrial membrane. It seems that this protein is essential for the formation of normal tubular cristae and cristae junctions because depletion of mitofilin resulted in a strong change in the organization of inner membrane and mitochondrial structural and functional abnormalities (John, 2005).

MTHSP75 (GRP75, mortalin) is a mitochondrial chaperone which interacts with different metabolic enzymes, proteins involved in cell survival and differentiation and protein p53. After activation of p53, this protein translocates to the mitochondria where it binds to anti-apoptotic proteins Bcl-2 and BclXL resulting in a release of cytochrome c (Mihara *et al.*, 2003). MTHSP75 operates as an inhibitor of p53 function and inhibits p53-targeted gene expression. Moreover, it is likely to be involved in p53 degradation (Czarnecka *et al.*, 2006).

Altogether, the levels of all these five mitochondrial proteins were enhanced by the prolonged TRH treatment suggesting the TRH exposure can support mitochondrial function and integrity. It can also prevent a release of cytochrome c from mitochondrial membrane to cytoplasm and thus inhibit induction of caspase activity. The support of mitochondrial function by TRH can be connected with

the increased levels of succinate dehydrogenase complex subunit A flavoprotein precursor, ubiquinol-cytochrome c reductase core protein I, NADH dehydrogenase (ubiquinone) Fe-S protein 3 and PMPCA protein which were detected by mass spectrometry in our study (Table 1).

TRH has been reported to induce apoptosis or have neuroprotective effects, which was investigated mainly in β pancreatic and neuronal cells. Treatment with 200 nM TRH for 1 h was shown to up-regulate a gene for small inducible cytokine subfamily A20 reported as a factor inhibiting NF-κB activation in islets and protecting from cytokine- and death receptor-mediated apoptosis (Luo and Yano, 2005). Contrarily, this exposure led to down-regulation of pro-apoptotic genes coding Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter (BAD) and Fas antigen (Luo and Yano, 2005). Another study observing the effect of TRH on rat pancreas revealed that TRH exposure stimulated pro-apoptotic gene p53 as well as Bcl-2-like protein 1 (BclX), which might indicate dual biological actions in different pancreatic cell types (Yano and Luo, 2004). On the other hand, treatment of human islet-derived precursor cells (hIPCs) with 1 μM TRH for 24 h decreased the number of viable cells and resulted in a release of cytochrome c from mitochondria (Mulla *et al.*, 2009).

TRH was shown to be involved in regulation of the activity of some proteins which may take part in the pathogenesis of Alzheimer's disease. It was proposed that a particular signalling pathway triggered by TRH is linked with the activity of glycogen synthase kinase 3β (GSK3-β) and tau phosphorylation in hippocampal neurons (Luo *et al.*, 2002). Tau is a phosphoprotein binding to microtubules and maintaining the cell cytoskeleton. When it is hyperphosphorylated by protein kinases (e.g. GSK3-β), it aggregates into paired helical filaments and becomes unable to bind to microtubules. TRH seems to trigger the MAPK signalling pathway to inhibit GSK3-β activity and prevent tau phosphorylation. Depletion of TRH resulted in reduction of MAPK signalling, increased GSK3-β activity and hyperphosphorylation of tau. Nevertheless, other signalling pathways are likely to be involved as well (Luo *et al.*, 2002).

Another study assessed TRH effects in primary neuronal cell culture (Jantas *et al.*, 2009). Pretreatment with 1 μM TRH for 24 h attenuated the neuronal cell death evoked by glutamate, H_2O_2 and staurosporine but it did not affect neuronal cell death induced by doxorubicin (agent activating death receptor FAS) or lactacystin (agent inhibiting proteasome function). Neither wortmannin, an inhibitor of the pro-survival PI3-K pathway, nor PD 098059, an inhibitor of the MAPK/ERK1/2 pathway, inhibited the neuroprotective effect of TRH in staurosportine evoked cell death. TRH neuroprotection was shown not to be connected with pro-apoptotic GSK3-β and JNK protein kinase signalling pathways. Nevertheless, the increase in spectrin α II product cleaved by calpains after glutamate treatment was blocked by TRH suggesting the engagement of calpains in the neuroprotective effect of this hormone (Jantas *et al.*, 2009).

Interestingly, another mechanism of TRH neuroprotection was reported in a study exploring TRH effects in human neuroblastoma SH-SY5Y differentiated by retinoic acid (RA) (Jaworska-Feil *et al.*, 2010). Contrary to the study of Jantas and co-workers (Jantas *et al.*, 2009), the neuroprotective effect of TRH on staurosporine-induced cell death was caspase-3-dependent and attenuated by the inhibitor of PI3-K pathway wortmannin. Treatment with TRH neuroprotective concentrations (1 μM and 10 μM) for 24 h increased the level of anti-apoptotic protein Bcl-2 (Jaworska-Feil *et al.*, 2010).

The Bcl-2/Bax expression ratio is determined to evaluate the relative sensitivity or resistance of cells to apoptotic stimuli (Li *et al.*, 2010; Patel and Brewer, 2008; Pirzadeh *et al.*, 2011; Reed, 1998). Bcl-2 and Bax proteins represent effectors of cell survival and cell death, respectively. In order to assess apoptosis in TRH-treated cells, the Bcl-2/Bax ratio was analysed by determination of Bcl-2 and Bax expression levels in control, TRH-treated, campthotecin-treated or campthotecin/TRH-treated cells (Fig. 5.6). Campthotecin treatment resulted in a decrease of the Bcl-2/Bax ratio compared to the corresponding control sample. Contrarily, the Bcl-2/Bax ratios of TRH-treated and campthotecin/TRH-treated cells were increased in comparison with these ratios in control and campthotecin-treated cells, respectively. This increase was caused by decline in the expression of Bax. Similar observation was reported in the study of Luo and co-workers (Luo and Yano, 2005) where the expression of Bax in differentiated rat immortalized β-cell line (INS-1) was attenuated three times after treatment with 200 nM TRH for 1 h.

In conclusion, the effects of TRH can differ dependently on TRH concentration as well as type of the observed cells. In any case, prolonged treatment with 10 μM TRH of HEK293 can trigger the cytoprotective processes. In addition, our findings suggest that TRH may have supportive effect on the structure of mitochondrial membrane, formation of cristae and mitochondrial function.

6.2. A study of TRH-R1 and $G_{q/11}$ protein complexes

Specific protein-protein interactions between signalling molecules determine which signalling pathways will become activated and involved in coordinating cellular responses. Information from multiple environmental inputs mediated through various signalling pathways is integrated and may bring about a number of different cell processes such as cell differentiation, cell cycle arrest, morphological changes and apoptosis. Interactions between proteins occur due to unique structural motifs (domains) in proteins. Nowadays, great attention is paid to protein-protein interactions regarding not only signalling molecules but also other proteins such as structural and transport proteins, transcription factors and enzymes. The term “interactome” stands for the map of all interactions in the cell.

There are different methods available for determining of protein-protein interactions. Among the most popular methods used in these days are methods based on fluorescence microscopy – fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET). The advantage of these methods lies in the precision of measurement and detection of interactions in living cells. These techniques certainly represent very useful tools for studying interactions of two chosen molecules. However, proteins can interact with several different partners at the same time and thus these techniques are not quite suitable for investigation of more complex protein interactions. Even if the incorporation of fluorescent proteins into molecules of chosen proteins would not influence interactions between those two proteins and these proteins can still interact physiologically, it is not clear whether interactions with other interacting protein partners would be affected under these conditions or not. In case that some protein interactions were altered by insertion of fluorescent tags into protein molecules, the labelled proteins under investigation may interact with each other under "unphysiological" conditions, because their interactions with some other natural partners can be perhaps disturbed at the same time. It is worth mentioning in this context, that not always FRET and BRET provide the same results. Whereas no interaction between α_{2A}-adrenergic receptor and $G_{i1}\alpha$ was observed by Hein and co-workers (Hein *et al.*, 2005) using FRET, Gales and co-workers (Gales *et al.*, 2006) reported that these two proteins can interact to some extent even under resting conditions in cells (assessed by BRET).

In our present study, we aimed to investigate the molecular complexes of TRH receptor and/or $G_{q/11}$ protein by clear native electrophoresis (CN-PAGE). By this method, it is possible to detect several complexes in the same experiment and find out the possible interacting proteins in detected complexes. We succeeded in detecting several molecular complexes of TRH receptor and/or $G_{q/11}$ protein in several gel regions using varied solubilisation conditions. Three complexes of TRH-R1 were found in gel regions with molecular sizes of 80 kDa, 140 kDa and 500 kDa and four complexes of $G_{q/11}\alpha$ protein in gel regions with molecular sizes of 70 kDa, 140 kDa, 300 kDa and 700 kDa. The complexes of $G_{q/11}\alpha$ protein with 140 kDa and 300 kDa molecular sizes were proved to include $G\beta\gamma$ heterodimer while the others are likely to contain only $G_{q/11}\alpha$ protein. These results suggest that TRH receptor forms several complexes which contain $G_{q/11}$ protein in some cases, and that the $G_{q/11}\alpha$ and $G\beta$ subunits can interact with other suitable partners.

Experiments with increased temperature (37 °C) during solubilisation revealed that protein complexes of $G_{q/11}\alpha$ and TRH-R dissociated into monomeric $G_{q/11}\alpha$ protein (~ 45 kDa) and TRH-R complex with molecular size of 80 kDa, respectively (Fig. 5.11). TRH-R1 has been reported to form homodimers or heterooligomers with TRH-R2 but not with other GPCRs such as GnRH-R or β_2-adrenergic receptor (Hanyaloglu *et al.*, 2002; Pfleger *et al.*, 2004). The TRH-R complex in the 80 kDa region most likely corresponds to TRH-R1 homodimer because HEK293 cell line does not express endogenous TRH receptors.

It seems that both TRH-R1 and $G_{q/11}$ complexes observed around 140 kDa would constitute one single complex which would represent the presumed pre-associated complex of the receptor with trimeric G-protein. Interactions between GPCRs and trimeric G-proteins in HEK293 cells were previously studied by using FRET, BRET or co-immunoprecipitation. It was shown in many cases that at least a certain amount of a particular receptor was directly connected with its cognate G-protein (Audet *et al.*, 2008; Ayoub *et al.*, 2007; Gales *et al.*, 2006; Martin Shreeve, 2002; Nobles *et al.*, 2005; Philip *et al.*, 2007). However, the current evidence preferring the existence of pre-associated receptor and G-protein complexes is not fully conclusive. Hein and co-workers failed to see direct connection of the α_{2A}-adrenergic receptor and $G_{i1}\alpha$ protein in living cells (Hein *et al.*, 2005) while the interaction between these signalling molecules was observed in a study of Gales and co-workers (Gales *et al.*, 2006).

The putative TRH-R–$G_{q/11}$ signaling complex was detected in the postnuclear supernatant as well as in the plasma membrane-enriched fraction after solubilisation with different detergents followed by CN-PAGE or CN/SDS-PAGE. The assumption that TRH-R1 forms pre-associated complex with $G_{q/11}$ protein was verified by experiments with increased solubilisation temperature, experiments with cells expressing different levels of $G_{q/11}$ and by co-immunoprecipitation (Fig. 5.11-5.14). Results of these experiments indicated that TRH-R and $G_{q/11}$ represent one complex and do not constitute two separated molecular complexes.

It must be kept in mind, however, that the existence of protein complexes in the gel does not necessarily reflect the situation in an intact living cell and that solubilisation arteifacts can occur. In order to exclude that the presumed TRH-R–$G_{q/11}$ signaling complex constitutes solubilisation artefact, a set of control experiments was conducted. Results of these experiments indicated that the solubilisation conditions apparently did not induce the formation of artificial complexes. Samples of the PNS from control cells were separated after solubilisation with different detergents (1% Brij, 1% digitonin or 1% Triton X-100) on 10-15% polyacrylamide gels and immunoblotted with specific antibodies against α_{2A}-adrenergic receptor (AR) or $G_{i1,2}\alpha$. Absolute majority of α_{2A}-AR migrated at 80 kDa, which apparently reflects the receptor dimers (Small *et al.*, 2006), and only a very faint signal appeared in the 140 kDa region, in which a strong signal of TRH receptor was found. Contrarily to $G_{q/11}\alpha$ protein, some signal of $G_{i1,2}\alpha$ was detected at 140 kDa, but a substantial amount of this protein was distributed in other bands, out of which the 40 kDa band was the most prominent (corresponding to monomeric $G_{i1,2}\alpha$). Our observation indicating that a fraction of α_{2A}-AR and $G_{i1}\alpha$ may form complexes is in accord with previous study of Gales and co-workers (Gales *et al.*, 2006). These results together with some earlier data (Gales *et al.*, 2006; Hein *et al.*, 2005; Qin *et al.*, 2008) suggest that G_i proteins and their cognate GPCRs may probably form only limited amount of pre-associated complexes and predominantly exist in an uncoupled state because of lower affinity of G_i proteins to receptors. By contrast, a significant amount of $G_{q/11}\alpha$ was observed just in the 140 kDa complexes. It

seems that $G_{q/11}\alpha$ proteins preferentially couple to their cognate GPCRs. These findings support the view that different G-proteins can significantly differ in their ability to form molecular complexes with their cognate receptors.

It is known that interactions between GPCRs and G-proteins in molecular complexes can be strongly affected by ligand binding, which can result in alterations in stability and composition of these complexes (Christopoulos and Kenakin, 2002; Tuteja, 2009). Therefore, it was of interest to investigate the consequences of short as well as prolonged hormone treatment on the identified TRH-R1–$G_{q/11}$ protein complex. HEK293-E2M11 cells were treated with TRH for different time intervals and the solubilised protein complexes were separated by CN-PAGE or CN/SDS-PAGE and identified by immunoblotting with antibodies against TRH-R1, $G_{q/11}\alpha$ or Gβ proteins (Fig. 5.15-5.17). The immunoblot patterns of the TRH-R1–$G_{q/11}$ protein complex after TRH treatment differed dependently on applied antibodies. The immunoblot signals of TRH-R1 declined after 10-min and 30-min treatment with TRH and prolonged treatment resulted in its restoration. In parallel, the immunoblot signals of $G_{q/11}\alpha$ or Gβ proteins markedly decreased after prolonged hormone treatment (2–16 h). This discrepancy in patterns of immunoblot signals and detection of two molecular complexes in the 140 kDa region containing $G_{q/11}\alpha$ protein but only one TRH-R1 complex in this region suggest that other endogenous GPCRs expressed in HEK293 cells (e.g. α_{1B}-adrenergic receptor, adenosine 2 receptor, acetylcholine muscarinic M3 receptor and δ-opioid receptor) (Atwood *et al.*, 2011) can also form similar receptor–G-protein complexes. This assumption was vindicated by detection of two molecular complexes in the 140 kDa area containing $G_{q/11}$-coupled α_{1B}-adrenergic receptor (α_{1B}-AR). The two detected bands formed a signal pattern which closely resembled the one seen for $G_{q/11}\alpha$ protein (Fig. 5. 19). Weak immunoblot signals in the 140 kDa region were also found for muscarinic M1 receptor and bradykinin B2 receptor (data not shown). This observation supports our hypothesis stated above that $G_{q/11}$ proteins are not discrete entities in non-stimulated cells and preferentially couple to their cognate GPCRs.

As mentioned above, the TRH-R1–$G_{q/11}$ protein complex dissociated after short-term hormone treatment (10–30 min). Similar phenomena were observed in cells expressing PARs-YFP (protease-activated receptors) and $G_{i1}\alpha$-RLuc by BRET (Ayoub *et al.*, 2007). A strong basal BRET signal assessed between these molecules under resting conditions was diminished after receptor activation with thrombin. The BRET signal totally disappeared after agonist treatment for 30 min.

In our study, the dissociation of the TRH-R1–$G_{q/11}$ protein complex was accompanied by increase in the levels of TRH-R1 homodimer (Fig. 5.18) and TRH-R1 complex in the 500 kDa region (Fig. 5.26). These results suggest that TRH-R1 dissociated from TRH-R1–$G_{q/11}$ protein complex is partly re-arranged into free TRH-R1 homodimer and partly into a high-molecular-weight complex in which TRH receptor could interact with signalling molecules conveying the signal to other components of different signalling pathways. Because there was found no increase in the level of

TRH-R1 homodimer and no signal of TRH-R1 monomer, it can be supposed that TRH-R1 can occur in the form of homodimer in the putative TRH-R–$G_{q/11}$ protein complex. It was reported earlier that one molecule of receptor dimers interacts with α subunit of G-protein while the other with Gβγ heterodimer (Nobles *et al.*, 2005). Therefore, it can be assumed that TRH receptor with heterotrimeric G-protein form a pentameric complex.

The increased level of $G_{q/11}α$ complex in the 700 kDa region observed after short-term treatment with TRH (Fig. 5.27) suggests that activated $G_{q/11}α$ could translocate into this complex from the pre-associated TRH-R1-$G_{q/11}$ protein complex. This supposition was confirmed by autoradiography with [^{35}S]GTPγS when basal activity of $G_{q/11}α$ was detectable in the 40 kDa region representing monomer of $G_{q/11}α$ as well as in the 700 kDa region representing a high-molecular-weight $G_{q/11}α$ protein complex (Fig. 5.28). After TRH was added into the reaction mixture used in [^{35}S]GTPγS binding experiment, the signals of [^{35}S]GTPγS in the 40 kDa and 700 kDa regions increased. Because the $G_{q/11}α$ complex in the 700 kDa region apparently does not contain TRH-R, it can be assumed that this complex containing activated $G_{q/11}α$ protein is constituted after short-term TRH treatment.

Prolonged treatment with TRH had an opposite effect on the stability of the presumed TRH-R1–$G_{q/11}$ protein complex as compared to short-term TRH treatment because it led to a partial re-association of this complex. This re-association was detected only after solubilisation of samples with Brij 56 or Triton X-100 followed by CN/SDS-PAGE and immunoblotting, but not after solubilisation with lauryl maltoside or digitonin. Nevertheless, these results are not very surprising because it is known that the effectiveness of solubilisation depends on the character of the detergent and on the detergent-to-protein ratio (Helenius and Simons, 1977; Wittig *et al.*, 2007). Next, the extent of dissociation/re-association of the TRH-R1–$G_{q/11}$ protein complex in the course of hormone treatment was assessed in the plasma membrane-enriched fraction (Fig. 5.16 and 5.17). Because the amount of membrane complexes in this fraction was higher than in the postnuclear supernatant, the ratio of the receptor complexes and detergent concentration was expected to increase as well. In this experiment, digitonin was used as detergent. When using CN/SDS-PAGE and immunoblotting, a decrease in the immunoblot signal of TRH-R was detected after short-term TRH treatment while long-term treatment led to increase of this signal. These results support our findings from experiments with samples of the postnuclear supernatant where re-association of the TRH-R1–$G_{q/11}$ protein complex occurred after prolonged TRH treatment. This observation seems to be in contradiction with a general concept that GPCRs are down-regulated after sustained agonist treatment (Li *et al.*, 2000). However, it is worth noting that TRH receptor has been shown to be up-regulated after long-term treatment with TRH (Cook and Hinkle, 2004; Drmota *et al.*, 1999), which was also confirmed by immunoblotting in our study. Therefore, it is not surprising that a partial restoration of the TRH-R1–$G_{q/11}$ protein complex was detected.

As mentioned above, prolonged TRH treatment resulted in strong diminution of the immunoblot $G_{q/11}$ signal in the 140 kDa region. A similar pattern was observed for $G_{q/11}$ protein complex in the 300 kDa region (Fig. 5.21 and 5.22). This complex was solubilised only by Brij 56 and neither TRH-R nor α_{1B}-AR was detected in this region suggesting that these receptors are not components of this complex. The TRH-R1–$G_{q/11}$ protein complex and $G_{q/11}$ protein complex in the 300 kDa region were proved to be membrane-bound. The decrease in immunoblot signals of both these membrane-bound complexes after prolonged TRH treatment can be explained by down-regulation and translocation of $G_{q/11}\alpha$ proteins (Drmota *et al.*, 1998). Contrarily to these complexes, the 70 kDa low-molecular-weight complexes of $G_{q/11}\alpha$ in the cytosol were formed during TRH exposure (Fig. 5.23 and 5.24). There was a significant rise of the immunoblot $G_{q/11}\alpha$ signal in the 70 kDa region after prolonged hormone treatment (2–16 h). These complexes were solubilised with all used detergents, which can be explained by their cytosolic localization. All these results indicate that the prolonged TRH treatment caused translocation of a portion of $G_{q/11}\alpha$ from membrane-bound complexes into cytosolic complexes.

Because a protein complex in the 300 kDa region was shown not to contain TRH receptor, it seems that this complex is not directly engaged in TRH-R signalling. The reason why the stability of this complex is affected by TRH treatment can be the assumption that the amounts of individual components involved in particular complex determine subsequently the amount of this complex. Therefore, the decreased amount of $G_{q/11}\alpha$ in the plasma membrane due to down-regulation and translocation of $G_{q/11}\alpha$ during the prolonged TRH treatment lowers the amount of individual membrane-bound complexes involving $G_{q/11}\alpha$.

Translocation of Gα proteins (mainly $G_s\alpha$ and $G_{q/11}\alpha$) from the plasma membrane into cytosol has been reported in many previous studies in which cell fractionation, immunofluorescence microscopy and cell live imaging were applied to investigate this process (Allen *et al.*, 2005; Hynes *et al.*, 2004;, Thiyagarajan *et al.*, 2002; Wedegaertne *et al.*r, 1996; Yu *et al.*, 2002; Ransnas, 1989; Miserey-Lenkei *et al.*, 2001; Kosloff *et al.*, 2003; Cronin *et al.*, 2004; Drmota *et al.*, 1998). This process was observed in HEK293, MCF7, C6 and S49 cell lines (Allen *et al.*, 2005; Hynes *et al.*, 2004; Thiyagarajan *et al.*, 2002; Wedegaertner *et al.*, 1996) whereas no redistribution of $G_s\alpha$ was found in COS-7 monkey kidney cells or MA104 cells derived from rhesus monkey kidney (Huang *et al.*, 1999; Jones *et al.*, 1997). It was shown that translocation of Gα protein is a rapid process, occurring within 1–20 min after addition of agonist (Marrari *et al.*, 2007). Subcellular redistribution of $G_s\alpha$ was observed to be connected with activation-dependent depalmitoylation of Gα subunit (Wedegaertner and Bourne, 1994). Nevertheless, the rate of translocation of $G_{q/11}\alpha$ proteins appears to be dependent on the type of activated receptor. Subcellular redistribution of these proteins was investigated after activation of rhodopsin in Drosophila eyes or after activation of angiotensin 2 receptor (AT2 receptor) and TRH-R1 in HEK293 cells (Marrari *et al.*, 2007). While the soluble form

of $G_q\alpha$ was detected in studies with rhodopsin and AT2 receptor within 5–20 min after stimulation with agonist (Cronin *et al.*, 2004; Kosloff *et al.*, 2003; Miserey-Lenkei *et al.*, 2001), $G_{q/11}\alpha$ translocation to the cytosol was not observed earlier than 2 h after TRH treatment (Drmota *et al.*, 1998). Hence, our findings concerning translocation of $G_{q/11}\alpha$ from membrane-bound complexes into cytosolic complexes after prolonged hormone treatment conform well to previous observations.

A relatively high TRH (10 μM) concentration, which was applied in our experiments dealing with the stability of receptor or G-protein complexes, cannot be considered physiological although it did not seem to induce apoptosis in our cell model (Drastichova *et al.*, 2010). Therefore, it was of interest to check whether translocation of $G_{q/11}\alpha$ from membrane-bound into cytosolic complexes would occur also at lower concentrations of TRH. Although dissociation of the membrane-bound $G_{q/11}$ protein complex in the 300 kDa region was observed mainly at higher concentrations of TRH (10^{-5} – 10^{-7} M, 16 h), dissociation of the membrane-bound TRH-R1–$G_{q/11}$ protein complex and formation of cytosolic complexes were observed at all applied TRH concentrations (10^{-5}–10^{-9} M, 16 h) (Fig. 5.25). However, these dissociation/association processes occurred to a greater extent at higher concentrations (10^{-5}–10^{-7} M) than at lower concentrations (10^{-8}–10^{-9} M) of TRH, which suggests that formation of low-molecular-weight complexes in the 70 kDa region is more likely to be connected with dissociation of the 140 kDa protein complex. Hence, it can be concluded that the stability and formation of protein complexes may be influenced not only by extremely high but also by relatively low physiological hormone concentrations, although the effect of lower concentrations is weaker.

The levels of some of the detected molecular complexes increased after TRH treatment suggesting a partial relocation of TRH-R or $G_{q/11}$ from the 140 kDa complexes to protein complexes containing other interacting partners of these proteins. These complexes are represented by a TRH-R1 complex in the 500 kDa region, $G_{q/11}\alpha$ complex in the 700 kDa region and $G_{q/11}\alpha$ complexes in the 70 kDa region. To understand which signalling pathways can be triggered after hormone treatment, it is important to determine molecular partners interacting with the proteins identified in the observed complexes. We selected some proteins which could possibly interact with TRH receptor or $G_{q/11}$ protein. These proteins are Gβ protein, PLCβ, GRK2, NHERF1, RGS2-5, clathrin, β-arrestin, caveolin-1 and flotillin-2.

It seems that Gβ protein could be a component of both high-molecular-weight TRH-R and $G_{q/11}\alpha$ complexes in the 500 kDa and 700 kDa regions, respectively (Fig. 5.29). Nevertheless, it is absolutely not clear if $G_{q/11}\alpha$ proteins uncouple from Gβγ heterodimers after activation by cognate receptors or if only re-arrangement of G-protein structure occurs (Digby *et al.*, 2006). Gβγ was shown to interact with the third intracellular loop of GPCRs (Oldham and Hamm, 2008). However, this interaction is required for stabilization of the receptor-Gα interface. Thus, it is necessary to verify interactions of Gβ with TRH-R and $G_{q/11}\alpha$ in protein complexes of 500 kDa and 700 kDa, respectively.

Among the above mentioned potential interacting partners of TRH-R and $G_{q/11}\alpha$ protein, only immunoblot signals of GRK2 and PLCβ were detected in gel regions in which molecular complexes of TRH-R and $G_{q/11}\alpha$ were found. Both these proteins were observed in the regions of 500 kDa and 700 kDa (Fig. 5.30). GRK2 is known to interact with GPCRs as well as Gα proteins. It interacts with GPCRs in phosphorylation-dependent as well as phosphorylation-independent manner during receptor desensitization. This interaction is also critical for full activity of GRK2 (Pao and Benovic, 2005; Ribas *et al.*, 2007). Moreover, the RH domain of GRK2 interacts with different residues in the switch I and switch III regions of $G_{q/11}\alpha$ (Day *et al.*, 2004; Sterne-Marr *et al.*, 2003). PLCβ has been shown to directly interact with $G_q\alpha$ and this interaction is stronger when $G_q\alpha$ is activated (Drin and Scarlata, 2007). PLCβ1 has been also reported to interact with $G_{q/11}$-coupled bradykinin B2 receptor via PDZ scaffold protein Par-3 (Choi *et al.*, 2010). Par-3 was originally identified as a protein involved in cell polarity (Munro, 2006) and found to function as a scaffold for atypical protein kinase Cζ and protein phosphatase 1α (PP1α) (Traweger *et al.*, 2008). Therefore, it appears that PLCβ and GRK2 could be components of the 700 kDa $G_{q/11}\alpha$ complex and the 500 kDa TRH-R complex. Nevertheless, this well-founded speculation must be verified by experiments.

7. Conclusions

The first part of this thesis is preoccupied with the identification of protein alterations in the membrane fraction of HEK293-E2M11 cells after prolonged TRH treatment. The isolated membrane fraction enriched in plasma membranes contained high amounts of Na^{+},K^{+}-ATPase, TRH receptor and G-proteins compared to the postnuclear supernatant. By using 2D electrophoresis and mass spectrometry, the levels of 42 proteins were identified to be altered in samples of PM-enriched fractions from TRH-treated (16 h; 10 µM) cells. Out of these proteins only ezrin and stomatin-like 2 are known to be localized in the plasma membrane. Five proteins (mitofilin, MTHSP75, prohibitin, stomatin like-2, peroxiredoxin III) whose levels were increased after the prolonged TRH treatment represent proteins localized in mitochondria. All of them are important for proper structure and function of mitochondria. The ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax was markedly higher in cells treated with TRH compared to control cells. Hence, it can be concluded that prolonged TRH treatment may significantly affect mitochondrial membrane and function of mitochondria.

The second part of this thesis deals with the identification of molecular protein complexes of TRH-R and/or $G_{q/11}$ protein. The presumed effects of TRH on the stability of these complexes have also been investigated. By native electrophoresis, three complexes of TRH-R and four complexes of $G_{q/11}\alpha$ protein were identified. The TRH-R complex found in the 80 kDa region corresponds to TRH-R dimer, which was proved by experiments analysing the effect of solubilisation at different temperature. The molecular complex detected in the 140 kDa region represents a pre-associated TRH-R–$G_{q/11}$ complex, which was verified by co-immunoprecipitation and experiments on cells with decreased levels of the Gα and Gβ subunits of $G_{q/11}$ proteins. Short-term (10–30 min) treatment with TRH led to dissociation of the pre-associated TRH-R–$G_{q/11}$ complex with concomitant increase in the level of TRH-R dimer while long-term TRH treatment resulted in partial re-association of TRH-R–$G_{q/11}$ complex apparently due to up-regulation of TRH-R. The immunoblot signal of $G_{q/11}$ protein in the 140 kDa region apparently corresponds not only to the pre-associated TRH-R–$G_{q/11}$ complex but also to molecular complexes of $G_{q/11}$ protein with other $G_{q/11}$-coupled receptors.

The $G_{q/11}$ complexes found in the 140 kDa and 300 kDa regions were observed to be membrane-bound while $G_{q/11}\alpha$ complexes in the 70 kDa region were cytosolic. The cytosolic $G_{q/11}\alpha$ complexes were observed to associate after prolonged TRH treatment which was connected with dissociation of membrane-bound $G_{q/11}$ complexes in the 140 kDa and 300 kDa regions and translocation of $G_{q/11}\alpha$ from the plasma membrane into the cytosol. The second reason for a decrease in the level of membrane-bound $G_{q/11}$ complexes is down-regulation of $G_{q/11}\alpha$ proteins after prolonged TRH treatment.

High-molecular-weight complexes of TRH-R or $G_{q/11}\alpha$ were observed in the 500 kDa or 700 kDa regions, respectively. The levels of both these complexes were found to increase after short-term

TRH treatment suggesting that TRH-R and $G_{q/11}\alpha$ can be translocated from the pre-associated TRH-R–$G_{q/11}$ complex. In case of $G_{q/11}\alpha$, this hypothesis was confirmed by [^{35}S]GTPγS binding assay followed by autoradiography. The signal of bound [^{35}S]GTPγS in the 700 kDa $G_{q/11}\alpha$ complex was markedly enhanced after TRH treatment. GRK2 and PLCβ were assessed as potential components of these high-molecular-weight complexes.

Altogether, our present studies have demonstrated that native electrophoresis can serve as a highly suitable method for identification of molecular complexes. By using this approach we were able to show that the TRH receptor may form a pre-associated complex with its cognate G-protein and therefore it can be included among other GPCRs that constitute such complexes. The stability of this TRH-R–$G_{q/11}$ complex as well as other resolved receptor or $G_{q/11}$ protein complexes were markedly influenced by TRH treatment, which indicates that hormones can modulate the interactions between proteins and re-arrange proteins within complexes in the plasma membrane.

8. References

Abramow-Newerly, M., Roy, A.A., Nunn, C. and Chidiac, P. (2006) RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. Cell Signal. 18:579-591.

Aittaleb, M., Boguth, C.A. and Tesmer, J.J. (2010) Structure and function of heterotrimeric G protein-regulated Rho guanine nucleotide exchange factors. Mol Pharmacol. 77:111-125.

Akgoz, M., Kalyanaraman, V. and Gautam, N. (2004) Receptor-mediated reversible translocation of the G protein βγ complex from the plasma membrane to the Golgi complex. J Biol Chem. 279:51541-51544.

Akgoz, M., Kalyanaraman, V. and Gautam, N. (2006) G protein βγ complex translocation from plasma membrane to Golgi complex is influenced by receptor γ subunit interaction. Cell Signal. 18:1758-1768.

Albert, A.D. and Litman, B.J. (1978) Independent structural domains in the membrane protein bovine rhodopsin. Biochemistry. 17:3893-3900.

Allen, J.A., Yu, J.Z., Donati, R.J. and Rasenick, M.M. (2005) β-adrenergic receptor stimulation promotes $G_s\alpha$ internalization through lipid rafts: a study in living cells. Mol Pharmacol. 67:1493-1504.

Angers, S., Salahpour, A. and Bouvier, M. (2002) Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. Annu Rev Pharmacol Toxicol. 42:409-435.

Atwood, B.K., Lopez, J., Wager-Miller, J., Mackie, K. and Straiker, A. (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics. 12:14.

Audet, N., Gales, C., Archer-Lahlou, E., Vallieres, M., Schiller, P.W., Bouvier, M. and Pineyro, G. (2008) Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing δ-opioid receptors and heterotrimeric G proteins. J Biol Chem. 283:15078-15088.

Ayoub, M.A., Maurel, D., Binet, V., Fink, M., Prezeau, L., Ansanay, H. and Pin, J.P. (2007) Real-time analysis of agonist-induced activation of protease-activated receptor 1/$G_{i1}\alpha$ protein complex measured by bioluminescence resonance energy transfer in living cells. Mol Pharmacol. 71:1329-1340.

Baldwin, J.M. (1993) The probable arrangement of the helices in G protein-coupled receptors. EMBO J. 12:1693-1703.

Ballou, L.M., Chattopadhyay, M., Li, Y., Scarlata, S. and Lin, R.Z. (2006) $G_q\alpha$ binds to p110α/p85α phosphoinositide 3-kinase and displaces Ras. Biochem J. 394:557-562.

Ballou, L.M., Lin, H.Y., Fan, G., Jiang, Y.P. and Lin, R.Z. (2003) Activated $G_q\alpha$ inhibits p110α phosphatidylinositol 3-kinase and Akt. J Biol Chem. 278:23472-23479.

Baneres, J.L. and Parello, J. (2003) Structure-based analysis of GPCR function: evidence for a novel pentameric assembly between the dimeric leukotriene B4 receptor BLT1 and the G-protein. J Mol Biol. 329:815-829.

Barthet, G., Carrat, G., Cassier, E., Barker, B., Gaven, F., Pillot, M., Framery, B., Pellissier, L.P., Augier, J., Kang, D.S., Claeysen, S., Reiter, E., Baneres, J.L., Benovic, J.L., Marin, P., Bockaert, J. and Dumuis, A. (2009) β-arrestin1 phosphorylation by GRK5 regulates G protein-independent 5-HT4 receptor signalling. EMBO J. 28:2706-2718.

Beaulieu, J.M., Sotnikova, T.D., Marion, S., Lefkowitz, R.J., Gainetdinov, R.R. and Caron, M.G. (2005) An Akt/β-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell. 122:261-273.

Benzing, T., Kottgen, M., Johnson, M., Schermer, B., Zentgraf, H., Walz, G. and Kim, E. (2002) Interaction of 14-3-3 protein with regulator of G protein signaling 7 is dynamically regulated by tumor necrosis factor-α. J Biol Chem. 277:32954-32962.

Benzing, T., Yaffe, M.B., Arnould, T., Sellin, L., Schermer, B., Schilling, B., Schreiber, R., Kunzelmann, K., Leparc, G.G., Kim, E. and Walz, G. (2000) 14-3-3 interacts with regulator of G protein signaling proteins and modulates their activity. J Biol Chem. 275:28167-28172.

Bjarnadottir, T.K., Gloriam, D.E., Hellstrand, S.H., Kristiansson, H., Fredriksson, R. and Schioth, H.B. (2006) Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse. Genomics. 88:263-273.

Blank, J.L., Ross, A.H. and Exton, J.H. (1991) Purification and characterization of two G-proteins that activate the β_1 isozyme of phosphoinositide-specific phospholipase C. Identification as members of the G_q class. J Biol Chem. 266:18206-18216.

Blomquist, A., Schworer, G., Schablowski, H., Psoma, A., Lehnen, M., Jakobs, K.H. and Rumenapp, U. (2000) Identification and characterization of a novel Rho-specific guanine nucleotide exchange factor. Biochem J. 352 Pt 2:319-325.

Blumer, J.B., Smrcka, A.V. and Lanier, S.M. (2007) Mechanistic pathways and biological roles for receptor-independent activators of G-protein signaling. Pharmacology & Therapeutics. 113:488-506.

Bockaert, J., Marin, P., Dumuis, A. and Fagni, L. (2003) The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. FEBS Lett. 546:65-72.

Bockaert, J. and Pin, J.P. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J. 18:1723-1729.

Bourova, L., Kostrnova, A., Hejnova, L., Moravcova, Z., Moon, H.E., Novotny, J., Milligan, G. and Svoboda, P. (2003) δ-Opioid receptors exhibit high efficiency when activating trimeric G proteins in membrane domains. J Neurochem. 85:34-49.

Bourova, L., Stohr, J., Lisy, V., Rudajev, V., Novotny, J. and Svoboda, P. (2009) Isolation of plasma membrane compartments from rat brain cortex; detection of agonist-stimulated G protein activity. Med Sci Monit. 15:BR111-122.

Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. and Nirenberg, M. (1986) Human cDNA clones for four species of $G_s\alpha$ signal transduction protein. Proc Natl Acad Sci U S A. 83:8893-8897.

Bryson-Richardson, R.J., Logan, D.W., Currie, P.D. and Jackson, I.J. (2004) Large-scale analysis of gene structure in rhodopsin-like GPCRs: evidence for widespread loss of an ancient intron. Gene. 338:15-23.

Bulenger, S., Marullo, S. and Bouvier, M. (2005) Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. Trends Pharmacol Sci. 26:131-137.

Bunemann, M. and Hosey, M.M. (1999) G-protein coupled receptor kinases as modulators of G-protein signalling. J Physiol. 517 (Pt 1):5-23.

Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P.J. and Gierschik, P. (1992) Isozyme-selective stimulation of phospholipase C-β 2 by G protein βγ-subunits. Nature. 360:684-686.

Cant, S.H. and Pitcher, J.A. (2005) G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. Mol Biol Cell. 16:3088-3099.

Cantrell, D.A. (2001) Phosphoinositide 3-kinase signalling pathways. J Cell Sci. 114:1439-1445.

Cao, T.T., Deacon, H.W., Reczek, D., Bretscher, A. and von Zastrow, M. (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2-adrenergic receptor. Nature. 401:286-290.

Carman, C.V., Lisanti, M.P. and Benovic, J.L. (1999) Regulation of G protein-coupled receptor kinases by caveolin. J Biol Chem. 274:8858-8864.

Caron, M.G., Srinivasan, Y., Pitha, J., Kociolek, K. and Lefkowitz, R.J. (1979) Affinity chromatography of the β-adrenergic receptor. J Biol Chem. 254:2923-2927.

Chakir, K., Xiang, Y., Yang, D., Zhang, S.J., Cheng, H., Kobilka, B.K. and Xiao, R.P. (2003) The third intracellular loop and the carboxyl terminus of β_2-adrenergic receptor confer spontaneous activity of the receptor. Mol Pharmacol. 64:1048-1058.

Chang, T.S., Cho, C.S., Park, S., Yu, S., Kang, S.W. and Rhee, S.G. (2004) Peroxiredoxin III, a mitochondrion-specific peroxidase, regulates apoptotic signaling by mitochondria. J Biol Chem. 279:41975-41984.

Chen, C., Shahabi, V., Xu, W. and Liu-Chen, L.Y. (1998) Palmitoylation of the rat μ opioid receptor. FEBS Lett. 441:148-152.

Chen, C.A. and Manning, D.R. (2001) Regulation of G proteins by covalent modification. Oncogene. 20:1643-1652.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K. and Stevens, R.C. (2007) High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. Science. 318:1258-1265.

Chien, E.Y., Liu, W., Zhao, Q., Katritch, V., Han, G.W., Hanson, M.A., Shi, L., Newman, A.H., Javitch, J.A., Cherezov, V. and Stevens, R.C. (2010) Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. Science. 330:1091-1095.

Choi, J.W., Lim, S., Oh, Y.S., Kim, E.K., Kim, S.H., Kim, Y.H., Heo, K., Kim, J., Kim, J.K., Yang, Y.R., Ryu, S.H. and Suh, P.G. (2010) Subtype-specific role of phospholipase C-β in bradykinin and LPA signaling through differential binding of different PDZ scaffold proteins. Cell Signal. 22:1153-1161.

Choi, S.Y., Gonzalvez, F., Jenkins, G.M., Slomianny, C., Chretien, D., Arnoult, D., Petit, P.X. and Frohman, M.A. (2007) Cardiolipin deficiency releases cytochrome c from the inner

mitochondrial membrane and accelerates stimuli-elicited apoptosis. Cell Death Differ. 14:597-606.

Christie, D.A., Lemke, C.D., Elias, I.M., Chau, L.A., Kirchhof, M.G., Li, B., Ball, E.H., Dunn, S.D., Hatch, G.M. and Madrenas, J. (2011) Stomatin-like protein 2 binds cardiolipin and regulates mitochondrial biogenesis and function. Mol Cell Biol. 31:3845-3856.

Christopoulos, A. and Kenakin, T. (2002) G protein-coupled receptor allosterism and complexing. Pharmacol Rev. 54:323-374.

Cismowski, M.J. (2006) Non-receptor activators of heterotrimeric G-protein signaling (AGS proteins). Semin Cell Dev Biol. 17:334-344.

Claing, A., Chen, W., Miller, W.E., Vitale, N., Moss, J., Premont, R.T. and Lefkowitz, R.J. (2001) β-Arrestin-mediated ADP-ribosylation factor 6 activation and β_2-adrenergic receptor endocytosis. J Biol Chem. 276:42509-42513.

Conti, M. and Beavo, J. (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem. 76:481-511.

Cook, L.B. and Hinkle, P.M. (2004) Agonist-dependent up-regulation of thyrotrophin-releasing hormone receptor protein. Biochem J. 380:815-821.

Cronin, M.A., Diao, F. and Tsunoda, S. (2004) Light-dependent subcellular translocation of $G_q\alpha$ in Drosophila photoreceptors is facilitated by the photoreceptor-specific myosin III NINAC. J Cell Sci. 117:4797-4806.

Czarnecka, A.M., Campanella, C., Zummo, G. and Cappello, F. (2006) Mitochondrial chaperones in cancer: from molecular biology to clinical diagnostics. Cancer Biol Ther. 5:714-720.

Daaka, Y., Luttrell, L.M. and Lefkowitz, R.J. (1997) Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. Nature. 390:88-91.

Day, P.W., Tesmer, J.J., Sterne-Marr, R., Freeman, L.C., Benovic, J.L. and Wedegaertner, P.B. (2004) Characterization of the GRK2 binding site of $G_q\alpha$. J Biol Chem. 279:53643-53652.

de la Pena, P., Delgado, L.M., del Camino, D. and Barros, F. (1992) Two isoforms of the thyrotropin-releasing hormone receptor generated by alternative splicing have indistinguishable functional properties. J Biol Chem. 267:25703-25708.

DeBlasi, A., O'Reilly, K. and Motulsky, H.J. (1989) Calculating receptor number from binding experiments using same compound as radioligand and competitor. Trends Pharmacol Sci. 10:227-229.

DeFea, K.A. (2011) β-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? Cell Signal. 23:621-629.

Deflorian, F., Engel, S., Colson, A.O., Raaka, B.M., Gershengorn, M.C. and Costanzi, S. (2008) Understanding the structural and functional differences between mouse thyrotropin-releasing hormone receptors 1 and 2. Proteins. 71:783-794.

Delcourt, N., Bockaert, J. and Marin, P. (2007) GPCR-jacking: from a new route in RTK signalling to a new concept in GPCR activation. Trends Pharmacol Sci. 28:602-607.

Digby, G.J., Lober, R.M., Sethi, P.R. and Lambert, N.A. (2006) Some G protein heterotrimers physically dissociate in living cells. Proc Natl Acad Sci U S A. 103:17789-17794.

DiNitto, J.P. and Lambright, D.G. (2006) Membrane and juxtamembrane targeting by PH and PTB domains. Biochim Biophys Acta. 1761:850-867.

Drastichova, Z., Bourova, L., Hejnova, L., Jedelsky, P., Svoboda, P. and Novotny, J. (2010) Protein alterations induced by long-term agonist treatment of HEK293 cells expressing thyrotropin-releasing hormone receptor and $G_{11}\alpha$ protein. J Cell Biochem. 109:255-264.

Drin, G. and Scarlata, S. (2007) Stimulation of phospholipase Cβ by membrane interactions, interdomain movement, and G protein binding--how many ways can you activate an enzyme? Cell Signal. 19:1383-1392.

Drmota, T., Novotny, J., Gould, G.W., Svoboda, P. and Milligan, G. (1999) Visualization of distinct patterns of subcellular redistribution of the thyrotropin-releasing hormone receptor-1 and $G_{q/11}\alpha$ induced by agonist stimulation. Biochem J. 340 (Pt 2):529-538.

Drmota, T., Novotny, J., Kim, G.D., Eidne, K.A., Milligan, G. and Svoboda, P. (1998) Agonist-induced internalization of the G protein $G_{11}\alpha$ and thyrotropin-releasing hormone receptors proceed on different time scales. J Biol Chem. 273:21699-21707.

Du, D., Raaka, B.M., Grimberg, H., Lupu-Meiri, M., Oron, Y. and Gershengorn, M.C. (2005) Carboxyl tail cysteine mutants of the thyrotropin-releasing hormone receptor type 1 exhibit constitutive signaling: role of palmitoylation. Mol Pharmacol. 68:204-209.

Durchankova, D., Novotny, J. and Svoboda, P. (2008) The time-course of agonist-induced solubilization of trimeric $G_{q/11}\alpha$ proteins resolved by two-dimensional electrophoresis. Physiol Res. 57:195-203.

Elorza, A., Sarnago, S. and Mayor, F., Jr. (2000) Agonist-dependent modulation of G protein-coupled receptor kinase 2 by mitogen-activated protein kinases. Mol Pharmacol. 57:778-783.

Escriba, P.V., Wedegaertner, P.B., Goni, F.M. and Vogler, O. (2007) Lipid-protein interactions in GPCR-associated signaling. Biochim Biophys Acta. 1768:836-852.

Evanko, D.S., Thiyagarajan, M.M., Takida, S. and Wedegaertner, P.B. (2005) Loss of association between activated $G_q\alpha$ and Gβγ disrupts receptor-dependent and receptor-independent signaling. Cell Signal. 17:1218-1228.

Evanko, D.S., Thiyagarajan, M.M. and Wedegaertner, P.B. (2000) Interaction with Gβγ is required for membrane targeting and palmitoylation of $G_s\alpha$ and $G_q\alpha$. J Biol Chem. 275:1327-1336.

Fan, G., Shumay, E., Malbon, C.C. and Wang, H. (2001) c-Src tyrosine kinase binds the β_2-adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. J Biol Chem. 276:13240-13247.

Fauvel, J., Chap, H., Roques, V., Levy-Toledano, S. and Douste-Blazy, L. (1986) Biochemical characterization of plasma membranes and intracellular membranes isolated from human platelets using Percoll gradients. Biochim Biophys Acta. 856:155-164.

Figueroa, M., Hinrichs, M.V., Bunster, M., Babbitt, P., Martinez-Oyanedel, J. and Olate, J. (2009) Biophysical studies support a predicted superhelical structure with armadillo repeats for Ric-8. Protein Sci. 18:1139-1145.

Fridmanis, D., Fredriksson, R., Kapa, I., Schioth, H.B. and Klovins, J. (2007) Formation of new genes explains lower intron density in mammalian Rhodopsin G protein-coupled receptors. Mol Phylogenet Evol. 43:864-880.

Gales, C., Van Durm, J.J., Schaak, S., Pontier, S., Percherancier, Y., Audet, M., Paris, H. and Bouvier, M. (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. Nat Struct Mol Biol. 13:778-786.

Gao, H., Sun, Y., Wu, Y., Luan, B., Wang, Y., Qu, B. and Pei, G. (2004) Identification of β-arrestin2 as a G protein-coupled receptor-stimulated regulator of NFκB pathways. Mol Cell. 14:303-317.

Gehret, A.U. and Hinkle, P.M. (2010) Importance of regions outside the cytoplasmic tail of G-protein-coupled receptors for phosphorylation and dephosphorylation. Biochem J. 428:235-245.

Gehret, A.U., Jones, B.W., Tran, P.N., Cook, L.B., Greuber, E.K. and Hinkle, P.M. (2010) Role of helix 8 of the thyrotropin-releasing hormone receptor in phosphorylation by G protein-coupled receptor kinase. Mol Pharmacol. 77:288-297.

Gentles, A.J. and Karlin, S. (1999) Why are human G-protein-coupled receptors predominantly intronless? Trends Genet. 15:47-49.

George, S.R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G. and O'Dowd, B.F. (2000) Oligomerization of μ- and δ-opioid receptors. Generation of novel functional properties. J Biol Chem. 275:26128-26135.

Gershengorn, M.C. and Osman, R. (2001) Minireview: Insights into G protein-coupled receptor function using molecular models. Endocrinology. 142:2-10.

Gilman, A.G. (1987) G proteins: transducers of receptor-generated signals. Annu Rev Biochem. 56:615-649.

Godeny, M.D., Sayyah, J., VonDerLinden, D., Johns, M., Ostrov, D.A., Caldwell-Busby, J. and Sayeski, P.P. (2007) The N-terminal SH2 domain of the tyrosine phosphatase, SHP-2, is essential for Jak2-dependent signaling via the angiotensin II type AT_1 receptor. Cell Signal. 19:600-609.

Granovsky, A.E. and Artemyev, N.O. (2001) Partial reconstitution of photoreceptor cGMP phosphodiesterase characteristics in cGMP phosphodiesterase-5. J Biol Chem. 276:21698-21703.

Grant, J.E., Guo, L.W., Vestling, M.M., Martemyanov, K.A., Arshavsky, V.Y. and Ruoho, A.E. (2006) The N terminus of GTPγS-activated transducin α-subunit interacts with the C terminus of the cGMP phosphodiesterase γ-subunit. J Biol Chem. 281:6194-6202.

Gurevich, E.V. and Gurevich, V.V. (2006) Arrestins: ubiquitous regulators of cellular signaling pathways. Genome Biol. 7:236. (Gurevich and Gurevich a, 2006)

Gurevich, V.V. and Gurevich, E.V. (2006) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol Ther. 110:465-502. (Gurevich and Gurevich b, 2006)

Hanyaloglu, A.C., Seeber, R.M., Kohout, T.A., Lefkowitz, R.J. and Eidne, K.A. (2002) Homo- and hetero-oligomerization of thyrotropin-releasing hormone (TRH) receptor subtypes. Differential regulation of β-arrestins 1 and 2. J Biol Chem. 277:50422-50430.

Hanyaloglu, A.C., Vrecl, M., Kroeger, K.M., Miles, L.E., Qian, H., Thomas, W.G. and Eidne, K.A. (2001) Casein kinase II sites in the intracellular C-terminal domain of the thyrotropin-releasing hormone receptor and chimeric gonadotropin-releasing hormone receptors contribute to beta-arrestin-dependent internalization. J Biol Chem. 276:18066-18074.

Haraguchi, K. and Rodbell, M. (1990) Isoproterenol stimulates shift of G proteins from plasma membrane to pinocytotic vesicles in rat adipocytes: a possible means of signal dissemination. Proc Natl Acad Sci U S A. 87:1208-1212.

Hargrave, P.A., Mcdowell, J.H., Curtis, D.R., Wang, J.K., Juszczak, E., Fong, S.L., Rao, J.K.M. and Argos, P. (1983) The Structure of Bovine Rhodopsin. Biophysics of Structure and Mechanism. 9:235-244.

Hawtin, S.R., Tobin, A.B., Patel, S. and Wheatley, M. (2001) Palmitoylation of the vasopressin V_{1a} receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. J Biol Chem. 276:38139-38146.

Hein, P., Frank, M., Hoffmann, C., Lohse, M.J. and Bunemann, M. (2005) Dynamics of receptor/G protein coupling in living cells. EMBO J. 24:4106-4114.

Helenius, A. and Simons, K. (1977) Charge shift electrophoresis: simple method for distinguishing between amphiphilic and hydrophilic proteins in detergent solution. Proc Natl Acad Sci U S A. 74:529-532.

Herlitze, S., Ruppersberg, J.P. and Mark, M.D. (1999) New roles for RGS2, 5 and 8 on the ratio-dependent modulation of recombinant GIRK channels expressed in Xenopus oocytes. J Physiol. 517 (Pt 2):341-352.

Herrick-Davis, K., Grinde, E., Harrigan, T.J. and Mazurkiewicz, J.E. (2005) Inhibition of serotonin 5-hydroxytryptamine 2c receptor function through heterodimerization: receptor dimers bind two molecules of ligand and one G-protein. J Biol Chem. 280:40144-40151.

Hilgenfeld, R. (1995) Regulatory GTPases. Curr Opin Struct Biol. 5:810-817.

Ho, M.K., Su, Y., Yeung, W.W. and Wong, Y.H. (2009) Regulation of transcription factors by heterotrimeric G proteins. Curr Mol Pharmacol. 2:19-31.

Huang, C., Duncan, J.A., Gilman, A.G. and Mumby, S.M. (1999) Persistent membrane association of activated and depalmitoylated G protein α subunits. Proc Natl Acad Sci U S A. 96:412-417.

Huynh, J., Thomas, W.G., Aguilar, M.I. and Pattenden, L.K. (2009) Role of helix 8 in G protein-coupled receptors based on structure-function studies on the type 1 angiotensin receptor. Mol Cell Endocrinol. 302:118-127.

Hynes, T.R., Mervine, S.M., Yost, E.A., Sabo, J.L. and Berlot, C.H. (2004) Live cell imaging of Gs and the β_2-adrenergic receptor demonstrates that both Gα and $G\beta_1\gamma_7$ internalize upon stimulation and exhibit similar trafficking patterns that differ from that of the β_2-adrenergic receptor. J Biol Chem. 279:44101-44112.

Ihnatovych, I., Novotny, J., Haugvicova, R., Bourova, L., Mares, P. and Svoboda, P. (2002) Opposing changes of trimeric G protein levels during ontogenetic development of rat brain. Brain Res Dev Brain Res. 133:57-67.

Iiri, T., Backlund, P.S., Jr., Jones, T.L., Wedegaertner, P.B. and Bourne, H.R. (1996) Reciprocal regulation of $G_s\alpha$ by palmitate and the βγ subunit. Proc Natl Acad Sci U S A. 93:14592-14597.

Ilsley, J.L., Sudol, M. and Winder, S.J. (2002) The WW domain: linking cell signalling to the membrane cytoskeleton. Cell Signal. 14:183-189.

Ishii, M., Inanobe, A. and Kurachi, Y. (2002) PIP3 inhibition of RGS protein and its reversal by Ca^{2+}/calmodulin mediate voltage-dependent control of the G protein cycle in a cardiac K^+ channel. Proc Natl Acad Sci U S A. 99:4325-4330.

Jaakola, V.P., Griffith, M.T., Hanson, M.A., Cherezov, V., Chien, E.Y., Lane, J.R., Ijzerman, A.P. and Stevens, R.C. (2008) The 2.6 angstrom crystal structure of a human A_{2A} adenosine receptor bound to an antagonist. Science. 322:1211-1217.

Jacoby, E., Bouhelal, R., Gerspacher, M. and Seuwen, K. (2006) The 7 TM G-protein-coupled receptor target family. ChemMedChem. 1:761-782.

Jantas, D., Jaworska-Feil, L., Lipkowski, A.W. and Lason, W. (2009) Effects of TRH and its analogues on primary cortical neuronal cell damage induced by various excitotoxic, necrotic and apoptotic agents. Neuropeptides. 43:371-385.

Jaworska-Feil, L., Jantas, D., Leskiewicz, M., Budziszewska, B., Kubera, M., Basta-Kaim, A., Lipkowski, A.W. and Lason, W. (2010) Protective effects of TRH and its analogues against various cytotoxic agents in retinoic acid (RA)-differentiated human neuroblastoma SH-SY5Y cells. Neuropeptides. 44:495-508.

Jimenez-Sainz, M.C., Murga, C., Kavelaars, A., Jurado-Pueyo, M., Krakstad, B.F., Heijnen, C.J., Mayor, F., Jr. and Aragay, A.M. (2006) G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits. Mol Biol Cell. 17:25-31.

John, G.B., Shang, Y., Li, L., Renken, C., Mannella, C.A., Selker, J.M., Rangell, L., Bennett, M.J. and Zha, J. (2005) The mitochondrial inner membrane protein mitofilin controls cristae morphology. Mol Biol Cell. 16:1543-1554.

Jones, T.L., Degtyarev, M.Y. and Backlund, P.S., Jr. (1997) The stoichiometry of $G_s\alpha$ palmitoylation in its basal and activated states. Biochemistry. 36:7185-7191.

Jordan, B.A. and Devi, L.A. (1999) G-protein-coupled receptor heterodimerization modulates receptor function. Nature. 399:697-700.

Kang, J., Shi, Y., Xiang, B., Qu, B., Su, W., Zhu, M., Zhang, M., Bao, G., Wang, F., Zhang, X., Yang, R., Fan, F., Chen, X., Pei, G. and Ma, L. (2005) A nuclear function of β-arrestin1 in GPCR signaling: regulation of histone acetylation and gene transcription. Cell. 123:833-847.

Karnik, S.S., Gogonea, C., Patil, S., Saad, Y. and Takezako, T. (2003) Activation of G-protein-coupled receptors: a common molecular mechanism. Trends Endocrinol Metab. 14:431-437.

Karoor, V. and Malbon, C.C. (1996) Insulin-like growth factor receptor-1 stimulates phosphorylation of the β_2-adrenergic receptor in vivo on sites distinct from those phosphorylated in response to insulin. J Biol Chem. 271:29347-29352.

Kilpatrick, E.L. and Hildebrandt, J.D. (2007) Sequence dependence and differential expression of Gγ5 subunit isoforms of the heterotrimeric G proteins variably processed after prenylation in mammalian cells. J Biol Chem. 282:14038-14047.

Kim, G.D., Carr, I.C., Anderson, L.A., Zabavnik, J., Eidne, K.A. and Milligan, G. (1994) The long isoform of the rat thyrotropin-releasing hormone receptor down-regulates G_q proteins. J Biol Chem. 269:19933-19940.

Kino, T., Kozasa, T. and Chrousos, G.P. (2005) Statin-induced blockade of prenylation alters nucleocytoplasmic shuttling of GTP-binding proteins γ_2 and β_2 and enhances their suppressive effect on glucocorticoid receptor transcriptional activity. Eur J Clin Invest. 35:508-513.

Kosloff, M., Elia, N., Joel-Almagor, T., Timberg, R., Zars, T.D., Hyde, D.R., Minke, B. and Selinger, Z. (2003) Regulation of light-dependent $G_q\alpha$ translocation and morphological changes in fly photoreceptors. EMBO J. 22:459-468.

Krasel, C., Dammeier, S., Winstel, R., Brockmann, J., Mischak, H. and Lohse, M.J. (2001) Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin. J Biol Chem. 2761911-1915.

Ladbury, J.E. and Arold, S. (2000) Searching for specificity in SH domains. Chem Biol. 7:R3-8.

Lagerstrom, M.C. and Schioth, H.B. (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov. 7:339-357.

Landry, Y., Niederhoffer, N., Sick, E. and Gies, J.P. (2006) Heptahelical and other G-protein-coupled receptors (GPCRs) signaling. Curr Med Chem. 13:51-63.

Lee, S.P., So, C.H., Rashid, A.J., Varghese, G., Cheng, R., Lanca, A.J., O'Dowd, B.F. and George, S.R. (2004) Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. J Biol Chem. 279:35671-35678.

Lee, T.W., Anderson, L.A., Eidne, K.A. and Milligan, G. (1995) Comparison of the signalling properties of the long and short isoforms of the rat thyrotropin-releasing-hormone receptor following expression in rat 1 fibroblasts. Biochem J. 310 (Pt 1):291-298.

Lefkowitz, R.J. (2004) Historical review: a brief history and personal retrospective of seven-transmembrane receptors. Trends Pharmacol Sci. 25:413-422.

Lei, B., Morris, D.P., Smith, M.P. and Schwinn, D.A. (2009) Lipid rafts constrain basal α_{1A}-adrenergic receptor signaling by maintaining receptor in an inactive conformation. Cell Signal. 21:1532-1539.

Lemmon, M.A. (2008) Membrane recognition by phospholipid-binding domains. Nat Rev Mol Cell Biol. 9:99-111.

Levoye, A., Balabanian, K., Baleux, F., Bachelerie, F. and Lagane, B. (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. Blood. 113:6085-6093.

Li, J.G., Benovic, J.L. and Liu-Chen, L.Y. (2000) Mechanisms of agonist-induced down-regulation of the human κ-opioid receptor: internalization is required for down-regulation. Mol Pharmacol. 58:795-801.

Li, J.G., Chen, C. and Liu-Chen, L.Y. (2002) Ezrin-radixin-moesin-binding phosphoprotein-50/Na^+/H^+ exchanger regulatory factor (EBP50/NHERF) blocks U50,488H-induced down-regulation of the human κ opioid receptor by enhancing its recycling rate. J Biol Chem. 277:27545-27552.

Li, W.J., Shin, M.K. and Oh, S.J. (2010) Time dependent bladder apoptosis induced by acute bladder outlet obstruction and subsequent emptying is associated with decreased MnSOD expression and Bcl-2/Bax ratio. J Korean Med Sci. 25:1652-1656.

Li, Z., Benard, O. and Margolskee, R.F. (2006) $G_{13}\gamma$ interacts with PDZ domain-containing proteins. J Biol Chem. 281:11066-11073.

Lin, F.T., Krueger, K.M., Kendall, H.E., Daaka, Y., Fredericks, Z.L., Pitcher, J.A. and Lefkowitz, R.J. (1997) Clathrin-mediated endocytosis of the β-adrenergic receptor is regulated by phosphorylation/dephosphorylation of β-arrestin1. J Biol Chem. 272:31051-31057.

Linder, M.E., Pang, I.H., Duronio, R.J., Gordon, J.I., Sternweis, P.C. and Gilman, A.G. (1991) Lipid modifications of G protein subunits. Myristoylation of $G_o\alpha$ increases its affinity for Gβγ. J Biol Chem. 266:4654-4659.

Lorenz, K., Lohse, M.J. and Quitterer, U. (2003) Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. Nature. 426:574-579.

Lu, Z.L., Saldanha, J.W. and Hulme, E.C. (2002) Seven-transmembrane receptors: crystals clarify. Trends Pharmacol Sci. 23:140-146.

Luo, L. and Yano, N. (2005) Thyrotropin releasing hormone (TRH) affects gene expression in pancreatic β-cells. Endocr Res. 31:185-198.

Luo, L., Yano, N., Mao, Q., Jackson, I.M. and Stopa, E.G. (2002) Thyrotropin releasing hormone (TRH) in the hippocampus of Alzheimer patients. J Alzheimers Dis. 4:97-103.

Lupi, R., Dani, N., Dietrich, A., Marchegiani, A., Turacchio, S., Berrie, C.P., Moss, J., Gierschik, P., Corda, D. and Di Girolamo, M. (2002) Endogenous mono-ADP-ribosylation of the free Gβγ prevents stimulation of phosphoinositide 3-kinase-γ and phospholipase C-β_2 and is activated by G-protein-coupled receptors. Biochem J. 367:825-832.

Luttrell, L.M., Ferguson, S.S., Daaka, Y., Miller, W.E., Maudsley, S., Della Rocca, G.J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D.K., Caron, M.G. and Lefkowitz, R.J. (1999) β-arrestin-dependent formation of β_2 adrenergic receptor-Src protein kinase complexes. Science. 283:655-661.

Luttrell, L.M., van Biesen, T., Hawes, B.E., Koch, W.J., Touhara, K. and Lefkowitz, R.J. (1995) Gβγ subunits mediate mitogen-activated protein kinase activation by the tyrosine kinase insulin-like growth factor 1 receptor. J Biol Chem. 270:16495-16498.

Lutz, S., Freichel-Blomquist, A., Yang, Y., Rumenapp, U., Jakobs, K.H., Schmidt, M. and Wieland, T. (2005) The guanine nucleotide exchange factor p63RhoGEF, a specific link between $G_{q/11}$-coupled receptor signaling and RhoA. J Biol Chem. 280:11134-11139.

Lynch, M.J., Baillie, G.S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., van Heeke, G. and Houslay, M.D. (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with ζ arrestin to control the protein kinase A/AKAP79-mediated switching of the β2-adrenergic receptor to activation of ERK in HEK293B2 cells. J Biol Chem. 280:33178-33189.

Ma, L. and Pei, G. (2007) β-arrestin signaling and regulation of transcription. J Cell Sci. 120:213-218.

Maggio, R., Innamorati, G. and Parenti, M. (2007) G protein-coupled receptor oligomerization provides the framework for signal discrimination. J Neurochem. 103:1741-1752.

Mahon, M.J. and Segre, G.V. (2004) Stimulation by parathyroid hormone of a NHERF-1-assembled complex consisting of the parathyroid hormone I receptor, phospholipase Cβ, and actin increases intracellular calcium in opossum kidney cells. J Biol Chem. 279:23550-23558.

Mao, J., Xie, W., Yuan, H., Simon, M.I., Mano, H. and Wu, D. (1998) Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by $G_{12/13}\alpha$. EMBO J. 17:5638-5646.

Marchese, A., Chen, C., Kim, Y.M. and Benovic, J.L. (2003) The ins and outs of G protein-coupled receptor trafficking. Trends Biochem Sci. 28:369-376.

Marchese, A., Paing, M.M., Temple, B.R. and Trejo, J. (2008) G protein-coupled receptor sorting to endosomes and lysosomes. Annu Rev Pharmacol Toxicol. 48:601-629.

Mariggio, S., Garcia-Hoz, C., Sarnago, S., De Blasi, A., Mayor, F., Jr. and Ribas, C. (2006) Tyrosine phosphorylation of G-protein-coupled-receptor kinase 2 (GRK2) by c-Src modulates its interaction with $G_q\alpha$. Cell Signal. 18:2004-2012.

Marion, S., Fralish, G.B., Laporte, S., Caron, M.G. and Barak, L.S. (2007) N-terminal tyrosine modulation of the endocytic adaptor function of the β-arrestins. J Biol Chem. 282:18937-18944.

Marrari, Y., Crouthamel, M., Irannejad, R. and Wedegaertner, P.B. (2007) Assembly and trafficking of heterotrimeric G proteins. Biochemistry. 46:7665-7677.

Martin Shreeve, S. (2002) Identification of G-proteins coupling to the vasoactive intestinal peptide receptor $VPAC_1$ using immunoaffinity chromatography: evidence for precoupling. Biochem Biophys Res Commun. 290:1300-1307.

Matousek, P., Novotny, J., Rudajev, V. and Svoboda, P. (2005) Prolonged agonist stimulation does not alter the protein composition of membrane domains in spite of dramatic changes induced in a specific signaling cascade. Cell Biochem Biophys. 42:21-40.

Matousek, P., Novotny, J. and Svoboda, P. (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. Physiol Res. 53:295-303.

Matsuda, T., Takao, T., Shimonishi, Y., Murata, M., Asano, T., Yoshizawa, T. and Fukada, Y. (1994) Characterization of interactions between transducin α/βγ-subunits and lipid membranes. J Biol Chem. 269:30358-30363.

Maurer, A., Wieland, T., Meissl, F., Niroomand, F., Mehringer, R., Krieglstein, J. and Klumpp, S. (2005) The β-subunit of G proteins is a substrate of protein histidine phosphatase. Biochem Biophys Res Commun. 334:1115-1120.

McClatchey, A.I. and Fehon, R.G. (2009) Merlin and the ERM proteins--regulators of receptor distribution and signaling at the cell cortex. Trends Cell Biol. 19:198-206.

McIntire, W.E. (2009) Structural determinants involved in the formation and activation of G protein βγ dimers. Neurosignals. 17:82-99.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P. and Moll, U.M. (2003) p53 has a direct apoptogenic role at the mitochondria. Mol Cell. 11:577-590.

Michineau, S., Alhenc-Gelas, F. and Rajerison, R.M. (2006) Human bradykinin B2 receptor sialylation and N-glycosylation participate with disulfide bonding in surface receptor dimerization. Biochemistry. 45:2699-2707.

Milligan, G. and Kostenis, E. (2006) Heterotrimeric G-proteins: a short history. Br J Pharmacol. 147 Suppl 1:S46-55.

Mirzadegan, T., Benko, G., Filipek, S. and Palczewski, K. (2003) Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. Biochemistry. 42:2759-2767.

Miserey-Lenkei, S., Lenkei, Z., Parnot, C., Corvol, P. and Clauser, E. (2001) A functional enhanced green fluorescent protein (EGFP)-tagged angiotensin II AT_{1a} receptor recruits the endogenous $G_{q/11}\alpha$ protein to the membrane and induces its specific internalization independently of receptor-G protein coupling in HEK-293 cells. Mol Endocrinol. 15:294-307.

Miyamoto-Matsubara, M., Chung, S. and Saito, Y. (2010) Functional interaction of regulator of G protein signaling-2 with melanin-concentrating hormone receptor 1. Ann N Y Acad Sci. 1200:112-119.

Mizuno, N. and Itoh, H. (2009) Functions and regulatory mechanisms of G_q-signaling pathways. Neurosignals. 17:42-54.

Montero, J., Mari, M., Colell, A., Morales, A., Basanez, G., Garcia-Ruiz, C. and Fernandez-Checa, J.C. (2010) Cholesterol and peroxidized cardiolipin in mitochondrial membrane properties, permeabilization and cell death. Biochim Biophys Acta. 1797:1217-1224.

Morales, J., Fishburn, C.S., Wilson, P.T. and Bourne, H.R. (1998) Plasma membrane localization of $G_z\alpha$ requires two signals. Mol Biol Cell. 9:1-14.

Morris, A.J. and Malbon, C.C. (1999) Physiological regulation of G protein-linked signaling. Physiol Rev. 79:1373-1430.

Morrison, N., Duthie, S.M., Boyd, E., Eidne, K.A. and Connor, J.M. (1994) Assignment of the gene encoding the human thyrotropin-releasing hormone receptor to 8q23 by fluorescence in situ hybridization. Hum Genet. 93:716-718.

Mulla, C.M., Geras-Raaka, E., Raaka, B.M. and Gershengorn, M.C. (2009) High levels of thyrotropin-releasing hormone receptors activate programmed cell death in human pancreatic precursors. Pancreas. 38:197-202.

Mullaney, I., Mitchell, F.M., McCallum, J.F., Buckley, N.J. and Milligan, G. (1993) The human muscarinic M1 acetylcholine receptor, when express in CHO cells, activates and downregulates both $G_q\alpha$ and $G_{11}\alpha$ equally and non-selectively. FEBS Lett. 324:241-245.

Munro, E.M. (2006) PAR proteins and the cytoskeleton: a marriage of equals. Curr Opin Cell Biol. 18:86-94.

Nobles, M., Benians, A. and Tinker, A. (2005) Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. Proc Natl Acad Sci U S A. 102:18706-18711.

Novi, F., Stanasila, L., Giorgi, F., Corsini, G.U., Cotecchia, S. and Maggio, R. (2005) Paired activation of two components within muscarinic M3 receptor dimers is required for recruitment of β-arrestin-1 to the plasma membrane. J Biol Chem. 280:19768-19776.

O'Dowd, B.F., Lee, D.K., Huang, W., Nguyen, T., Cheng, R., Liu, Y., Wang, B., Gershengorn, M.C. and George, S.R. (2000) TRH-R2 exhibits similar binding and acute signaling but distinct regulation and anatomic distribution compared with TRH-R1. Mol Endocrinol. 14:183-193.

Oh, P. and Schnitzer, J.E. (2001) Segregation of heterotrimeric G proteins in cell surface microdomains. G_q binds caveolin to concentrate in caveolae, whereas G_i and G_s target lipid rafts by default. Mol Biol Cell. 12:685-698.

Okamoto, Y., Ninomiya, H., Tanioka, M., Sakamoto, A., Miwa, S. and Masaki, T. (1997) Palmitoylation of human endothelinB. Its critical role in G protein coupling and a differential requirement for the cytoplasmic tail by G protein subtypes. J Biol Chem. 272:21589-21596.

Oldham, W.M. and Hamm, H.E. (2006) Structural basis of function in heterotrimeric G proteins. Q Rev Biophys. 39:117-166.

Oldham, W.M. and Hamm, H.E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. Nat Rev Mol Cell Biol. 9:60-71.

Omori, K. and Kotera, J. (2007) Overview of PDEs and their regulation. Circ Res. 100:309-327.

Osman, C., Merkwirth, C. and Langer, T. (2009) Prohibitins and the functional compartmentalization of mitochondrial membranes. J Cell Sci. 122:3823-3830.

Ovchinnikov, Y.A., Abdulaev, N.G., Feigina, M.Y., Artamonov, I.D., Zolotarev, A.S., Kostina, M.B., Bogachuk, A.S., Miroshnikov, A.I., Martinov, V.I. and Kudelin, A.B. (1982) The Complete Amino-Acid-Sequence of Visual Rhodopsin. Bioorganicheskaya Khimiya. 8:1011.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M. and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science. 289:739-745.

Palomero, T., Barros, F., del Camino, D., Viloria, C.G. and de la Pena, P. (1998) A G protein β γ dimer-mediated pathway contributes to mitogen-activated protein kinase activation by thyrotropin-releasing hormone receptors in transfected COS-7 cells. Mol Pharmacol. 53:613-622.

Pandey, K.N. (2009) Functional roles of short sequence motifs in the endocytosis of membrane receptors. Front Biosci. 14:5339-5360.

Pao, C.S. and Benovic, J.L. (2005) Structure/function analysis of α_{2A}-adrenergic receptor interaction with G protein-coupled receptor kinase 2. J Biol Chem. 280:11052-11058.

Park, D., Jhon, D.Y., Lee, C.W., Lee, K.H. and Rhee, S.G. (1993) Activation of phospholipase C isozymes by G protein βγ subunits. J Biol Chem. 268:4573-4576.

Patel, J.R. and Brewer, G.J. (2008) Age-related differences in NFκB translocation and Bcl-2/Bax ratio caused by TNFα and Aβ42 promote survival in middle-age neurons and death in old neurons. Exp Neurol. 213:93-100.

Peeters, M.C., van Westen, G.J., Li, Q. and AP, I.J. (2011) Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. Trends Pharmacol Sci. 32:35-42.

Penela, P., Murga, C., Ribas, C., Lafarga, V. and Mayor, F. (2010) The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. British Journal of Pharmacology. 160:821-832.

Penela, P., Ribas, C. and Mayor, F., Jr. (2003) Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. Cell Signal. 15:973-981.

Peng, Y.W., Robishaw, J.D., Levine, M.A. and Yau, K.W. (1992) Retinal rods and cones have distinct G protein β and γ subunits. Proc Natl Acad Sci U S A. 89:10882-10886.

Pertoft, H. (2000) Fractionation of cells and subcellular particles with Percoll. J Biochem Biophys Methods. 44:1-30.

Petaja-Repo, U.E., Hogue, M., Laperriere, A., Walker, P. and Bouvier, M. (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. J Biol Chem. 275:13727-13736.

Petrak, J., Ivanek, R., Toman, O., Cmejla, R., Cmejlova, J., Vyoral, D., Zivny, J. and Vulpe, C.D. (2008) Deja vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. Proteomics. 8:1744-1749.

Peverelli, E., Mantovani, G., Calebiro, D., Doni, A., Bondioni, S., Lania, A., Beck-Peccoz, P. and Spada, A. (2008) The third intracellular loop of the human somatostatin receptor 5 is crucial for arrestin binding and receptor internalization after somatostatin stimulation. Mol Endocrinol. 22:676-688.

Pfleger, K.D., Kroeger, K.M. and Eidne, K.A. (2004) Receptors for hypothalamic releasing hormones TRH and GnRH: oligomerization and interactions with intracellular proteins. Semin Cell Dev Biol. 15:269-280.

Pfreimer, M., Vatter, P., Langer, T., Wieland, T., Gierschik, P. and Moepps, B. (2012) LARG links histamine-H1-receptor-activated G_q to Rho-GTPase-dependent signaling pathways. Cell Signal. 24:652-663.

Philip, F., Sengupta, P. and Scarlata, S. (2007) Signaling through a G Protein-coupled receptor and its corresponding G protein follows a stoichiometrically limited model. J Biol Chem. 282:19203-19216.

Pirzadeh, A., Mammen, A., Kubin, J., Reade, E., Liu, H., Mendoza, A., Greeley, W.J., Wilson, D.F. and Pastuszko, A. (2011) Early regional response of apoptotic activity in newborn piglet brain following hypoxia and ischemia. Neurochem Res. 36:83-92.

Popov, S.G., Krishna, U.M., Falck, J.R. and Wilkie, T.M. (2000) Ca^{2+}/Calmodulin reverses phosphatidylinositol 3,4, 5-trisphosphate-dependent inhibition of regulators of G protein-signaling GTPase-activating protein activity. J Biol Chem. 275:18962-18968.

Poppleton, H., Sun, H., Fulgham, D., Bertics, P. and Patel, T.B. (1996) Activation of $G_s\alpha$ by the epidermal growth factor receptor involves phosphorylation. J Biol Chem. 271:6947-6951.

Prossnitz, E.R. (2004) Novel roles for arrestins in the post-endocytic trafficking of G protein-coupled receptors. Life Sci. 75:893-899.

Qin, K., Sethi, P.R. and Lambert, N.A. (2008) Abundance and stability of complexes containing inactive G protein-coupled receptors and G proteins. FASEB J. 22:2920-2927.

Ransnas, L.A., Svoboda, P., Jasper, J.R. and Insel, P.A. (1989) Stimulation of β-adrenergic receptors of S49 lymphoma cells redistributes the α subunit of the stimulatory G-protein between cytosol and membranes. Proc Natl Acad Sci U S A 86:7900-7903

Rasmussen, S.G., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., Chae, P.S., Pardon, E., Calinski, D., Mathiesen, J.M., Shah, S.T., Lyons, J.A., Caffrey, M., Gellman, S.H., Steyaert, J., Skiniotis, G., Weis, W.I., Sunahara, R.K. and Kobilka, B.K. (2011) Crystal structure of the β_2 adrenergic receptor-G_s protein complex. Nature. 477:549-555.

Rebois, R.V., Warner, D.R. and Basi, N.S. (1997) Does subunit dissociation necessarily accompany the activation of all heterotrimeric G proteins? Cell Signal. 9:141-151.

Reed, J.C. (1998) Bcl-2 family proteins. Oncogene. 17:3225-3236.

Ribas, C., Penela, P., Murga, C., Salcedo, A., Garcia-Hoz, C., Jurado-Pueyo, M., Aymerich, I. and Mayor, F., Jr. (2007) The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. Biochim Biophys Acta. 1768:913-922.

Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M. (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanylnucleotides in glucagon action. J Biol Chem. 246:1877-1882.

Roy, A.A., Baragli, A., Bernstein, L.S., Hepler, J.R., Hebert, T.E. and Chidiac, P. (2006) RGS2 interacts with G_s and adenylyl cyclase in living cells. Cell Signal. 18:336-348.

Sadeghi, H. and Birnbaumer, M. (1999) O-Glycosylation of the V2 vasopressin receptor. Glycobiology. 9:731-737.

Salim, S., Sinnarajah, S., Kehrl, J.H. and Dessauer, C.W. (2003) Identification of RGS2 and type V adenylyl cyclase interaction sites. J Biol Chem. 278:15842-15849.

Sarnago, S., Roca, R., de Blasi, A., Valencia, A., Mayor, F., Jr. and Murga, C. (2003) Involvement of intramolecular interactions in the regulation of G protein-coupled receptor kinase 2. Mol Pharmacol. 64:629-639.

Sengupta, P., Philip, F. and Scarlata, S. (2008) Caveolin-1 alters Ca^{2+} signal duration through specific interaction with the $G_q\alpha$ family of G proteins. J Cell Sci. 121:1363-1372.

Shenoy, S.K. and Lefkowitz, R.J. (2011) β-Arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol Sci. 32:521-533.

Shenoy, S.K., McDonald, P.H., Kohout, T.A. and Lefkowitz, R.J. (2001) Regulation of receptor fate by ubiquitination of activated β_2-adrenergic receptor and β-arrestin. Science. 294:1307-1313.

Shiina, T., Arai, K., Tanabe, S., Yoshida, N., Haga, T., Nagao, T. and Kurose, H. (2001) Clathrin box in G protein-coupled receptor kinase 2. J Biol Chem. 276:33019-33026.

Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G.W., Kobayashi, T., Stevens, R.C. and Iwata, S. (2011) Structure of the human histamine H1 receptor complex with doxepin. Nature. 475:65-70.

Schertler, G.F., Villa, C. and Henderson, R. (1993) Projection structure of rhodopsin. Nature. 362:770-772.

Schioth, H.B. and Fredriksson, R. (2005) The GRAFS classification system of G-protein coupled receptors in comparative perspective. Gen Comp Endocrinol. 142:94-101.

Schutzer, W.E., Reed, J.F. and Mader, S.L. (2005) Decline in caveolin-1 expression and scaffolding of G protein receptor kinase-2 with age in Fischer 344 aortic vascular smooth muscle. Am J Physiol Heart Circ Physiol. 288:H2457-2464.

Siderovski, D.P. and Willard, F.S. (2005) The GAPs, GEFs, and GDIs of heterotrimeric G-protein α subunits. Int J Biol Sci. 1:51-66.

Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Diversity of G proteins in signal transduction. Science. 252:802-808.

Singer, A.U., Waldo, G.L., Harden, T.K. and Sondek, J. (2002) A unique fold of phospholipase C-β mediates dimerization and interaction with $G_q\alpha$. Nat Struct Biol. 9:32-36.

Skiba, N.P., Yang, C.S., Huang, T., Bae, H. and Hamm, H.E. (1999) The α-helical domain of $G_t\alpha$ determines specific interaction with regulator of G protein signaling 9. J Biol Chem. 274:8770-8778.

Small, K.M., Schwarb, M.R., Glinka, C., Theiss, C.T., Brown, K.M., Seman, C.A. and Liggett, S.B. (2006) α_{2A}- and α_{2C}-adrenergic receptors form homo- and heterodimers: the heterodimeric state impairs agonist-promoted GRK phosphorylation and β-arrestin recruitment. Biochemistry. 45:4760-4767.

Smith, J., Yu, R. and Hinkle, P.M. (2001) Activation of MAPK by TRH requires clathrin-dependent endocytosis and PKC but not receptor interaction with β-arrestin or receptor endocytosis. Mol Endocrinol. 15:1539-1548.

Smrcka, A.V., Hepler, J.R., Brown, K.O. and Sternweis, P.C. (1991) Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q. Science. 251:804-807.

Smrcka, A.V. and Sternweis, P.C. (1993) Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C β by G protein α and βγ subunits. J Biol Chem. 268:9667-9674.

Song, X., Coffa, S., Fu, H. and Gurevich, V.V. (2009) How does arrestin assemble MAPKs into a signaling complex? J Biol Chem. 284:685-695.

Stanasila, L., Abuin, L., Diviani, D. and Cotecchia, S. (2006) Ezrin directly interacts with the α_{1b}-adrenergic receptor and plays a role in receptor recycling. J Biol Chem. 281:4354-4363.

Sterne-Marr, R., Tesmer, J.J., Day, P.W., Stracquatanio, R.P., Cilente, J.A., O'Connor, K.E., Pronin, A.N., Benovic, J.L. and Wedegaertner, P.B. (2003) G protein-coupled receptor Kinase 2/$G_{q/11}\alpha$ interaction. A novel surface on a regulator of G protein signaling homology domain for binding G α subunits. J Biol Chem. 278:6050-6058.

Strotmann, R., Schrock, K., Boselt, I., Staubert, C., Russ, A. and Schoneberg, T. (2011) Evolution of GPCR: change and continuity. Mol Cell Endocrinol. 331:170-178.

Suh, P.G., Hwang, J.I., Ryu, S.H., Donowitz, M. and Kim, J.H. (2001) The roles of PDZ-containing proteins in PLC-β-mediated signaling. Biochem Biophys Res Commun. 288:1-7.

Sun, Y., Lu, X. and Gershengorn, M.C. (2003) Thyrotropin-releasing hormone receptors -- similarities and differences. J Mol Endocrinol. 30:87-97.

Sunahara, R.K. and Taussig, R. (2002) Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. Mol Interv. 2:168-184.

Svoboda, P., Kim, G.D., Grassie, M.A., Eidne, K.A. and Milligan, G. (1996) Thyrotropin-releasing hormone-induced subcellular redistribution and down-regulation of $G_{11}\alpha$: analysis of agonist regulation of coexpressed $G_{11}\alpha$ species variants. Mol Pharmacol. 49:646-655.

Tall, G.G., Krumins, A.M. and Gilman, A.G. (2003) Mammalian Ric-8A (synembryn) is a heterotrimeric Gα protein guanine nucleotide exchange factor. J Biol Chem. 278:8356-8362.

Tesmer, J.J. (2009) Structure and function of regulator of G protein signaling homology domains. Prog Mol Biol Transl Sci. 86:75-113.

Tewari, R., Bailes, E., Bunting, K.A. and Coates, J.C. (2010) Armadillo-repeat protein functions: questions for little creatures. Trends Cell Biol. 20:470-481.

Thiyagarajan, M.M., Bigras, E., Van Tol, H.H., Hebert, T.E., Evanko, D.S. and Wedegaertner, P.B. (2002) Activation-induced subcellular redistribution of $G_s\alpha$ is dependent upon its unique N-terminus. Biochemistry. 41:9470-9484.

Tilakaratne, N. and Sexton, P.M. (2005) G-Protein-coupled receptor-protein interactions: basis for new concepts on receptor structure and function. Clin Exp Pharmacol Physiol. 32:979-987.

Tobin, A.B. (2008) G-protein-coupled receptor phosphorylation: where, when and by whom. Br J Pharmacol. 153 Suppl 1:S167-176.

Tobin, A.B., Butcher, A.J. and Kong, K.C. (2008) Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. Trends Pharmacol Sci. 29:413-420.

Torrecilla, I., Spragg, E.J., Poulin, B., McWilliams, P.J., Mistry, S.C., Blaukat, A. and Tobin, A.B. (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. J Cell Biol. 177:127-137.

Traweger, A., Wiggin, G., Taylor, L., Tate, S.A., Metalnikov, P. and Pawson, T. (2008) Protein phosphatase 1 regulates the phosphorylation state of the polarity scaffold Par-3. Proc Natl Acad Sci U S A. 105:10402-10407.

Tsutsui, H., Kinugawa, S. and Matsushima, S. (2009) Mitochondrial oxidative stress and dysfunction in myocardial remodelling. Cardiovasc Res. 81:449-456.

Tuteja, N. (2009) Signaling through G protein coupled receptors. Plant Signal Behav. 4:942-947.

Unger, V.M., Hargrave, P.A., Baldwin, J.M. and Schertler, G.F. (1997) Arrangement of rhodopsin transmembrane α-helices. Nature. 389:203-206.

van Ham, M., Croes, H., Schepens, J., Fransen, J., Wieringa, B. and Hendriks, W. (2003) Cloning and characterization of mCRIP2, a mouse LIM-only protein that interacts with PDZ domain IV of PTP-BL. Genes Cells. 8:631-644.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. and Waterfield, M.D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci. 22:267-272.

Venkatakrishnan, G. and Exton, J.H. (1996) Identification of determinants in the α-subunit of G_q required for phospholipase C activation. J Biol Chem. 271:5066-5072.

Wang, P., Wu, Y., Ge, X., Ma, L. and Pei, G. (2003) Subcellular localization of β-arrestins is determined by their intact N domain and the nuclear export signal at the C terminus. J Biol Chem. 278:11648-11653.

Wang, X., Zeng, W., Soyombo, A.A., Tang, W., Ross, E.M., Barnes, A.P., Milgram, S.L., Penninger, J.M., Allen, P.B., Greengard, P. and Muallem, S. (2005) Spinophilin regulates Ca^{2+} signalling by binding the N-terminal domain of RGS2 and the third intracellular loop of G-protein-coupled receptors. Nat Cell Biol. 7:405-411.

Wang, Y., Tang, Y., Teng, L., Wu, Y., Zhao, X. and Pei, G. (2006) Association of β-arrestin and TRAF6 negatively regulates Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol. 7:139-147.

Wang, Y.H., Jue, S.F. and Maurer, R.A. (2000) Thyrotropin-releasing hormone stimulates phosphorylation of the epidermal growth factor receptor in GH3 pituitary cells. Mol Endocrinol. 14:1328-1337.

Wang, Y.H. and Maurer, R.A. (1999) A role for the mitogen-activated protein kinase in mediating the ability of thyrotropin-releasing hormone to stimulate the prolactin promoter. Mol Endocrinol. 13:1094-1104.

Warne, T., Serrano-Vega, M.J., Baker, J.G., Moukhametzianov, R., Edwards, P.C., Henderson, R., Leslie, A.G., Tate, C.G. and Schertler, G.F. (2008) Structure of a β1-adrenergic G-protein-coupled receptor. Nature. 454:486-491.

Wedegaertner, P.B. and Bourne, H.R. (1994) Activation and depalmitoylation of $G_s\alpha$. Cell. 77:1063-1070.

Wedegaertner, P.B., Bourne, H.R. and von Zastrow, M. (1996) Activation-induced subcellular redistribution of $G_s\alpha$. Mol Biol Cell. 7:1225-1233.

Weinman, E.J., Hall, R.A., Friedman, P.A., Liu-Chen, L.Y. and Shenolikar, S. (2006) The association of NHERF adaptor proteins with g protein-coupled receptors and receptor tyrosine kinases. Annu Rev Physiol. 68:491-505.

Weinman, E.J., Steplock, D., Tate, K., Hall, R.A., Spurney, R.F. and Shenolikar, S. (1998) Structure-function of recombinant Na/H exchanger regulatory factor (NHE-RF). J Clin Invest. 101:2199-2206.

Weis, W.I. and Kobilka, B.K. (2008) Structural insights into G-protein-coupled receptor activation. Curr Opin Struct Biol. 18:734-740.

Welch, H.C., Coadwell, W.J., Ellson, C.D., Ferguson, G.J., Andrews, S.R., Erdjument-Bromage, H., Tempst, P., Hawkins, P.T. and Stephens, L.R. (2002) P-Rex1, a PtdIns(3,4,5)P3- and Gβγ-regulated guanine-nucleotide exchange factor for Rac. Cell. 108:809-821.

Wess, J., Han, S.J., Kim, S.K., Jacobson, K.A. and Li, J.H. (2008) Conformational changes involved in G-protein-coupled-receptor activation. Trends Pharmacol Sci. 29:616-625.

Wettschureck, N. and Offermanns, S. (2005) Mammalian G proteins and their cell type specific functions. Physiol Rev. 85:1159-1204.

Wheatley, M. and Hawtin, S.R. (1999) Glycosylation of G-protein-coupled receptors for hormones central to normal reproductive functioning: its occurrence and role. Hum Reprod Update. 5:356-364.

Wittig, I., Karas, M. and Schagger, H. (2007) High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Mol Cell Proteomics. 6:1215-1225.

Wolf, G., Aumann, N., Michalska, M., Bast, A., Sonnemann, J., Beck, J.F., Lendeckel, U., Newsholme, P. and Walther, R. (2010) Peroxiredoxin III protects pancreatic ss cells from apoptosis. J Endocrinol. 207:163-175.

Wu, B., Chien, E.Y., Mol, C.D., Fenalti, G., Liu, W., Katritch, V., Abagyan, R., Brooun, A., Wells, P., Bi, F.C., Hamel, D.J., Kuhn, P., Handel, T.M., Cherezov, V. and Stevens, R.C. (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science. 330:1066-1071.

Wyatt, D., Malik, R., Vesecky, A.C. and Marchese, A. (2011) Small ubiquitin-like modifier modification of arrestin-3 regulates receptor trafficking. J Biol Chem. 286:3884-3893.

Yan, K., Kalyanaraman, V. and Gautam, N. (1996) Differential ability to form the G protein βγ complex among members of the β and γ subunit families. J Biol Chem. 271:7141-7146.

Yano, N. and Luo, L. (2004) Effect of thyrotropin releasing hormone (TRH) on gene expressions in rat pancreas: approach by microarray hybridization. JOP. 5:193-204.

Yeung, W.W. and Wong, Y.H. (2010) Gα16 interacts with Class IA phosphatidylinositol 3-kinases and inhibits Akt signaling. Cell Signal. 22:1379-1387.

Yu, R., Ashworth, R. and Hinkle, P.M. (1998) Receptors for thyrotropin-releasing hormone on rat lactotropes and thyrotropes. Thyroid. 8:887-894.

Yun, H.M., Kim, S., Kim, H.J., Kostenis, E., Kim, J.I., Seong, J.Y., Baik, J.H. and Rhim, H. (2007) The novel cellular mechanism of human 5-HT6 receptor through an interaction with Fyn. J Biol Chem. 282:5496-5505.

Zhang, W. and Neer, E.J. (2001) Reassembly of phospholipase C-β2 from separated domains: analysis of basal and G protein-stimulated activities. J Biol Chem. 276:2503-2508.

Zhu, C.C., Cook, L.B. and Hinkle, P.M. (2002) Dimerization and phosphorylation of thyrotropin-releasing hormone receptors are modulated by agonist stimulation. J Biol Chem. 277:28228-28237.

9. Supplement