SUBCELLULAR REDISTRIBUTION OF TRIMERIC G-PROTEINS - POTENTIAL MECHANISM OF DESENSITISATION OF HORMONE RESPONSE; *internalisation, solubilization, down-regulation*.

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Summary

Agonist-induced subcellular redistribution of G-protein coupled receptors (GPCR) and of trimeric guanine-nucleotide binding regulatory proteins (G-proteins) represent mechanisms of desensitisation of hormone response, which have been studied in our laboratory since 1989. This review brings a short summary of these results and also presents information about related literature data covering at least small part of research carried out in this area. We have also mentioned sodium plus potassium dependent adenosine triphosphatase (Na, K-ATPase) and ^{3}H-ouabain binding as useful reference standard of plasma membrane purity in the brain.

Key words

Brain · Subcellular fractionation · trimeric G-proteins · desensitisation · Na, K-ATPase

Introduction

Hormones or neurotransmitters bind to the stereo-specific site of the receptor at the cell surface. This binding reaction represents the first step in complicated sequence of

molecular events transmitting the signal into the cell interior and initiating the ultimate physiological response. In all eukaryotic organisms, a family of heterotrimeric GTP-binding and hydrolysing proteins (G-proteins) plays an essential transducing role in linking many cell-surface receptors to effector proteins at the plasma membrane (Hepler and Gilman 1992). In G-protein-mediated pathway (Gilman 1987), the hormone/neurotransmitter binding induces conformational change of receptor molecule, which, in the next step, induces dissociation of trimeric G-protein complex (non-active) into the free Gα (active) and Gβγ (also active) subunits. Subsequently, both Gα and Gβγ activate the number of enzyme activities (effectors) or ionic channels, which then regulate the intracellular concentrations of secondary messengers.

Switching on and off the G protein cycle

The activation of receptor and dissociation of the non-active trimeric G-protein complex into individual free (active) Gα and Gβ subunits is quickly followed by switching off the signal transmission. This is caused by hormone (agonist)-induced stimulation of endogenous high-affinity GTPase located in the Gα subunit. This enzyme activity hydrolyses the terminal γ-phosphate of Gα-GTP; the non-active Gα-GDP is formed; Gα-GDP subunits then exhibit high-affinity binding capacity towards the free Gβγ subunits, the two subunits bind together and the (non-active) trimeric G-protein complex Gαβγ is thus reassembled again and, in this way, the G-protein molecule is prepared for another round of an activation/deactivation cycle. The activation/deactivation cycle of trimeric G-proteins is rapid process, its turn - over rate ranges among different G-protein families from *mseconds* (transducin) to *seconds* range (Stryer 1991).

Desensitisation of hormone response

Besides the short-term switching on and off the signal initiated by agonist binding to GPCR, there are further types of dampening of the hormone response (Lefkowitz *et al*. 1980,

Hausdorff *et al*. 1990, Dohlman *et al*. 1991, Lohse *et al.* 1993, Geshengorn 1994). These mechanisms proceed in seconds-minutes time-scale and are followed by even longer "negative feedback" regulatory loops proceeding within hours-days. The former type of desensitisation is represented by receptor phosphorylation, sequestration and internalisation. In this way, receptors are physically removed from the plasma (cell) membrane and transferred into the cell interior - **internalised**. Thus, the receptor is excluded from functional pool located at the cell surface and transferred into an inactive pool located in the cell interior. The latter type of negative feedback regulation of GPCR action proceeds within hours-days. It is represented by **down-regulation** of receptor molecules and represents the terminal stage of receptor "life". In this way, the total number of receptors in the cell, which has been stimulated for a long time by respective agonist, is decreased.

The short-term desensitisation of GPCR action is primarily based on serine- or threonine-oriented phosphorylation at the C-terminus of receptor protein. Phosphorylation of receptor is followed by arrestin-induced functional uncoupling of receptor from G protein (Lefkowitz *et al*. 1980, Collins *et al*. 1992). Subsequently, the sequestration and internalisation of receptors occurs within time-scale of minutes. Major part of the agonist-induced internalisation of GPCR proceeds via clathrin-coated vesicles (Hausdorff *et al*. 1990).

G protein-based mechanisms of desensitisation

Besides receptor-based mechanisms of desensitisation of hormone response, changes in subcellular localisation of trimeric G-proteins have been suggested as potential mechanism of desensitisation of hormone response (Svoboda and Novotný 2002). These mechanisms were revealed by studies of subcellular localisation of G proteins in cells exposed to an agonist for relatively long periods of time (>30 min). The long-term stimulation of target cells is associated with internalisation, solubilisation and "down-regulation" of trimeric G-proteins $G_s\alpha$ and $G_q\alpha/G_{11}\alpha$ (Ransnas *et al*. 1989, 1991, 1992, Levis and Bourne 1992, Svoboda *et al.*

1992, 1994, 1996a, Kvapil *et al*. 1994, 1995a,b, Novotný *et al.* 1995, Milligan *et al*. 1995, Novotný and Svoboda 1998, Novotny *et al.* 2001, Durchánková *et al*. 2007). The internalisation of $G_q\alpha/G_{11}\alpha$ proceeds independently from internalisation of their cognate membrane receptor, TRH-R and vice versa (Drmota *et al*. 1998, 1999, Yu and Hinkle 1999). The agonist-induced internalisation of trimeric G proteins is not confined to $G_q\alpha/G_{11}\alpha$ but has been also demonstrated in other signalling cascades, such as β2-adrenergic receptor and $G_s\alpha$ protein in S49 lymphoma cells (Ransnas *et al*. 1991, 1992, Yu and Rasenick 2002). It seems likely, that G-protein internalisation and/or solubilisation represents the general homeostatic mechanism protecting the target cell against excessive, long-term stimulation. Considering the generally accepted view that many GPCR act through the same class of G-proteins, the internalisation or solubilisation of given type of G-protein represents the basis for ***heterologous*** desensitisation of hormone response. Over-expression of G11α prevented the homologous desensitisation of Ca^{2+} response to thyrotropin-releasing hormone (Novotný *et al*. 1999a). The simultaneous presence of both *homologous* and *heterologous* types of desensitisation was recently noticed in cells expressing (endogenously) angiotensin II receptors and large amount of TRH-R and G11α protein (Ostasov *et al*. 2007).

The mechanism of desensitisation of hormone response was studied in our laboratory not only in various cell lines such as S49 lymphoma, CHO or HEK293 cells, but we have also studied this phenomenon in natural tissue – brown adipose tissue (Svartengren *et al*. 1982, 1984, Svoboda *et al.* 1984a,b, 1993, 1996b). Methodological experience collected over the years has been more recently applied for studies of brain tissue.

Subcellular fraction of the brain tissue

Original subcellular fractionations of the brain tissue were based on combination of differential and density gradient centrifugation in sucrose media (DePierre and Karnovsky 1973, Whittacker 1984). These procedures resulted in separation of myelin, nerve-endings

(synaptosomes) and mitochondrial fractions mutually contaminated to larger or smaller extent. Bearing in mind that in brain and other tissues such as brown adipose tissue or heart-muscle (Svoboda and Lodin 1972, Ihnatovych *et al*. 2001, 2002a,b, Hejnova *et al*. 2002, Stohr *et al.* 2004, 2005, Svoboda *et al*. 1984a,b, 1993, 1996b, Bourova *et al*. 1999a,b, 2000, Novotny *et al.* 1999b, 2001, Hrbasova *et al.* 2003), the major part of subcellular membrane structures is represented by mitochondria, we have applied Percoll^R medium with the aim to achieve the clear separation of plasma membrane and mitochondrial fractions (for review of Percoll^R applications, see Pertoft 2000).

In the beginning of our experimental efforts, clear separation of upper (plasma membranes, PM) and lower (mitochondria, MITO) bands was not achieved. The total amount of protein applied per Percoll^R gradient as well as homogenisation intensity had to be decreased. The tightly - fitting homogenisers (Brown, clearance 0.002-0.004 mm) used in our previous fractionations of HEK293 cells (Bourova *et al*. 2003) had to be changed for loosely-fitting ones and homogenisation had to be performed mildly with the aim to prevent degradation of mitochondria. The starting brain homogenate was filtered through a nylon nets of decreasing pore size (330, 110 and 75 μm) and centrifuged at low speed to sediment nuclear fraction and cell debris (5 min at 3000xg). Optimum results were found when applying filtered post-nuclear supernatant containing 33-50 mg of protein on top of 30 ml of 30% Percoll^R in STE medium. After centrifugation in self-forming Percoll^R gradient, the upper layer of plasma membranes was separated from lower layer of mitochondria. Electron microscopy revealed very pure mitochondrial preparation. The upper layer of PM consisted of mixed population of large and small vesicular structures, synaptosomes and myelin (**Fig. 1, A, B** and **C**).

Subsequent sub-fractionation of plasma membrane fraction was performed by flotation in step-wise 15/20/25/30/35/40% sucrose gradient designed in similar way as in our previous

studies dealing with membrane domains/caveolae (Moravcova *et al*. 2004). Low-density plasma membrane fragments in 20/25% sucrose (**Fig. 1D,** LPM) were separated from the bulk of plasma membranes (**Fig. 1E**, BPM). LPM were enriched in large vesicular structures of synaptosomal origin. Major part of plasma membrane markers Na, K-ATPase, β-AR, DOR and $GABA_B$-R, caveolin 1,2, flotillin and $G_i1/G_i2\alpha$ proteins were co-localized in BPM. Majority of PM protein was also recovered in BPM.

Subcellular fractionation of transfected cell lines stably expressing defined signalling molecules

The best methodological approach how to isolate plasma (cell) membrane fragments is represented by density gradient centrifugation. Our earlier studies used the "sedimentation" types of continuous and later discontinuous sucrose gradients. When using this protocol, cell homogenate or post-nuclear supernatant was applied on the top of sucrose layer of the lowest density and subcellular particles like microsomes, plasma (cell) membrane derived vesicles, mitochondria, lysosomes and Golgi fragments migrated down through sucrose layers of increasing density. In this case, centrifugation was performed for relatively short period of time: 30-60 min in Beckman SW28 rotor. This type of centrifugation is denominated as *rate-zonal* mode of centrifugation (Svoboda *et al.* 1992, 1993, 1994, 1996a,b).

The second version of centrifugation, which was used in our more recent studies of ***membrane domains*** and had originally been successfully used for preparation of Golgi compartments, is much more time-consuming. It is represented by "flotation" of membrane fragments or vesicles ***up*** into equilibrium density zones, density of which is equal to buoyant density of a given membrane structure/vesicle (Pesanova *et al*. 1999, Bourova *et al*. 2003, Moravcova *et al*. 2004, Matousek *et al.* 2003a,b, 2005a,b, Rudajev *et al.* 2005). All these "flotation gradients" were performed with cell homogenate or crude membrane preparation applied to the bottom of the tube and subsequently, overlayed with sucrose layers of

decreasing density. In the future, we would like to use the same procedure, however, with purified PM as starting material. Therefore we applied Percoll^R-medium for resolution of plasma membrane and mitochondrial fractions from HEK293 cells stably expressing δ-opioid receptor, DOR (Moon *et al*. 2001), TRH-R (Kim *et al.* 1994), TRH-R plus large amount of $G_{11}\alpha$ (Svoboda *et al*. 1996a) or VSV-TRH-R-GFP hybrid molecule (Drmota *et al*. 1999). In this case, an optimum separation of PM and MITO bands was achieved when applying post-nuclear supernatant prepared from 15 x 80 cm2 flasks on the top of Percol^R-gradient in one Beckman Ti60 tube. Surprisingly, the efforts to use swing-out rotors for separation of PM and MITO bands were not successful. Angle rotors have to be used for proper separation of PM and MITO fractions when using Percoll^R medium for subcellular fractionation.

As shown in **Figs. 2 and 3**, purification of PM is not just methodological perfectionism of a biochemist obsessed with purity. Separation of PM-enriched fraction allows detection of physiologically significant functions such as agonist-stimulated trimeric G-protein activity with high efficiency (**Fig. 2**); it also allows detailed analysis of membrane proteins by 2-D electrophoresis (**Fig. 3**). It increases specificity of immunoblot assays when detecting very small amounts of membrane-bound signalling molecules such as trimeric G proteins.

Detection of Na, K-ATPase as internal standard of plasma membrane purity

Receptor number (B_{max}) or an intensity of an immunoblot signal of G-protein in a given PM preparation is related to the amount of protein present in a given sample. Thus, in studies of plasma membrane-bound signalling molecules such as receptors, G-proteins or adenylyl cyclase molecules, the contaminating proteins decrease the specific binding capacity or the specific content of signalling molecule. This does not mean a great problem if:

-i) the extent of contamination is the same,

-ii) protein is determined by the same method,

-iii) the same protein standard is used over the whole set of experiments,

-iiii) data are collected from the linear range of calibration curve.

These criteria, though appearing trivial, are not easy to be met in reality of physiological research and bear strong impact on interpretation of receptor or G-protein studies. Studies of ontogenetic development, effects of nutrition, long-term hormonal or drug exposure, long-term mechanisms of desensitisation of hormone response or drug addiction, environmental effects such as cold-acclimation and many others, require comparison of PM preparations of constant protein composition. In reality this means the work with PM preparation **contaminated** by a non-plasma membrane proteins to different extent. So, what sort of internal standard, inherently and exclusively bound/attached to and located in PM under all ***imaginable*** conditions may be used?

The sodium plus potassium activated, magnesium dependent adenosine-triphosphatase (EC 3.6.1.3) (Jorgensen 1975, 1988) is localised exclusively in the plasma membrane, represents an integral membrane protein strongly bound into PM (because of multiple transmembrane segments) and is expressed in central nervous system in high quantity. Therefore, detection of this enzyme activity might serve as a satisfactory tool for characterisation of purity of PM. However, there is a better, more quantitative and simple way how to estimate the purity of PM preparation with the help of Na, K-ATPase: the usage of radioactively labelled inhibitor, ^{3}H-ouabain.

Na, K-ATPase activity is selectively inhibited by cardioactive glycosides and its brain isoform was studied in our laboratory for a prolonged period of time (Svoboda and Mosinger 1981a,b, Svoboda *et al.* 1984c,d, 1986, Vyskočil *et al.* 1983, 1987, Amler *et al.* 1987, 1988a,b, Teisinger *et al.* 1992). Among wide range of cardiac glycosides, ouabain (strophantin G), as the water-soluble derivative, was often used when detecting this enzyme in PM preparations. We have also used it for characterisation of membrane polarity of Na, K-

ATPase molecule in brain microsomes (Svoboda *et al.* 1988). Surprising feature of this radioligand is very low non-specific binding, at least in brain.

It could be mentioned in this respect that [^{3}H]ouabain is by far the most specific ligand used by us in the course of 20 years of laboratory practice. The non-specific binding of radioactively labelled antagonists or agonists of GPCR [^{3}H-dihydroalprenolol (β-AR antagonist), ^{3}H-CGP12157 (β-AR antagonist), ^{125}I-azidobenylcarazolol (β-AR antagonist), ^{125}I-cyanopindol (β-AR antagonist), ^{3}H-TRH (TRH-R agonist), ^{3}H-CGP54566 ($GABA_B$-R antagonist), ^{3}H-baclofen ($GABA_B$-R agonist), ^{3}H-naltrindol (DOR agonist), ^{3}H-DADLE (DOR agonist), ^{3}H-DAMGO (MOR agonist), etc.] was significantly higher.

We have used ^{3}H-ouabain for comparison of PM isolated from brown adipose tissue of control and cold-acclimated hamsters (Svoboda *et al.* 1993, 1996b). Cold-acclimation induces dramatic increase of respiratory chain enzyme activities per cell; consequently, PM isolated from brown adipose tissue of cold-acclimated hamsters were contaminated by mitochondrial fragments to higher degree than PM isolated from control animals. Introduction of ^{3}H-ouabain as reference standard of PM purity, helped to overcome this problem. It is therefore logical to use this radioligand as "internal standard" of various PM compartments isolated from brain tissue in the future research as one should not forget that brain is the most oxidative tissue in the body of man and mammals and contains numerous (too many) mitochondria.

Acknowledgements

The authors thank to Graeme Milligan (Glasgow University, Scotland, UK), Paul A. Insel (School of Medicine, University of California, San Diego, USA), Lennart A. Ransnas (University of Goteborg, Sweden) and Barbara Cannon (The Wenner-Gren Institute, University of Stockholm, Sweden) for support, help and long lasting friendship, which made our work in the last 20 years possible. Experimental data presented in this work were

supported by Centrum of Neuroscience (project of MSMT LC554) and Grant Agency of Czech Republic (309/06/0121).

Legends to figures

Fig. 1 Subcellular fractionation of rat brain cortex

A, ***Separation of plasma membrane and mitochondrial fractions in Percoll^R gradient.*** Post-nuclear supernatant was prepared from cerebral cortex of the rat and fractionated in Percoll^R gradient. The upper layer of plasma membranes (PM) was separated from lower layer of mitochondria (MITO). **B**, plasma membrane fraction represented mixture of large and small vesicular structures together with sheets of myelin; **C**, in mitochondrial fraction, pure mitochondria were detected.

D, ***Flotation of plasma membrane fraction in sucrose gradient.*** The upper layer collected from Percoll^R gradient (PM) was fractionated by flotation in 15/20/25/30/35/40% w/v sucrose gradient. LDM, represented by hazy area in 15/20% sucrose (fractions 3-5), were resolved from bulk of PM observed as distinct, optically dense band in 35% sucrose or at 35/40% sucrose interface (fractions 7-8). **E**, LDM were composed from large synaptosomal membrane particles and myelin; **F**, BPM contained heterogeneous mixture of small vesicular structures (magnification 11700x).

Fig. 2

Detection of TRH-stimulated G-protein functional activity in purified PM fraction;

comparison with post-nuclear supernatant

HEK293 cells stably expressing TRH-R and $G_{11}\alpha$ protein were collected from 15x80 cm^2 flasks, homogenised, centrifuged at a low-speed and the post-nuclear supernatant (**PNS**) was applied on the top of 30% Percoll. Centrifugation was performed for 30 min at 65000xg and resulted in separation of upper (plasma membrane, **PM**) and mitochondrial fractions.

Agonist (TRH)-stimulated G-protein activity was measured by [^{35}S]GTPγS binding assay and compared in **PNS** and **PM** fractions.

Fig. 3

Resolution of plasma membrane proteins by 2-D electrophoresis

PM fraction was isolated from HEK293 cells stably expressing TRH-R and $G_{11}\alpha$ protein as described in legend to Fig. 2. PM proteins were resolved by 2-D electrophoresis and visualised by Sypro Ruby stain according to manufacturers protocol (Amersham). In parallel sample, the membrane proteins were transferred to nitrocellulose membrane and Gq/G11α proteins detected by immubloting with specific antibodies.

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Fig. 1

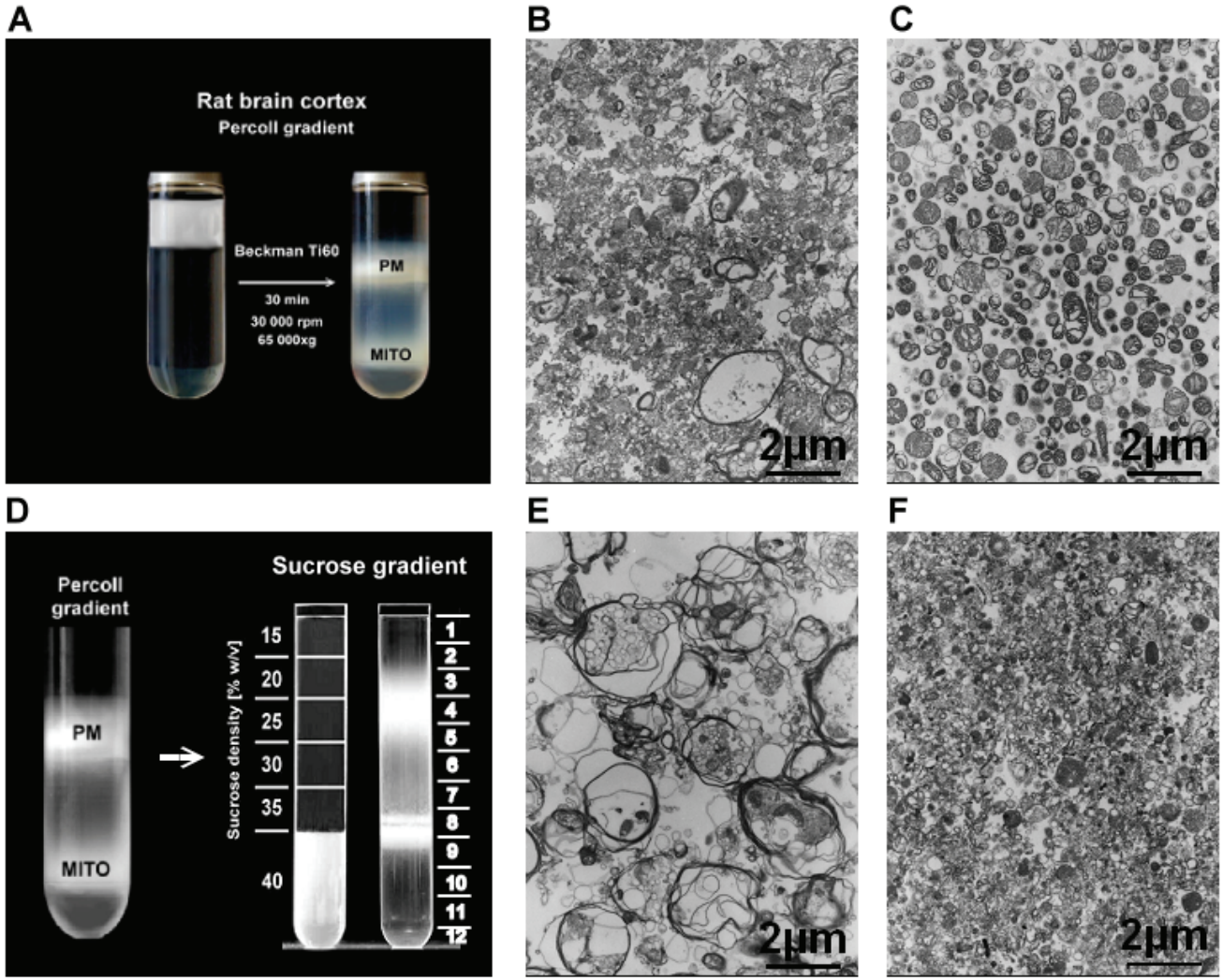
A
Rat brain cortex
Percoll gradient
Beckman Ti60
30 min
30 000 rpm
65 000xg
PM
MITO
B
2µm
C
2µm
D
Percoll gradient
PM
MITO
Sucrose gradient
Sucrose density [% w/v]
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
11
12
E
2µm
F
2µm

Fig. 2

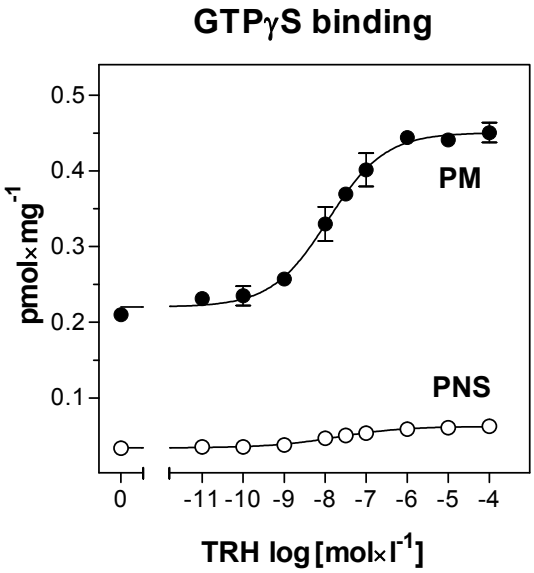
GTPγS binding
0.5
0.4
0.3
0.2
0.1
pmol×mg-1
PM
PNS
0
-11
-10
-9
-8
-7
-6
-5
-4
TRH log [mol×l-1]

Fig. 3

Percoll^{R}-purified PM
HEK293 cells
Total protein and $G_q/G_{11}\alpha$
±TRH (10^{-5}M, 16 hours)