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"Integrazione temporale del segnale mediato da recettori accoppiati a proteine G: il potenziale ruolo di G15 nella cancerogenesi"

"Temporal Integration of the signal mediated by G protein coupled receptors: the potential role of G15 in cancerogenesis"

S.S.D. **MED04**

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Abbreviations

AlF_3	Aluminum Fluoride
AVP	Arginine VasoPressin
β2AR	β2 Adrenergic Receptor
C5aR	C5a Chemotactic Receptor
DAG	DiAcylGlycerol
DOR	δ opiod receptor
DPDPE	[D-Pen^{2,5}]-enkephalin
CAM	Constitutively Active Mutant
EDG-6	Endothelial Differentiation, G-protein-coupled 6
ER	Endoplasmic Reticulum
FAK	Focal Adhesion Kinase
FPR	Formyl Peptide Receptors
GAP	GTPase Activating Proteins
GEF	Guanine nucleotide Exchange Factor
GDI	Guanine nucleotide Dissociation Inhibitor
GPCR	G Protein Coupled Receptor
GRK	G protein-coupled Receptor Kinase
HCMV	Human CytoMegaloVirus
IP3	Inositol (1,4,5)-trisPhosphate
Iso	Isoproterenol
MAPK	Mitogen Activated Protein Kinase
PKC	Protein Kinase C
PHA	PhytoHaemAgglutinin
PIP2	PhosphatidylInositol (4,5)-bisPhosphate
PLC	PhosphoLipase C
RGS	Regulator of G protein Signalling
SDF-1	Stromal Derives factor 1
SOCE	Store-Operated Ca^{2+} Entry
STAT	Signal Transducers and Activators of Transcription
TEC	Thymic medullary Epithelial Cells
TRPC	Transient Receptor Potential non-selective ion Channels
V2R	V2 Vasopressin Receptor
WT	Wild Type

Introduction

G-proteins and G-protein Coupled Receptors

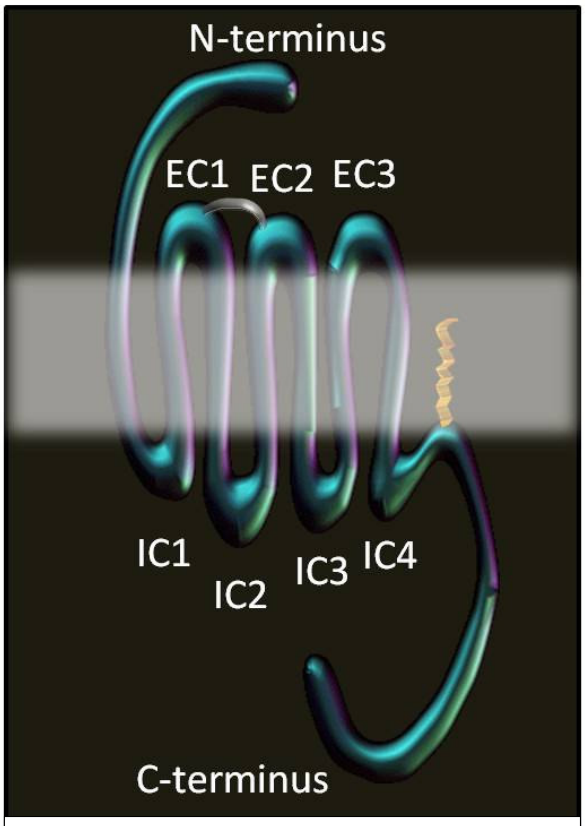


Figure 1 - "GPCR Topology": GPCR aminoacidic sequence typically includes seven hydrophobic alpha-helices that create seven transmembrane domains. This arrangement produces an extracellular N-terminus often glycosylated, three extracellular loops (EC), three intracellular loops (IC) and a fourth loop created by the palmytoylation of cysteine residues that become an anchor for the C-terminus. In most cases the C-terminus is particularly enriched in serines and threonines. A disulphide bridge usually links two cysteines present in EC 1 and EC2.

Any biological organism processes enormous amounts of data to survive and to adapt to the environment. Cells perceive the presence of nutrients, noxious agents, hormones, etc. by recognizing extremely heterogeneous arrays of external stimuli (including light, ions, peptides, sugars, lipids) and in many cases can follow the stimulus by trailing its concentration gradient. This implies the ability to detect the direction from where the "signal" originates and therefore, not only an integration of the information in three dimensions, but also an integration over time while moving toward a target or, as opposite, while stirring away from a repellent agent.

In multicellular organisms, communications evolved and became reciprocal. Stimuli are transmitted from one cell to another thanks to "ligand molecules". The ligand produced by a cell is set free to diffuse and specifically recognize transmembrane receptors on target cells. Receptors are molecules specifically committed to recognize a stimulus and to transmit its signal across the plasma membrane. The passage of information across the plasma membrane in most cases already implies a first

integration of the information (see below).

G-protein-coupled receptors (**GPCR**s) represent the largest family of proteins accounting for over 800 distinct genes, that correspond to more than 1% of the human genome and almost 3% of the proteome (Fredriksson et al., 2003; Vassilatis et al., 2003). GPCRs are expressed by almost all living organisms including fungi and viruses. It has been estimated that each individual eukaryotic cell expresses mRNA for perhaps over 100 different GPCRs (Hakak et al., 2003; Tang and Insel, 2004).

The members of this family of proteins mediate most of the signal transduction occurring across the plasma membrane. GPCRs not only respond to hormones, growth factors, neurotransmitters and chemokines but also respond to sensorial stimuli of sight, smell and taste. In order to mediate such a diversified number of tasks, specific GPCRs evolved to respond to structurally very diverse ligands including: peptides (chemokines, vasopressin, bradikinin, etc.), lipids (prostaglandin, tromboxane, lysophosphatidic acid, etc.), glycoproteins (follicle-stimulating hormone, thyroid-stimulating hormone, glucagon,etc.) aminoacids (glutamate, histidine,etc.) and their derivatives like bioamines (adrenalin, dopamine,etc.), ions (calcium and protons), photons (exciting retinal), aromatic molecules (odorant receptors), etc.

GPCRs are involved in the regulation of virtually all physiological functions. In the periphery of our organism, they modulate endocrine or exocrine secretion, pain transmission, smooth muscle and cardiac contraction, fluid homeostasis, blood pressure, immune response etc. In the central nervous system GPCR regulate body temperature, behavior, satiety, sleep, etc.

The development of a novel organism is likely the moment when cell-cell communications become more dynamic. Cells migrate from one district to another proliferating or dying in a coordinated fashion leading to the marvelous complexity of the entire organism. GPCRs like Frizzled (in the Wnt/catenin pathway) and Smoothened (in the Hedgehog pathway) are determinant key-factors in “cell development”. In many cases, the same receptor involved in the formation of the organ remains functional after cell differentiation is completed and in the adult cell it acquires tissue specific functions. A good example is represented by olfactory receptors. Each olfactory sensory neuron expresses a single olfactory GPCR after silencing several

hundreds homologs. It is this same GPCR that, trailing a still undefined chemotactic signal, drives the projection of the axon to a specific glomerulus in the olfactory bulb. In such glomerulus converge all, and only, projections of olfactory sensory neurons expressing the same GPCR (Mombaerts, 2004). As a result, in adult life, all olfactory neurons sensing a specific molecule will project to the same glomerulus.

The plethora of biological functions combined to the potential for pharmacological intervention intrinsic to the specificity of the ligand-receptor interaction (usually in the range of the low nanomolar) generated considerable interest in the mechanisms by which GPCRs exert their function. Not surprisingly, GPCRs are today the most successful pharmaceutical target and more than 30% of prescribed drugs are molecules that simulate or prevent natural GPCR ligands (Rozengurt, 2007c). This quota represents approximately 9% of global pharmaceutical sales (Rovati et al., 2007). However, therapeutic drugs act only on less than 40 distinct GPCR leaving a tremendous potential for drug discovery applied to this superfamily of proteins.

GPCR conformations

All GPCRs share a common molecular topology spanning seven times the plasma membrane (Figure 1). Typically, the interaction of the ligand with the extracellular domains triggers a conformational change that is transferred to the receptor intracellular domains and, on turn, specifically activates intracellular interactors. GPCRs derive their name by their ability to act as guanine nucleotide exchange factors (GEFs) for heterotrimeric (α, β, γ) G proteins (Figure 3). The occupied receptor thereby promotes the exchange of GTP for GDP in the alpha subunit of the G protein and, as a consequence, the activation of "downstream" signalling components.

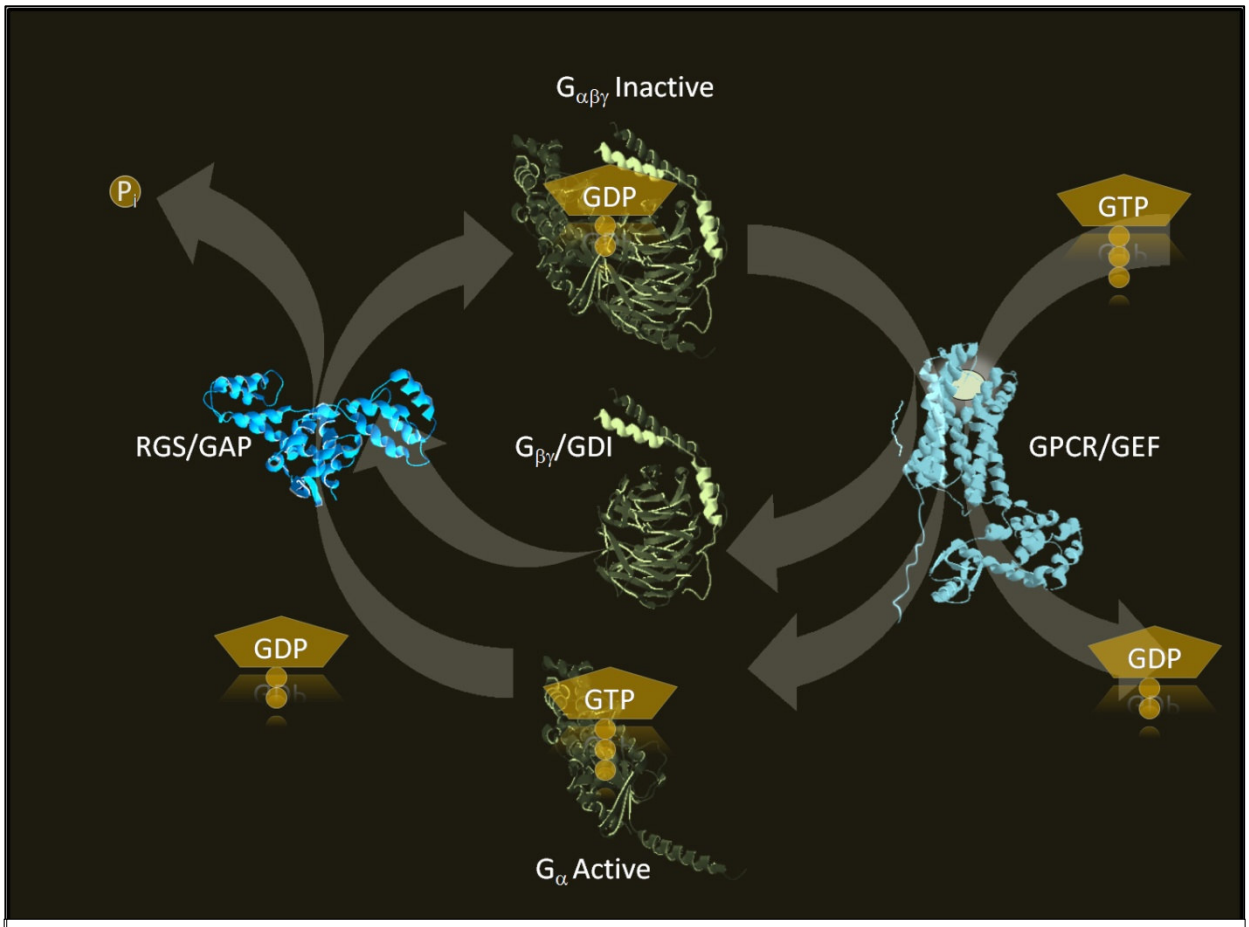


Figure 2 - G-protein cycle of activation: In the heterotrimeric form (α, β and γ subunits), G proteins are inactive with the α subunit bound to GDP. Active GPCRs act as exchange factors (**GEF**) favoring the release of GDP in exchange for GTP and the consequent separation of the βγ subunit. βγ acts therefore as guanine nucleotide dissociation inhibitor (**GDI**). Once separated, α and βγ subunits are now free to interact with their downstream effectors. The hydrolysis of GTP is catalyzed by proteins acting as Regulators of G protein signalling (**RGS**) also named GTPase Activating Proteins (**GAP**). The α subunit remaining bound to GDP associates again to the βγ subunit returning to the inactive state.

Mammals express 16 distinct α subunits genes grouped in four classes according to sequence similarity and effectors regulation:

- Gq class components activate phospholipases (**PLC**s) to hydrolyze phosphatidylinositol (4,5)-bisphosphate (**PIP2**) to diacylglycerol (**DAG**) and inositol (1,4,5)-trisphosphate (**IP3**).

- Gs class components activate adenylyl cyclases (**AC**) to increase the concentration of cAMP.

- Gi class components inhibit AC.

- G12 class components activate small GTPases (i.e. RhoA).

The released βγ subunit displays also functional activity. The identity of the components of the dimer determines which downstream effectors becomes activated (Birnbaumer, 2007).

GPCRs are remarkably versatile signalling molecules. An individual GPCR can signal through several G protein subtypes or even activate G-protein independent pathways (Lefkowitz and Shenoy, 2005; Brzostowski and Kimmel, 2001). This functional plasticity is likely due to their structural flexibility that allows each receptor to assume alternative specific conformations.

Initially, GPCR have been described as bimodal switches shifting from the "off state" to the "on state" in response to the interaction with the ligand. Accordingly, less effective agonists (partial agonists) were believed to vary in signal strength, nonetheless, all agonists were assumed to qualitatively reproduce the same effect as the endogenous agonist(s).

A similar scenario may apply to rhodopsin, with basal signalling virtually silent until the absorption of a single photon of light induces maximal activation. However, in many cases, GPCRs display significant levels of spontaneous activity. The extent of basal activity can be enhanced by single-point mutations in various structural domains. In several cases, mutations that disrupt the interactions between transmembrane domains also increase the 'flexibility' of the protein (movement of transmembrane domains relative to each other) and thus the probability that the receptor can assume an active conformation. Some of the best-characterized examples of constitutively active mutants (**CAM**s) are those that disrupt the highly conserved (D/E)R(Y/W/H) amino acid sequence (also called DRY motif) present in 72% of GPCRs belonging to the rhodopsin family (http://lmc.uab.cat/gmos/). This motif forms non-covalent interactions with the cytoplasmic end of the sixth transmembrane domains creating the so-called "ionic lock", which stabilizes the receptor in the basal state. The disruption of this network of interactions by mutating the DRY motif was shown to lead to constitutive activity (spontaneous activation) (Kobilka and Deupi, 2007).

Spectroscopic studies on purified receptors (Kobilka and Deupi, 2007) have also suggested that agonist binding and activation occur through a series of conformational intermediates. Possibly, the binding of structurally different agonists entails the disruption of specific combinations of intramolecular interactions. On the intracellular side, also the G protein in some cases was proven to interact with the inactive receptor (Figure 4) contributing to modify the conformation and the affinity of the receptor for the ligand (Birnbaumer, 2007). Therefore, at any time, agonists and intracellular direct interactors simultaneously contribute to shape the receptor

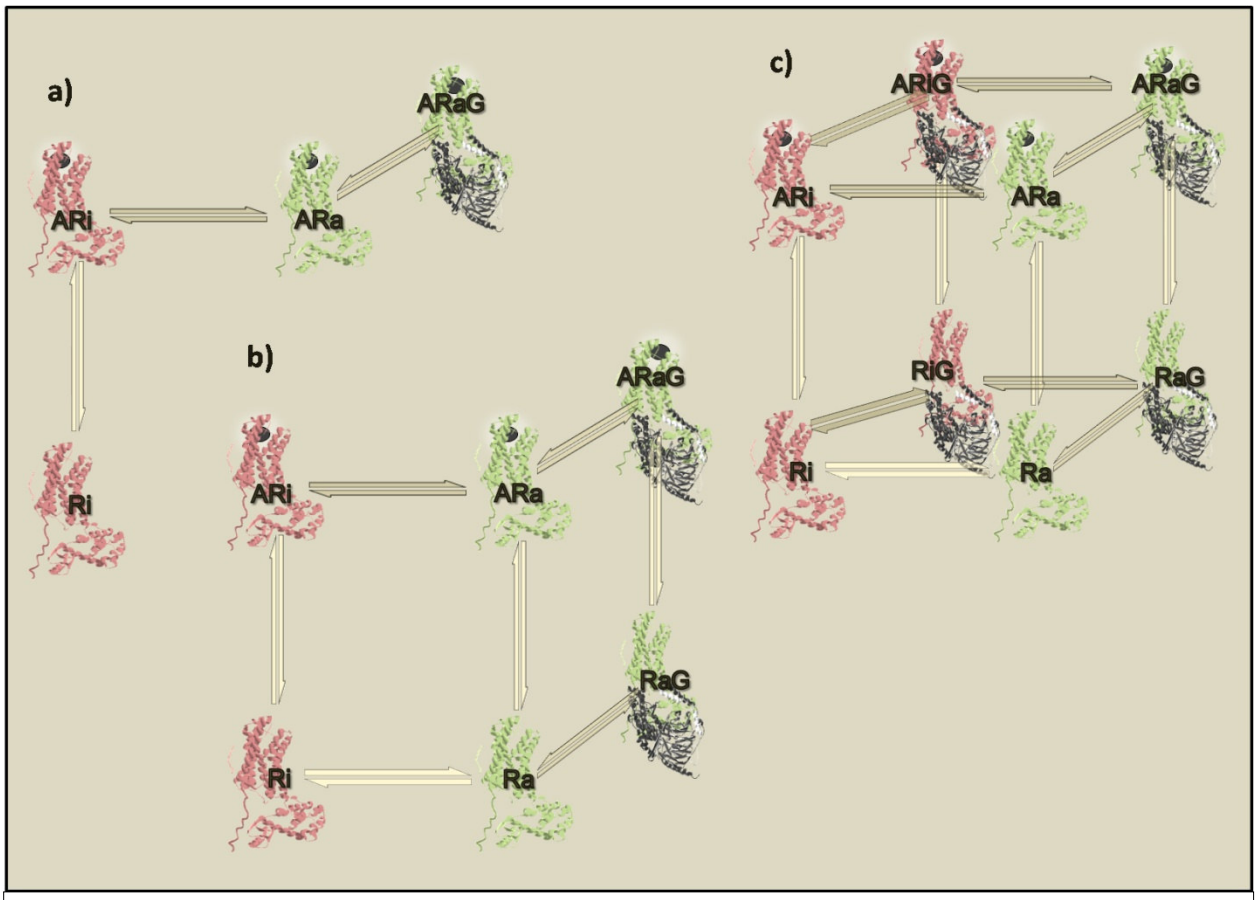


Figure 4 - GPCR pharmacological models - Free agonists (A) bind to the receptor (R) that on turn interacts with the G protein (G). All interactions follow the law of mass action (K1, K2, L, M = equilibrium dissociation constants). (**a**) 1980: 'Ternary complex model' by De Lean et al. (De Lean et al., 1980). Agonists bind to the receptors (R) and the (AR) complexes bind to G proteins to form active ternary (ARG) complexes. (**b**) 1993: 'Extended ternary complex model' by Samama et al. (Samama et al., 1993). Receptors must undergo a conformational transition from the basal, inactive state (Ri) into the active state (Ra) to bind to, and activate G proteins. Agonists favor this transition but it can also occur spontaneously. To be a true two-state model, α and β must be equal to 1. (**c**) 1996: 'Cubic ternary complex model' by Weiss et al. (Weiss et al., 1996). In this model are contemplated non-signalling complexes formed by inactive receptors and G proteins. Actually, the latter model is further expanded by biased agonism theories (see text).

conformation shifting the equilibrium of coexisting different states. In the model described above, agonists or the inverse agonists re-modulate the quotes of the different conformations. As a result, the message is transferred through the signalling network reducing or increasing the activity of effectors associated to one conformation or another, with the final result of producing a well defined physiological impact. This model could explain how most GPCRs simultaneously activate multiple signalling cascades sometime even bypassing the same G-protein (Brzostowski and Kimmel, 2001). In addition, the model explains how the final perturbation elicited by an agonist may depend on the intensity of the stimulation, not only in quantitative terms, but also qualitatively (Sun et al., 2007).

The existence of alternative activation states could explain paradoxes described for decades in pharmacology literature. For instance, the fact that different ligands for a given GPCR can show different efficacy profiles when distinct signalling pathways are compared (Kenakin, 2003; Kobilka and Deupi, 2007), in other words, different functional responses described by dose-response curves are not necessarily comparable when the ligand changes.

Another aspect that can be explained by the existence of distinct GPCR conformations is the existence of partial agonists, which, as mentioned above, are ligands that cannot produce the same maximal effect of a full agonist (even once all available receptors are occupied).

The complexity of the transduction process

During the normal life of a cell, dozens of different receptors are active at any given moment. As a result, multiple signals depart the plasma membrane to travel inside the cell. The diffusion of the signal occurs through shared effectors converging on a relatively limited number of common knots (Figure 5). The activation scheme is further complicated by the fact that GPCR can oligomerize. Compelling evidence shows in fact that omo- or hetero- dimers form *in vivo* (Park et al., 2004). Yet, besides very few exceptions, it remains obscure how the partners influence each other signalling properties.

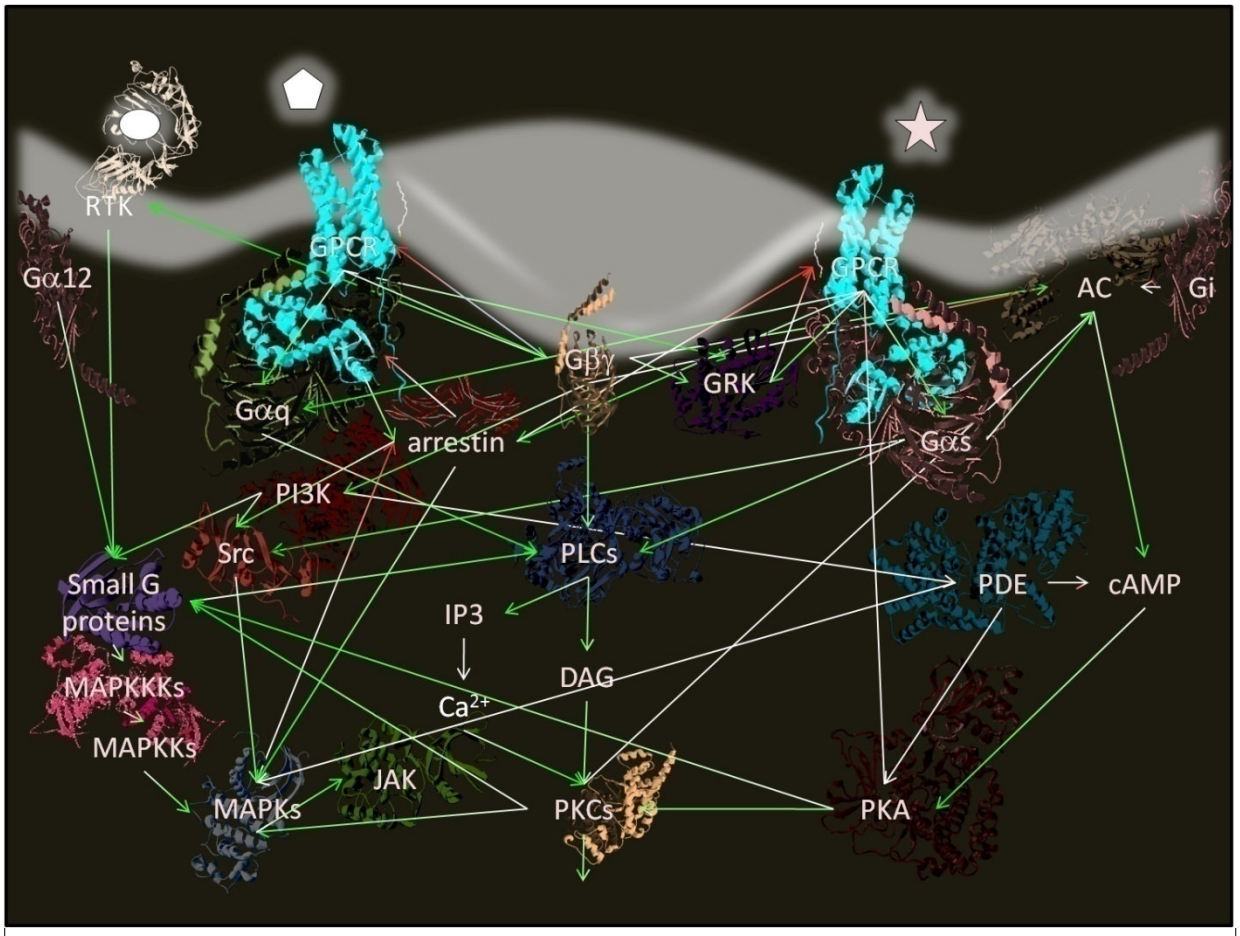


Figure 5 - Highly generalized scheme of the "network" of transduction pathways activated by GPCRs –This figure is an attempt to exemplify the most recurrent functional relationships among the major classes of signalling molecules. The arrows are representative of activatory (green) or inhibitory (red) interactions. Almost a thousand GPCR isoforms signal through a relatively limited number of highly shared effectors such us 16 Gα subunits, 2 non visual arrestins, 7 GRK.

The entire human "kinome" is estimated to comprehend approximately 500 members (Manning et al., 2002), usually a stimulus travels through the cytosol by branching sequences of phosphorylatory events and an apparently very limited number of second messengers. As a result, the signal converges on common knots often shared with other receptor types, like tyrosine kinase receptors.

GPCRs can utilize different scaffolds to activate the small G-protein/MAPK cascade, employing at least three different classes of Tyr kinases:

1) Src family kinases are recruited following activation of PI3K by βγ subunits. They are also recruited by receptor internalization, 2) cross-activation of receptor Tyr kinases, 3) signaling through an integrin scaffold involving Pyk2 and/or FAK. GPCRs can also employ PLCβ to mediate activation of PKC and CaMKII, which can have either stimulatory or inhibitory consequences for the downstream MAPK pathway.

It remains largely unclear how so many receptors process meaningful information by acting simultaneously through such a small number of effectors. How many G proteins are required to sort the appropriate response to a ligand? Is spatial organization of the molecules (enzymes, second messengers,etc.) what establishes autonomous circuits and therefore specificity? How does the signalling of each receptor adapt to different types of cell differentiation? All these questions are just beginning to be answered as technology develops the tools with the necessary resolution. For the moment however, earlier models of signalling cascades created by linear chains of interactions between exclusive partners appear today inadequate.

The regulation of the heartbeat, the modulation of synaptic transmission, a neutrophil chasing a bacterium in the bloodstream are just some examples of the final effect of coordinated GPCR activation. Though we begin to understand which factors are involved in transducing the signal we are likely still missing "the syntax". The emerging picture sees GPCR activation far more complicate than molecular pharmacologists imagined a decade ago. Rather than switches, may be receptors should be depicted more like the keys of a piano. Like single notes played simultaneously and timely generate music, a network of harmonic coordinated signals is likely produced once a GPCR agonist reaches the cell surface, and in the next paragraph we will see how the intensity and duration of the stimuli is also taken into account and integrated by these molecules.

GPCR regulation over time

GPCR stimuli are almost never presented to the cell as an "all or nothing" event, rather, the cell has usually to track continuous variations of the intensity of the stimulus. For example, a principal cell, determining how much water the kidney should recover by concentrating urine, is regulated by variation of the concentration of the antidiuretic hormone in the blood; the glucose receptor in yeast continuously monitors the sugar concentration in the environment, etc.

Over time, cells finely tune receptor efficiency by matching the cellular responsiveness to the intensity of the stimulation. Even when the concentration of the stimuli varies by many logs, an appropriate response can be obtained adjusting the sensitivity threshold. One example is represented by cells following gradients of stimuli such as leukocytes chasing bacteria or

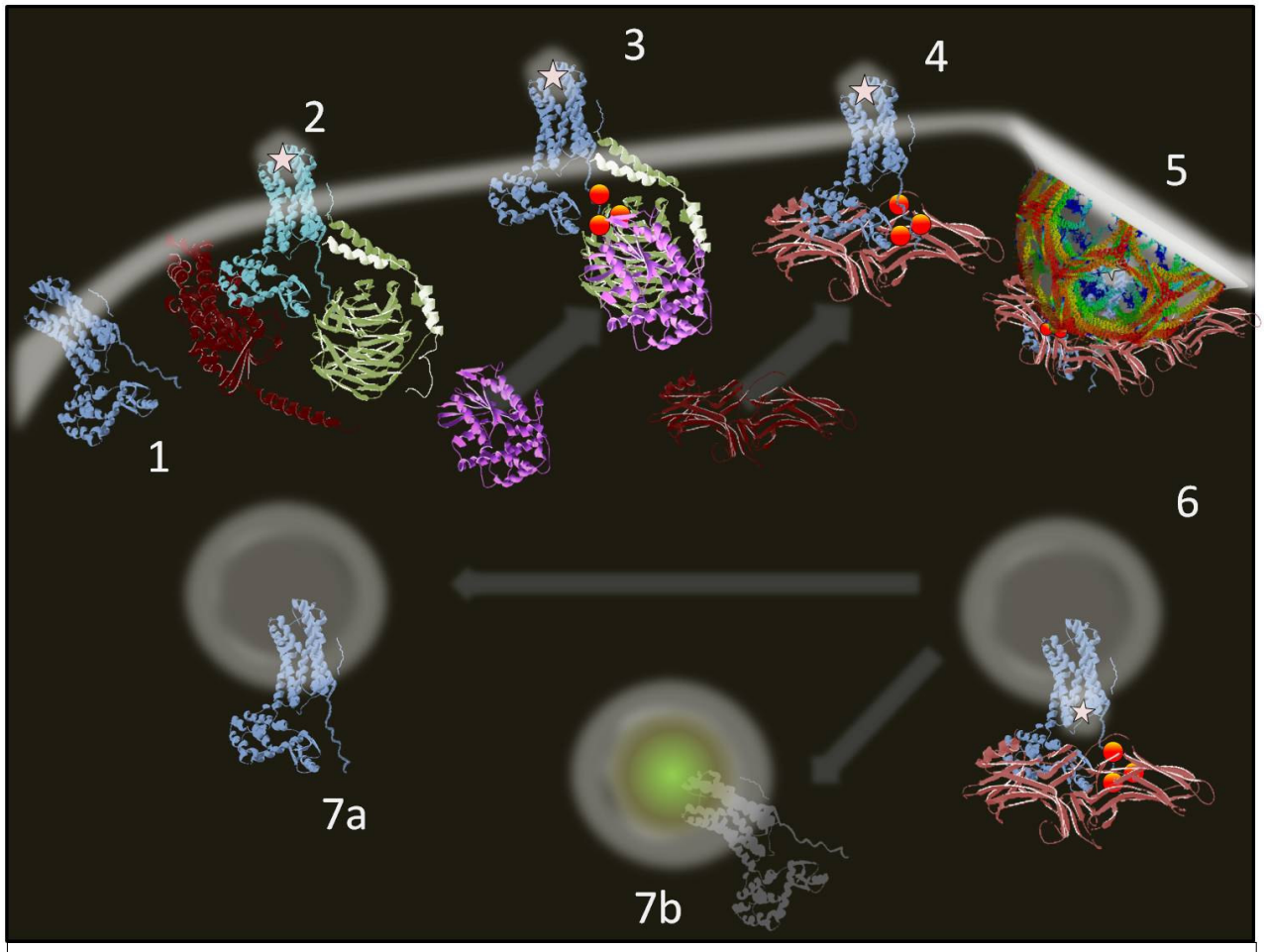


Figure 6 - GPCR Regulation - Schematic representation of the steps leading to GPCR desensitization, internalization and recycling. 1) Free GPCR is recognized by the cognate ligand. 2) The occupied receptor activates the G protein. 3) GRK is targeted to the plasma membrane by the interaction of its PH domain with βγ. Simultaneously, it recognizes the activated GPCR and catalyzes its phosphorylation on multiple sites. 4) The receptor is recognized by arrestin that binds to it and obstructs further interaction with the G protein. 5) The complex receptor-arrestin binds to other adaptors (AP2, NSF) and is recruited to clathrin coated pits. 6) GPCRs are endocytosed in early endosomes from where they can be targeted either to 7a) recycling compartments and from there back to the plasma membrane, or to 7b) lysosomes for degradation.

migrating toward the inflammation site while trailing chemokine signals.

An efficient regulation of the receptor potency is crucial not only under normal conditions, but also under pharmacological treatment of many diseases when GPCR agonists typically produce prolonged stimulation.

The process by which repeated or prolonged stimulation progressively reduces receptor efficiency and sensitivity is defined as "desensitization" and it includes regulatory mechanisms acting directly on the GPCR.

Within seconds of becoming occupied by the agonist and activating the G-protein, the receptor becomes phosphorylated by kinases named GPCR kinases (**GRKs**) (Figure 6). GRKs are activated by their own substrate, the occupied receptor. For this reason GRK activity is defined as homologous phosphorylation as opposed to the heterologous phosphorylation mediated by kinases activated by 2nd messengers (PKA and PKC). In the latter case, the substrate is not necessarily the same receptor that triggered the signal but can be another GPCR (ligand free) even of a different subtype. Normally the phosphorylation level is proportional to the number of receptors occupied by the ligand and remains sustained as long as the ligand is not removed (Innamorati et al., 1997). However in rare cases, the phenomenon is transient and rapidly declines even in continuous presence of saturating concentrations of ligand (Innamorati et al., 1998).

GRK phosphorylation usually occurs on multiple serines and threonines usually all located in the carboxyl terminal cytoplasmic tail. Over a threshold of 2-3 phosphate groups per receptor, phosphorylation promotes the interaction with a second class of proteins, named arrestins. Like the G proteins and GRKs, two ubiquitous arrestins recognize hundreds of GPCRs conditionally to their activation state (other two arrestin isoforms are specifically expressed in the retina). The interaction with arrestin interdicts the interaction with the G-protein and therefore limits the duration of G-protein signal by steric exclusion (Lefkowitz, 2007). β-arrestins also scaffold receptors to the membrane-trafficking machinery (NSF, AP-2, clathrin, sorting nexins,etc.) promoting receptor recruitment to coated pits and its internalization away from the cell surface and from G proteins. The rate of internalization can vary depending on the number and type of receptor. GPCR structural heterogeneity likely modulates the relative affinity for endocytic

adaptors (Yang and Xia, 2006) and therefore the intracellular routing followed by endocytosed receptors via multiple endocytic mechanisms, including caveolae. The same stability of the interaction with arrestin depends on the number and the pattern of phosphorylated sites; it therefore varies depending on the GPCR taken into consideration (Oakley et al., 2001). Arrestin can detach with clathrin just upon the vesicle is released from the plasma membrane, otherwise it can follow the receptor while trafficking to endosomal compartments (Innamorati et al., 2001).
The physiological role played by the concerted action of GRKs and arrestins in maintaining appropriate levels of GPCR signalling has been documented by transgenic and knockout animals (Premont and Gainetdinov, 2007). It should also be mentioned that while β-arrestins turn off G-protein coupling, they simultaneously turn on other sets of signals scaffolding numerous signalling molecules (such as MAPKs, Src) (Lefkowitz et al., 2006). The time extent of the interaction is likely to have important implications in this context. What described above, was mostly discovered studying the activation of "prototypical" GPCRs, such as the β2 adrenergic receptor (**β2AR**) and the V2 vasopressin receptor (**V2R**), that display very low spontaneous activation.
However, other GPCRs with higher spontaneous activity show tonic phosphorylation also in the absence of the ligand, possibly correlated to a higher basal turn-over (Innamorati et al., 2006).

Constitutive receptor activity and constitutive desensitization

As reported above, GPCRs can display robust constitutive activity due to their spontaneous activation or to activatory mutations in the case of CAMs. In some cases, such ligand independent activation is associated to the phosphorylation of the receptor and to a reduced efficiency. The effect of the mutation is however difficult to reconcile with a model accounting for only two receptor conformations (active and inactive). Most likely also the desensitization process is calibrated depending on the conformation of the receptor. An emblematic example is the V2R.
The substitution to cysteine or leucine of the central arginine of the DRY motif (DRH in the V2R) produces a mutant that displays higher activity as compared to the wild type (**WT**). As a result, patients carrying these mutations are affected by "nephrogenic syndrome of inappropriate

antidiuresis" (Feldman et al., 2005). These individuals concentrate urine as under the intense effect of the antidiuretic hormone Arg-Vasopressin (**AVP**) that exerts its antidiuretic action by binding to the V2R. However, the effect is due to the constitutive action of the receptor since in the blood AVP is virtually absent due to the hyponatrimia that induces the hypophisis to block its release.

As opposite, the substitution to histidine of the same arginine, stabilizes the receptor in a conformation that remains stably associated to β-arrestin (Barak et al., 2001). Because of the enhanced arrestin function, the V2R-R137H is substantially incapable of activating the natural V2R effector, Gs, and adenylyl cyclase as demonstrated by heterologous expression in HEK-293T (Barak et al., 2001) and L- (Rosenthal et al., 1993) cells. Consistently, patients carrying the R137H mutation suffer of nephrogenic diabetes insipidus, manifesting the same symptoms of patients carrying V2R mutations that prevent the synthesis of a functional receptor or its expression to the cell surface.

GPCR mediated mitogenic signalling

The cells of multicellular organisms must efficiently communicate with each other to coordinate and integrate their functions, including their reproduction. GPCRs agonists of different nature (aminoacidic, peptidic, lipidic,etc.) can act as potent cellular growth factors and relay mitogenic signals to the nucleus promoting cell proliferation.

Consistently, heterotrimeric G proteins have been implicated as mitogenic signal transmitters. The discovery of activating G protein mutations in various disease states highlights their roles in normal and aberrant growth (Vallar, 1996). To date, a number of G proteins subunits have been shown to stimulate mitogenesis and to induce neoplastic growth via initiation of intracellular signalling cascades that lead to the activation of transcription factors (Figure 7). Several intracellular effectors are responsible for creating the connection the nucleus. The best characterized regulators of DNA transcription are likely mitogen-activated protein kinases (**MAPK**s) (Dorsam and Gutkind, 2007). In addition, other critical molecules, such as signal transducers and activators of transcription (**STAT**s), have been shown to participate in the

transduction of proliferative signals (Ram et al., 2000). STATs were also reported as substrates of MAPKs. Intermediate steps in these signalling cascades include phospholipases (A2, C, D), small GTPases (Ras, Rho,etc.), kinases (Src, focal adhesion kinases (**FAK**), protein kinase C (**PKC**)) (Chiu and Rozengurt, 2001). As a first step, many mitogenic GPCR agonists activate Gq/11 family members. Four members of the Gq/11 subfamily (Gαq, Gα11, Gα14, and Gα15) couple GPCR to inositol lipid signalling via phospholipase C-β isozymes 1-4 (**PLC-β**1-4) and other protein binding partners (Hubbard and Hepler, 2006). Activated Gα subunits stimulate the enzymatic activity of PLC-β (Rhee, 2001). As a result, the breakdown of the membrane lipid phosphatidylinositol (4,5)-bisphosphate (**PIP2**) produces second messengers inositol (1,4,5)-trisphosphate (**IP3**) and diacylglycerol (**DAG**).

The Gβγ subunit is also active on PLC isoforms (PLCβ2 and PLCβ3), the lower affinity, is compensated by the large abundance of Gi in many cellular membranes that makes "Gi coupled receptors" also effective activators of PLC. IP3 binding to specific receptors on the endoplasmic

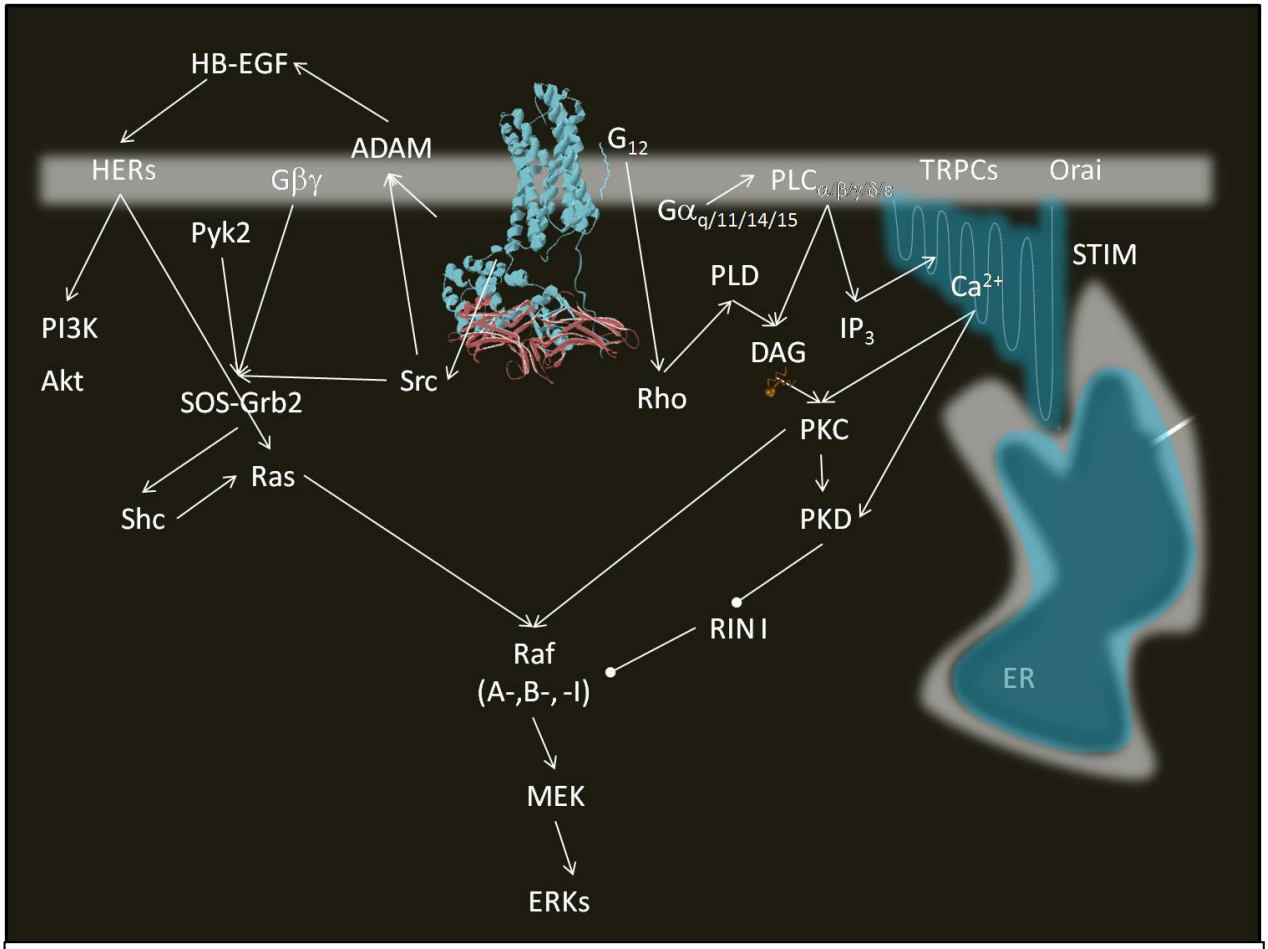


Figure 7 - GPCR mediated mitogenic signalling. This scheme summarizes the best described signalling pathways triggered by GPCRs and leading to the nucleus. Signalling mediated by PLC and Ras converge on MAPKs aimed to regulate cell cycle.

reticulum (**ER**) produces the release of large quantities of Ca^{2+} that are rapidly reabsorbed producing the typical transient increase. The depletion of the intracellular ER stores on turn triggers the opening of plasma membrane Ca^{2+} channels leading to the long-lasting plateau phase determined by the influx of extracellular ions. This process is known as "store-operated Ca^{2+} entry" (**SOCE**) and it is relevant to mitogenic stimuli (Charlesworth and Rozengurt, 1994). SOCE molecular bases remain poorly understood, in particular, it is still unclear how the depletion of the ER stores is sensed and transmitted to the plasma membrane. STIM is a ER resident protein strongly suspected to be the Ca^{2+} sensor (Liou et al., 2005; Roos et al., 2005; Lopez et al., 2008) while the strongest suspects on the plasma membrane are the transient receptor potential non-selective ion channels (**TRPC**) (Smyth et al., 2006) and the tetra-spanning Orai.

Sub-maximal GPCR stimulations are most of the time associated to oscillatory Ca^{2+} concentration, the frequency of the waves is proportional to the intensity of the stimulus (agonist concentration) and strongly depends on extracellular ions. SOCE is therefore responding to GPCR occupancy in a very coordinate manner transforming the message carried by the agonist in Ca^{2+} waves. Frequency and amplitude of the waves can regulate gene transcription and cell proliferation in a very precise manner. Calcium signalling can therefore be considered as a yet undeciphered language utilizing a syntax based on periodical variations of Ca^{2+} concentration.

DAG also contributes to mitogenic signals. Exogenously added to Swiss 3T3, it is sufficient to promote cell division (Rozengurt et al., 1984). DAG best established target is PKC, a regulatory domain interacts directly with DAG in all "novel" PKC isoforms (δ, ε, η, θ) and in all "classic" (α, β, γ) isoforms where it synergizes with Ca^{2+} binding to a distinct region (Rozengurt, 2007c). DAG effect can be stimulated by phorbol esters like PMA that are also potent carcinogens.

PKC involvement in the regulation of cell proliferation is very well established including for stimuli generated by GPCRs. Its effect can promote or inhibit cell proliferation or survival depending on the isoforms and on the context.

As the signal travels from the plasma membrane toward the nucleus, many other effectors become involved, not only strictly providing the connection but also integrating and modulating it.

PKD (a PKC related kinase activated by DAG and PKC phosphorylation) can act prolonging the duration of GPCR signalling (bombesin and AVP receptors) via MAPK (MEK→ERK→p90RSK (Rozengurt, 2007b)) aimed to promote DNA synthesis and cell proliferation in Swiss 3T3 cells. In fact, shortening sustained ERK activation prevents the effect on DNA synthesis. By modulating the time of the stimulation, PKD is therefore crucial to define gene products induced by GPCR activation. A number of scaffolding proteins and inhibitors is being discovered, these key-factors are likely responsible for making the stimulation more rapid, potent, specific or for modulating its duration.

For instance, PKC and PKD can relieve the action of inhibitors, like RKIP (Raf kinase inhibitor protein) and RIN1 (Rab Interactor 1) respectively (Wang et al., 2002), synergizing with other pathways.

RKIP and RINI phosphorylation by PKC sets Raf free from the inhibitor that relocate its action to GRK thus preventing GPCR homologous desensitization (Lorenz et al., 2003).

Cytosolic β-arrestins translocate on the plasma membrane to scaffold MAPKs to the stimulated GPCR once they have been initiated to the desensitization process (see page 12). In addition, β-arrestins have nuclear localization signals. In the case of β-arrestin1, this ensures nuclear and cytosolic distribution. In the case of β-arrestin 2, a prevailing nuclear export signal prevents nuclear accumulation (Scott et al., 2002). Massive GPCRs activation can deplete cytosolic/nuclear arrestin inducing the massive redistribution of MAPKs (Scott et al., 2002; Tohgo et al., 2003). Despite the astonishing number of interactions revealed so far, the role of arrestin in carcinogenesis remains elusive and could go beyond cell proliferation. Arrestin promoted ERK activity has been involved in cell migration and chemotaxis (Ge et al., 2004) and PGE2-induced metastasis in colon cancer cells (Buchanan et al., 2006).

As in any other physiological response triggered by GPCR, the pathways promoting cell proliferation are extensively interconnected by synergistic and antagonistic cross-talks. PLCβ2 and PLCε are activated by small GTPases (Rho, Ras) (Bunney and Katan, 2006).

The signal can also take less conventional paths, after crossing a first time the plasma membrane via GPCR, it can cross back exiting the cell to re-enter by activating tyrosin kinase receptors like the EGF receptor in a process named transactivation. GPCR effectors in fact activate metalloproteases (ADAM family) that cleave precursor forms of EGF, TGF-α, amphiregulin (Rozengurt, 2007a). Transactivation can also occur intracellularly or possibly by direct interaction between the receptors (Buchanan et al., 2006; Hunyady and Catt, 2006). In distinct cell types, transactivation mediates GPCR induced Ras->Raf->MEK->ERK->p90RSK and PI3K->Akt activation (Santiskulvong et al., 2001; Santiskulvong and Rozengurt, 2003; Chiu et al., 2005). Transactivation may therefore contribute to explain how GPCRs control cell cycle progression.

G15

G15 is a heterotrimeric G protein member of the Gq class, that also includes Gq, G11 and G14. Because of its restricted distribution and because of its peculiar signalling characteristics G15 is probably the most peculiar member of the Gq family.

G15 distribution

Among Gq class members, Gαq and Gα11 are ubiquitously distributed across tissues as they have been detected in every screened cell type (Hubbard and Hepler, 2006). However in a few cases one of the two prevails: differential expression was observed in certain T-cells types and other leukocytes while platelets selectively express Gαq but not Gα11. The other Gq family members, G15 and G14, have a much more limited distribution. The tissue expression pattern of Gα15 has been widely reported as the most restricted. An initial characterization of Gα15 distribution was based on cell lines and depicted a profile limited to cell types of hematopoietic origin (Amatruda, III et al., 1991). Further analysis localized Gα15 to tissues that are rich in hematopoietic cells (Wilkie et al., 1991). In lymphoma patients undergone to chemotherapy, Gα15 mRNA expression was associated to the presence of CD34 antigen during hematopoietic recovery (Pfeilstocker et al., 2000). CD34 antigen is a reliable surface marker for quantification of hematopoietic cells but its mRNA expression is restricted to the Go phase (quiescent cells) (Pfeilstocker et al., 2000). By contrast, Gα15 mRNA is expressed independently of cell cycle stage. In particular, Gα15 is enriched in cells in the earlier stages of differentiation. Various myeloid and lymphoid cells in the progenitor stages express Gα15 at high levels, but these levels decrease sharply as the cells become differentiated (Tenailleau et al., 1997). Gα15 is down

regulated also during megakaryocytopoiesis (den Dekker et al., 2001), however, in one report, Gα15 was found in the cytosolic fraction of platelets enriched of secretory granules (Giesberts et al., 1997). In HL60 cells, Gα15 expression is downregulated during terminal differentiation induced by dimethyl sulfoxide as the cells acquire morphological features characteristic of neutrophils and lose the ability to proliferate (Amatruda, III et al., 1991).
Under very specific circumstances Gα15 expression is restored, i.e. upon activation of quiescent blood lymphocytes in T (Lippert et al., 1997) and likely in B (Rosskopf et al., 2003) cells, as previously suggested by the presence of Gα15 mRNA in human B lymphoblast cell lines derived from peripheral blood lymphocytes and immortalized with Epstein-Barr virus (Rosskopf et al., 2003).

G15 signalling

Gq class members share functional abilities but exhibit limited amino acid sequence identity. Overall identity is only 57% (compared with 90% for Gs and 85% for Gi) that gets to 30% within the first 40 amino acids. As compared to Gαq, Gα11 is 83% identical in this region while for Gα14 and Gα15 this value decreases to only 65% and 35% respectively. The extensive sequence diversity of Gα14 and Gα15, suggests that these proteins may be functionally divergent from Gαq and Gα11, or at least may be regulated by different biochemical mechanisms.
G15 restricted expression pattern suggests tissue-specific signalling functions. Consistently, Gα15 expression has been shown to be important for multiple aspects of hematopoietic physiology including erythroid differentiation and T-cell activation (Lippert et al., 1997; Ghose et al., 1999). In particular, G15 was demonstrated to play a role in regulating TCR/CD3-induced production of two important cytokines, IL-2 and IL-10 (Zhou et al., 1998). G15 signalling could be associated to intense cellular expansion (activated T and B cells). Accordingly, the downregulation or upregulation of G15 functional activity affected growth rates and differentiation of human erythroleukemia cells, a model of erythroid differentiation.

However, knock out approach demonstrated that in mice Gα15 can be dispensed under normal conditions. Transgenic mice are in fact viable and fertile. Furthermore, Gα15 -/- mice show

normal hematopoiesis. In *ex-vivo* experiments, macrophages derived from these mice were challenged with GPCR agonists considered to couple to G15 *in vivo*: C5a, UTP, PAF. Inositol phosphate production and Ca^{2+} mobilization were affected only in the case of the anaphylatoxin C5a, with a partial reduction of the responses (likely simultaneously supported also by Gi (Davignon et al., 2000a)), the effects of chemokines on Ca^{2+}signalling in myeloid cells are in fact largely PTX-sensitive and therefore mediated by Gβγ derived from resident Gi rather than G15 (Offermanns and Simon, 1995; Vanek et al., 1994; Baggiolini and Clark-Lewis, 1992).

G15, and to a lesser extent G14, exhibit a surprising ability to couple to GPCRs that are not reported to be naturally linked to inositol lipid signalling. Followed by many other GPCR, Gi/o-linked C5a chemotactic receptor (**C5aR**) was the first one shown to couple to recombinant G15 (Amatruda, III et al., 1993; Buhl et al., 1993) in mammalian cells. A number of receptors (β2 and α1b adrenergic, M2 muscarinic, V2 AVP, D1 dopamine, adenosine A, serotonin, opioid, various chemotactic receptors and metabotropic glutamate receptors just to cite a few) have been found to interact promiscuously with Gα15 when reconstituted into COS-7 cells (Offermanns and Simon, 1995; Zhu and Birnbaumer, 1996). Despite such high promiscuity, some degree of specificity persists. The receptor promiscuity of G15 is in fact less apparent when co-transfected with the α1a and α1d adrenergic receptors that couple to other G-proteins but not to G15 (Hubbard and Hepler, 2006; Xie et al., 1997) or some C-C chemokine receptors although normally co-expressed with Gα15 (Arai and Charo, 1996; Kuang et al., 1996).

Given their role in hematopoiesis, chemokine receptors are traditionally considered the most likely upstream activators of G15 signalling, however, it is quite possible that GPCRs other than chemokines receptors are the main activators of G15 signalling *in vivo*. As a matter of fact, chemokine receptors are expressed at the highest levels in mature cells (Hubbard and Hepler, 2006) while, as mentioned above, Gα15 is highly expressed in progenitor cells and decreases during differentiation (Amatruda, III et al., 1991) as shown in HL-60 cells (Perez et al., 1992; Boulay et al., 1991; Minisini et al., 2003). Many other GPCRs could act via G15. Hematopoietic cells expresses hundreds of them, including the δ opioid (Steidl et al., 2004) (**DOR**) and the β2 adrenergic (Muthu et al., 2007) (**β2AR**) receptors. A number of signals could thus be amplified by this G protein including exogenous and constitutively active forms of pUS28, a human cytomegaloviral protein that encodes a chemokine-like GPCR, that was shown to induce ligand-independent, pertussis toxin-insensitive inositol lipid signalling, presumably mediated by Gα15

(Kostenis et al., 1997; Billstrom et al., 1999; Minisini et al., 2003). G15 could therefore become relevant under pathological conditions.

Owing to its promiscuity, G15 has been widely exploited to reveal the signalling of GPCR by a still unknown downstream pathway. Typically, this occurs in the case of orphan receptors (i.e. genes that have the characteristics of a GPCR but it is still unknown to which ligand/s they bind). Pharmaceutical research has developed high throughput screenings for potential ligands utilizing cells cotransfected with an orphan receptor and G15 as a platform in which [Ca^{2+}] or IP3 accumulation can be measured as a read out.

When G15 level of expression was induced to "physiological" levels, promiscuous coupling was still observed (Offermanns et al., 2001) but hardly any data is available in endogenous systems.

Specificity determinants on G proteins.

The molecular bases allowing G15 broad specificity are still elusive.
Generally speaking, the GPCR–G-protein interface must encode important information to determine which G proteins can interact with a particular receptor but, despite many of the contact sites at the interface have been mapped, the connections that define coupling selectivity remain unclear.
Upon activation, the receptor opens a cytosolic pocket for the Gα C terminus (Oldham and Hamm, 2008). C-terminal chimaeras of Gα subunits have been frequently used to switch receptor–effector coupling (Kostenis et al., 1997).
Two domains in Gα15 C-terminus are unique among Gαq family members and contribute to coupling, however, transferring these sequences into Gα11 failed to link Gα11 to C5aR unless additional upstream sequences were relocated (Lee et al., 1995). Other poorly defined N-terminal Gα15 sequences are also sufficient for promiscuous receptor interaction (Lee et al., 1995). Taken together, these studies indicate that various discrete regions of Gα15 independently contribute to

the non-selective coupling. If multiple regions on Gα15 confer capacities for promiscuous receptor coupling, probably it is only the subtle cooperation of multiple interactions that produces the correct selectivity.

In addition to the structural determinants on Gα other understudied factors could contribute to define the specificity of the interaction. Specific isoforms of Gβ have been shown to interact preferentially with specific receptors and in some cases the composition of the βγ subunits may preclude Gα binding to the receptor (Birnbaumer, 2007). Finally, other factors like the type of lipid modification could influence the affinity for the receptor (see γ geranygeranylation as compared to farnesylation).

Specificity determinants on GPCRs

Much of the difficulty in understanding coupling specificity between receptors and G proteins arises from the poor sequence homology of the intracellular loops that comprise the G protein binding site. Even closely related receptors that activate the same G protein can have dissimilar ICLs, making it impossible to determine coupling based on primary structure alone. Extensive studies have shown that IC2 and IC3 (see figure 1) are the most common selectivity determinants in receptors, although IC1 and IC4 can occasionally intervene. Frequently, mutations in other regions of the receptor (distant from the G protein binding site) can affect coupling, which suggests that the global conformation of the receptor or changes in its dynamics may be just as important as specific side chain interactions in determining receptor–G-protein selectivity (Oldham and Hamm, 2008). The α1b adrenergic receptor has been extensively mutagenized in order to define the molecular determinants driving the interaction of Gα15. However, only the deletion of half of the second intracellular loop could effectively prevent the interaction (Wu et al., 1995). Given the extent of the deletion, concerns raise about the integrity of the overall receptor conformation.

As opposite, CCR2A and CCR2B are two alternative splicing variants and they differ only in their C-terminal intracellular domains. However, only CCR2B is coupled to G15 in transfected

COS-7 cells (Kuang et al., 1996). Interestingly, replacement of the CCR2A C-terminus with the homologous region in CCR1 created a chimeric receptor capable of coupling to G15, as if the C-terminus of CCR2 was preventing the interaction.

The C-terminus of the receptor is palmitoylated and phosphorylated. There is the possibility that subcellular distribution might play an important role in defining coupling specificity and thus contribute specificity.

In summary, but a few exceptions, the literature describes for G15 a peculiar flexibility that allows the recognition of a structural domain common to most GPCRs that in each case only becomes available in the active conformation.

G15 in cancer

The selective expression of Gα15 in hematopoietic cells and in activated T lymphocytes suggests that its presence could be associated to highly proliferating states. Several signal transducers involved in cancer development are among G15 effectors. In human lymphoblastoma Reh, endogenously expressed G15 stimulated by the α1 adrenergic receptor activated a signalling cascade leading to the activation of NF-κB. The effect was mediated by a combination of Gα15 and Gβ1γ2 signalling aimed to IKK (Liu and Wong, 2004b). Gα15 activated the classical PLCβ/PKC/CaMKII pathway, likely directed toward the α and ε PKC isoforms. In parallel, the βγ branch of G15 signalling is directed toward c-Src (Liu and Wong, 2004b; Chan et al., 2002), a proto-oncogene prototype of an entire family of tyrosine kinase involved in malignant progression of tumors.

As described above, upon agonist stimulation, multiple signals diverge from a GPCR (as when α and βγ subunits separate) often converging downstream at specific integration knots (Chan et al., 2000; Lowes et al., 2002). In the case of G15 generated signals one potential locus for integration is the small GTPase Ras (Ito et al., 1995). The connection to Ras is provided for Gα15 by an adaptor protein named tetratricopeptide repeat 1 (Marty et al., 2003b) and for the Gβγ through Src, as long been shown in HEK 293 cells (Ito et al., 1995). Ras is known to initiate the Raf-1/MEK/ERK signalling cascade with a plethora of functional consequences including

IKK activation through direct interaction (Zhao and Lee, 1999) and through STAT3 (Lo et al., 2003).

A similar activation of MAPK signalling through G15, Src and Ras was also suggested in response to melatonin (Chan et al., 2002). All MAPK isoforms (ERK, JNK, p38) were shown to be activated by the constitutively active mutant form of G15 (Higashita et al., 1997; Chan et al., 2002; Heasley et al., 1996) and, in addition to activate NF-kB (Yang et al., 2001), G15 was shown to produce STAT1-dependent c-Fos transcriptional activation (Lo and Wong, 2006).

G15 and other Gq family members have previously been functionally analyzed for their potential role in cells transformation by utilizing vascular smooth muscle cells (Peavy et al., 2005) and Swiss-3T3 (Qian et al., 1994). The issue has been approached bypassing the GPCR and using a mutant form (Gα Q212L) of the G protein that becomes constitutively active because incapable of efficient GTP hydrolysis. Exogenous expression of the constitutively active form of Gα15 leads to inhibition of cell growth in Swiss 3T3 fibroblasts (Qian et al., 1994)and to cell differentiation in PC12 cells (Heasley et al., 1996). However, these experiments should be interpreted with caution. The signal departing from a constitutively active Gα subunit is very different from the signal produced by a receptor activating the heterotrimer, the most compelling reason is that the latter does not release βγ subunit and thus does not activates βγ downstream effectors. Furthermore, coordinated signalling generated by the receptor toward other signalling cascades (see above) is likely missing. In addition, cells growing in culture could activate compensating mechanisms to counteract the expression of a constitutive active mutant. Finally, a strong and uncorrelated signal could activate cell death, as observed for Gq and G14 (Qian et al., 1994; Peavy et al., 2005). Constitutively active Gq/11 produced PLCβ signals capable of inducing cell transformation at low levels of infection becoming toxic at higher levels (Kalinec et al., 1992). Therefore, the impact that G15 activation may play on neoplastic transformation remains to be determined when the effect is physiologically produced by a GPCR.

Materials and Methods

Phosphoinositol accumulation

Accumulation ofinositol phosphatewas measured by a modification of the method by Hung et al. (Zhu et al., 1994). COS-7 cells were grown in 12-wells tissue culture plates and 36 h after transfection, each well was supplemented with 2 μCi/ml of myo-[^{3}H]inositol. Following overnight labeling, cells were rinsed three times at room temperature with 1 ml of washing buffer [Dulbecco's phosphate buffered saline (D-PBS), supplemented with 5.5 mM glucose, 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$]. Cells were then incubated at 37^{o}C for 30 min in 0.5 ml D-PBS supplemented with 5 mM LiCl to inhibit inositol monophosphatase. The incubation was continued for 1 h at 37^{o}C. Agonists were added 10 min after LiCl. At the end of the incubation, the supernatant was removed and 0.75 ml ice-cold 20 mM formic acid was added to each well to extract the producedinositol phosphate.inositol phosphate were separated from myo-[^{3}H]inositol by a simplified ion exchange chromatographic procedure (Zhu et al., 1994). Briefly, after 1 hour on ice, the 20 mM formic acid extracts were applied to Dowex AG 1-X8, 100-200 mesh, formate form columns (0.6 cm diameter, 1.0 ml bed volume; BioRad, Hercules, CA) that had been sequentially pre-rinsed with 2 M ammonium formate/0.1 M formic acid, water, and 20 mM ammonium hydroxide adjusted to pH 9.0 with formic acid. Immediately after sample loading, 3 ml of 40 mM ammonium hydroxide, pH 9.0, were added to each column and the eluates collected into vials containing 10 ml of scintillation fluid (ULTIMA-FLO AF; Packard Instruments Inc., Palo Alto, CA). These first eluates were previously shown to recover the vast majority (98%) of myo-[^{3}H] inositol present in the samples (Zhu et al., 1994). The columns were then washed three times with 4 ml of 40 mM ammonium formate andinositol phosphate were eluted with 5 ml of 2 M ammonium formate/0.1 M formic acid into scintillation vials containing 15 ml of scintillation fluid. To normalize the accumulation ofinositol phosphate over total [^{3}H]inositol incorporated, the counts per minute of [^{3}H]IP (last eluate) were divided by the sum of c.p.m. of myo-[^{3}H]inositol (first eluate) plus c.p.m. of [^{3}H]IP and expressed as percentages.

Western immunoblotting

COS-7 cells were transfected as described in the previous paragraph. After removal of culture medium cells were lysed in Nonidet P-40 (NP40) buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1% v/v NP40) containing protease inhibitor cocktail (Sigma Aldrich, Saint Louis, MO) and spun in a microcentrifuge at 11,000 x g. Alternatively 3-5 slices of frozen tumoral or healthy tissues were solubilized in the same buffer and treated according to the same procedure.

Protein contents of supernatants were measured by the BCA assay (Pierce, Rockford, IL) and equal amounts of proteins were resolved by SDS-PAGE (10% acrylamide) and transferred to a Hybond-P membrane (GE Healthcare-Amersham, Piscataway, NJ). Membranes were blocked by incubation with 5% (w/v) nonfat dry milk and hybridized with primary antibodies.

Anti-EE monoclonal antibody was obtained from Covance, Princeton, NJ. Anti-HA rabbit polyclonal antibody was from Abcam. Anti-HA mouse monoclonal antibody was produced from 12CA5 hybridoma. The anti-Ga16 Ab was obtained from Torrey Pines Biolabs (San Diego, CA). To detect the phosphorylated state of Ser744 and Ser748 located in the activation loop of PKD two different antibodies were used. One antibody (anti-pS744/pS748), obtained from Cell Signalling Technology (Beverly, MA), was raised against a peptide phosphorylated on serines equivalent to Ser744 and Ser748 of PKD but predominantly detects the phosphorylated state of Ser744 (Jacamo et al., 2008). Consequently, we refer to this antibody as anti-pS744. A second antibody, obtained from Abcam (ab17945), detects the phosphorylated state of Ser748. A third antibody was used to detect PKD (C20, Abcam).

Secondary anti-mouse or anti-rabbit antibodies were horseradish peroxidase-conjugated (Pierce, Rockford, IL). Antigen-antibody complexes were detected using SuperSignal West Dura chemiluminescent substrates (Pierce, Rockford, IL) according to the manufacturer's instructions, and visualized with Kodak Image Station 440 or X-ray film.

cAMP accumulation

48 hours after transfection cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS, incubated 15 min at 37°C in PBS supplemented with 4 mM EDTA, and scraped. After centrifugation cell pellets were resuspended at a density of 10^6 cells/90 µl in D-PBS supplemented with 5.5 mM 3-isobutyl-1-methylxanthine. Samples were equilibrated for 15 min at 37°C and treated for 1 h with agonists or assay buffer (basal) at 37°C. cAMP accumulation was stopped by placing the tubes in liquid nitrogen and subsequent boiling for 5 min. Samples were then spun for 8 min at 12,000 rpm in a microcentrifuge and supernatants were immediately used for the assay. cAMP content was quantified by means of a competitive binding cAMP assay kit (GE Healthcare-Amersham) following manufacturer's instructions.

Determination of cytosolic free Ca^{2+} levels

Determination of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) was performed as previously described (Capra et al., 2005). Briefly, HEK-293T cells were seeded on sterile coverslips coated with poly-D-lysine. After an incubation for 30 minutes at 30°C in the dark with 2 µM Fura 2/AM, the dye was removed and the cells were further incubated for 30 min at 30°C to complete the Fura 2/AM hydrolysis. After loading, cells were washed twice with PBS and transferred to the spectrofluorimeter, where fluorescence was monitored at 37°C (505 nm emission, 340 and 380 nm excitation). To extrapolate Ca^{2+} concentration from the fluorescence recording, the system was calibrated as follows: Fmax was obtained by adding 2 µM ionomycin and 100 µM digitonin, and Fmin was obtained by adding 5 mM EGTA and 60 mM Tris base.

Co-immunoprecipitation

COS-7 cells were grown and transfected in 100 mm tissue culture dishes using 4 µg of plasmid DNA encoding for HA tagged V2R-R137H, EE-tagged Gα subunits and β-arrestin 1. 48 h post-transfection, cells were lysed in 1 ml of 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, supplemented with protease inhibitor cocktail (Sigma Aldrich). After 1 h at 4°C, the particulate was removed from the samples by centrifugation at 21,000 x g. Immunoprecipitation was

performed for 16 h at 4°C using anti-HA monoclonal Ab previously crosslinked to CNBr-activated Sepharose 4B beads (GE Healthcare-Amersham). Immune complexes were washed three times with 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% (v/v) NP-40 and eluted in Laemmli sample buffer. Samples were separated by SDS-PAGE (12% acrylamide) and analyzed by immunoblotting as described.

Receptor internalization

HA epitope tagged receptors in 12-well plates were incubated with or without agonist for 30 min in serum-free medium at 37°C. Cell surface receptors were labeled with 12CA5 mAb, and Alexa 488-conjugated goat antibody against mouse IgG as a secondary antibody. Receptor internalization was quantified as loss of cell surface receptors as measured by fluorescence-assisted cell sorting.

Quantitative PCR analysis of mRNA

Total mRNA was isolated from: tumoral cell lines, frozen tumor slices, healthy tissue or samples derived from xenografts. mRNA was extracted in Trizol, precipitated (isopropanol followed by 70% ethanol) and random priming retrotranscribed by RT-PCR. The messenger expression level was next quantified by TaqMan PCR. Real-time analysis was performed on an ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA,USA) using the TaqMan PCR Master Mix (Applied Biosystems). The TaqMan assays were chosen from the list of the Assays-on-Demand (Applied Biosystems). The PCR reactions contained primers and probe diluted 1:20 and 5 ng c-DNA (total RNA equivalent) in 25 µL total volume, and samples were analyzed in triplicate. Thermal cycling included an initial incubation at 50°C for 10 minutes followed by 95°C for 10 minutes then 50 cycles of 15 seconds at 95°C for denaturation and 1 minute at 60°C for extension. Fluorescence emission of 6-carboxyfluorescein (FAM) was automatically measured during PCR run. A cycle threshold value of 45 was considered the end of the PCR run. The expression of each mRNA was calculated by relative quantification using the average of GAPD

(glyceraldehyde-3-phosphate dehydrogenase) (Hs99999905_m1) transcript level as reference. Data were analyzed as indicated in User Bulletin no. 2 (Applied Biosystems).

Statistical Analysis

Data were evaluated using GraphPad Prism version 11. Statistical comparison of multiple groups was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Data are expressed as means ± S.E.M unless otherwise indicated.

Results

G15 expression in human tissues

Circulating cells

Human blood cells precursors were analyzed for the presence of Gα15. Hematopoietic derivatives were extracted from cord blood utilizing anti-CD34 immunomagnetic beads. Samples were lysated and examined by Western blot (Figure 8a). As expected (Lippert et al., 1997), a band corresponding to Gα15 appeared at 43 kDa. The presence of the protein in CD34positive

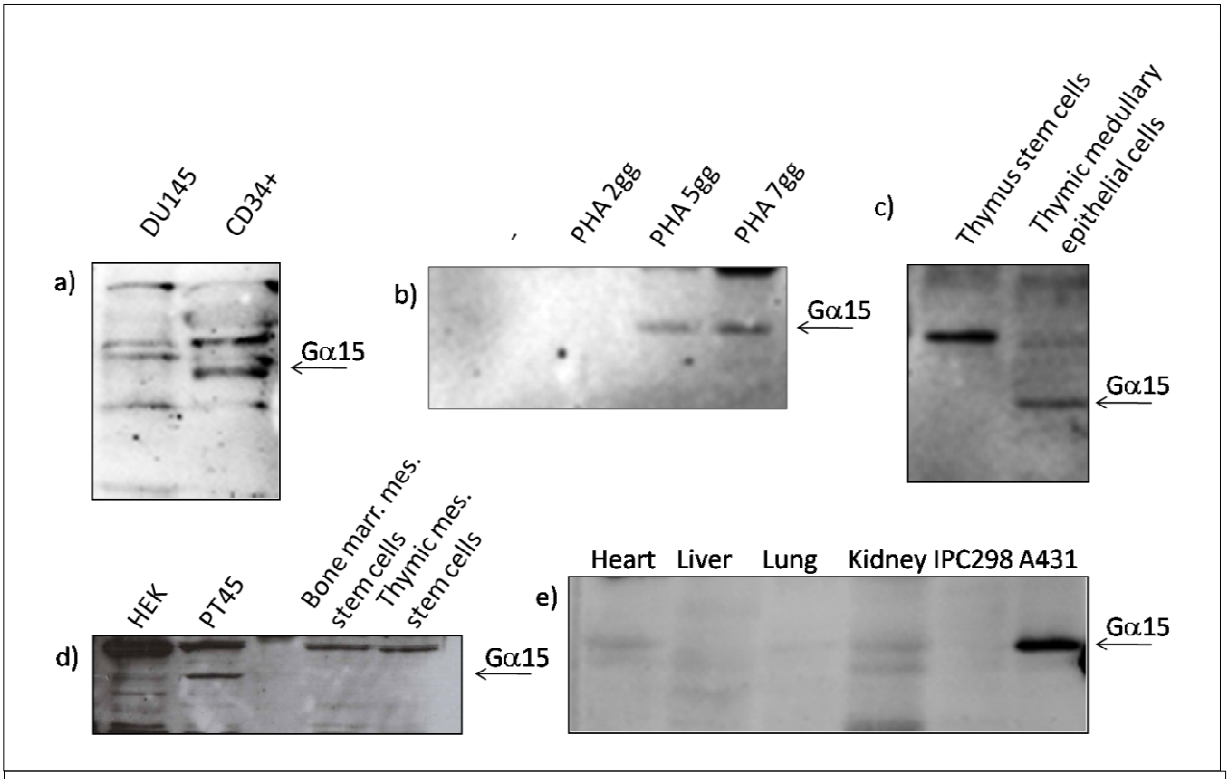


Figure 8 - Expression of Gα15 by Western blot in cells derived from various human tissues. The 43 kDa band corresponding to Gα15 is indicated by the arrow. **a)** CD34 positive cells were compared to an irrelevant cell line. **b)** peripheral blood mononucleated cells (containing CD34, CD8, CD4 positive cells, NK and dendritic cells) were stimulated with PHA for 2 to 7 days as indicated. **c)** epithelial cells derived from thymus. **d)** mesenchymal stem cells and **e)** various human tissues. A431 and PT45 represent positive controls (see below) while HEK (hamster embryonic kidney cells) represent negative control.

cells was associated to the correspondent mRNA (Figure 9) as assessed by TaqMan PCR. By contrast, Ficoll purified peripheral mononucleate cells obtained from the blood of a healthy donor did not display the 43 kDa band (Figure 8b). As reported for mature T cells by Lippert et al. (Lippert et al., 1997), elevated expression levels were obtained after 5 or more days of stimulation with phytohaemagglutinin (**PHA**).

These experiments confirmed that Gα15 is present in immature lymphocytes, it becomes silenced upon maturation and quiescence but its expression can be restored when lymphocytes are activated and resume proliferation.

Solid tissues

The analysis performed in circulating cells was extended to other tissues, including lymphoid organs. While virtually no mRNA signal could be detected in spleen, in thymus Gα15 expression was significantly higher (Figure 9). A strong positive signal was also observed in primary cell cultures from thymic medullary epithelial cells (**TEC**) (Figure 9). Also in this case, high mRNA levels were associated to a detectable amount of protein (Figure 8c). The robust presence of Gα15 in TEC suggests that the signal that we and others (Wilkie et al., 1991) observed in samples derived from whole thymus could be related to the endothelial component

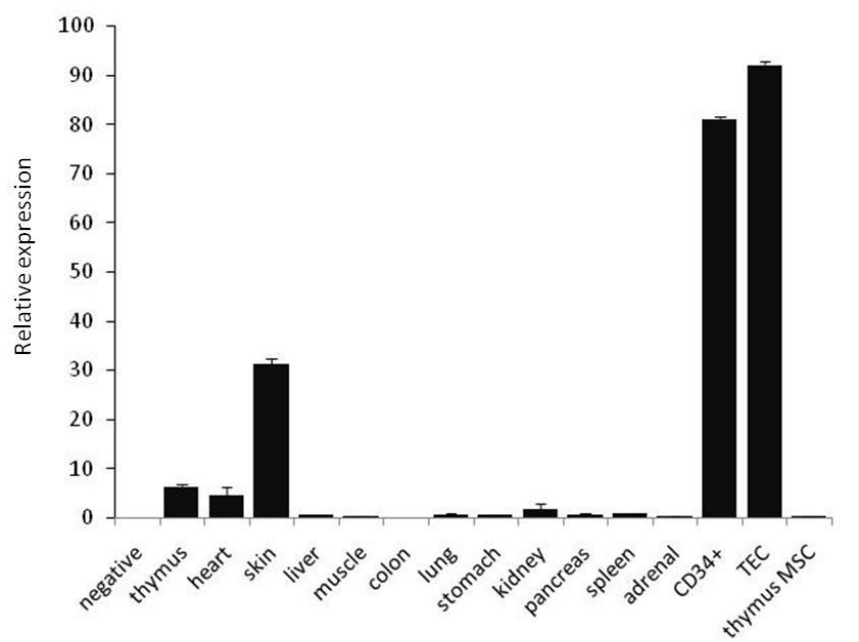


Figure 9 - Gα15 mRNA distribution in the human organism. The distribution of Gα15 mRNA was assessed by real time PCR in different tissues and cell preparations (as indicated). Data are expressed as relative expression (see material and methods) means ± SE.

rather than to the presence of immature lymphocytes in the lymphoid organ. In most human tissues a weak (but significantly higher than background level) signal was present, particularly in the skin (as previously reported (Rock et al., 1997a)) and in the heart. Contrary to CD34positive and TEC primary cells, specimens collected from various tissues were derived from a heterogeneous population. The contribution of few positive cells could therefore be masked by the background. Under these circumstances, substantially no protein was detected by Western blot. At this stage, it is not clear if the absence of a signal in the immunoblot is due to a lack of sensitivity of the antibody or rather to mRNA levels insufficient for a significant translation.

Because Gα15 is expressed by immature stages of hematopoiesis, the question arose as to, in solid tissues, Gα15 could be analogously associated to remnants stem-like cells (i.e. mesenchymal stem cells). To address this issue, primary cultures of adult stem cells were derived from human thymus and from bone marrow. After confirming the presence of staminal markers (negative for CD31, CD45, CD73, CD80, CD86, HLADR2, VCAM, CD80; positive for CD29, CD44, CD105, MHCI), by flow cytometry, Gα15 mRNA was measured by PCR (Figure 9) and by Western blot (Figure 8d), however, no significant signal could be revealed.

This argues against G15 mediating stem cell specific signalling, rather suggesting that Gα15 could be present in cells with high proliferation potential, such as TEC, keratinocytes, CD34positive and activated T lymphocytes. Overall these results suggest that G15 may play a broader role than it was previously anticipated. Among Gq family members, G15 remains the one with the most restricted expression profile being virtually absent in most tissues.

G15 signalling peculiarities

G15 promiscuity

To verify that G15 couples multiple GPCRs to PLCβ, we co-expressed its α subunit in COS-7

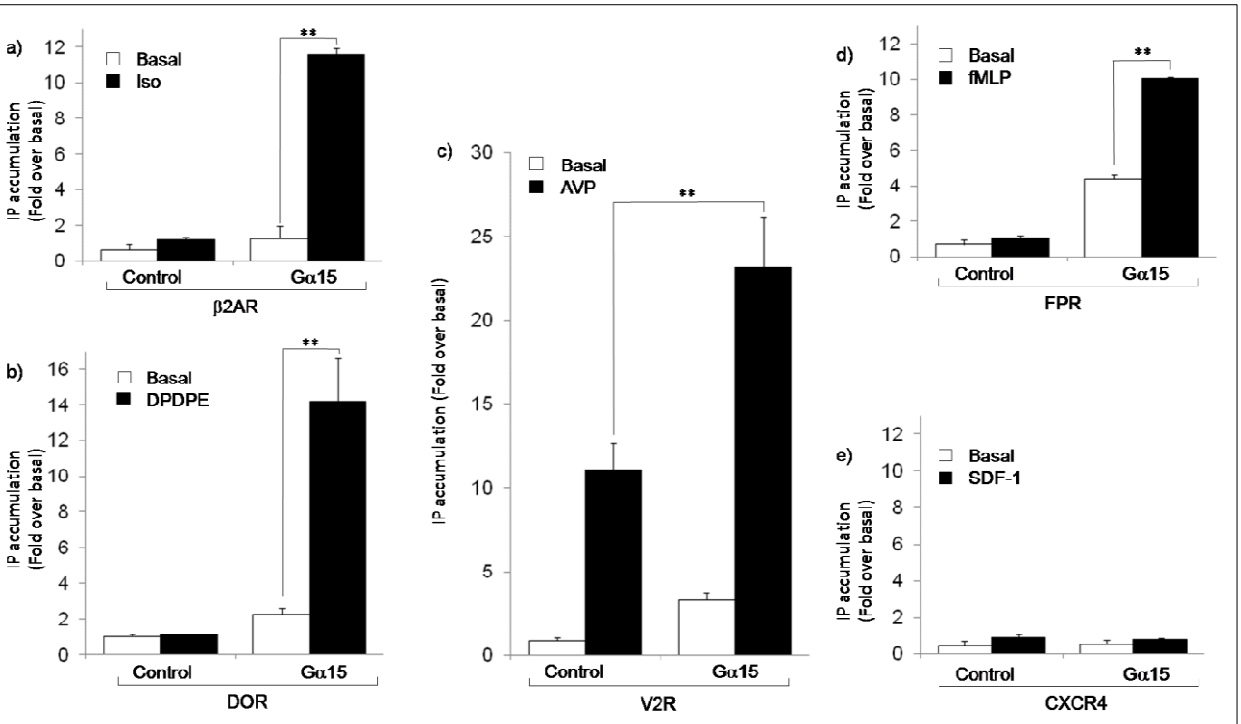


Figure 10 –G15 signalling promiscuity in COS-7 cells. COS-7 cells were transfected with plasmids containing cDNAs as indicated and prelabeled with myo[^{3}H]inositol overnight. Accumulation of [^{3}H]inositol phosphates was measured in presence of 5 mM LiCl as described. The inositol phosphate isolated was normalized for the total amount of myo[^{3}H]inositol incorporated (see materials and methods). Values are expressed as fold increase over the basal PLC activity level of cells transfected with an irrelevant plasmid (average±S.E.M, n=3 independent experiments performed in triplicate, ** P<0.005). **a)** Cells co-expressing Gα15 and β2AR stimulated by 10 μM Iso **b)** Cells co-expressing Gα15 and DOR stimulated by 10 μM DPDPE. **c)** Cells co-expressing Gα15 and V2R stimulated by 100 nM AVP. **d)** Cells co-expressing Gα15 and FPR stimulated by 100 nM fMLP. **e)** Cells co-expressing Gα15 and CXCR4 stimulated by 100 nM stromal derived factor 1 (SDF-1).

cells with different GPCRs, namely the β2AR, DOR, V2R, fPR, CXCR4.

The β2AR is normally coupled to Gs and Gi, therefore it does not efficiently couple to PLC. In fact, as shown in Figure 10a, addition of 10μM Isoproterenol (**Iso**) to cultured COS-7 cells did not induce any significant increase in inositol phosphate production. The expression of Gα15 with β2AR produced only a slight increase in inositol phosphate concentration, unless Iso was added inducing a dramatic increase above the background. Thus, Gα15 transfection made PLCβ responsive to Iso in COS-7 cells.
A similar result was obtained with the DOR that is primarily coupled to Gi (Lee et al., 1998). Like in the case of the β2AR, inositol phosphate production could be stimulated by an opioid agonist (10μM [D-Pen^{2,5}]-enkephalin (**DPDPE**)) provided that COS-7 cells were previously co-transfected with Gα15 (Figure 10b).

The V2R is naturally coupled to Gs (Rosenthal et al., 1993) and to Gq/11 (O'Connor and Cowley, Jr., 2007; Zhu et al., 1994). Accordingly, there was a significant increase in PLC activity in response to AVP. The expression of Gα15 enhanced V2R mediated PLC signalling (Figure 10c).
G15 can also collect the signal of receptors displaying high basal activity. In fig. 9d it can in fact be noticed how, in the presence of Gα15, the chemotactic Gi coupled formyl peptide receptors (**FPR**) significantly promoted PLC activity already in the absence of the ligand. The signal is further increased upon addition of formyl Met-Leu-Phe, one of its natural agonists, consistent with the idea that only a minor fraction of FPR spontaneously acquires an active conformation (see introduction and Figure 4).

Not all GPCRs activate G15 signalling. Among the few exceptions, are present chemokines receptors like CCR2 (see introduction), CCR5 and CXCR4 (Tian et al., 2008). PLCβ activity upon coexpression in COS-7 cells confirmed Gα15 refractoriness to CXCR4 activation.

It can also be noticed how, in most cases, inositol phosphate accumulation was slightly increased by the presence of G15, even in the absence of GPCR co-expression. That could be possibly due to exogenous G15 spontaneous activity or to G15 collecting signals generated by active endogenous GPCRs. However, the low basal signal was present also without any cotransfected GPCR (data not shown) confirming that the β2AR, DOR and V2R do not display significant spontaneous activity.

β2AR signalling to G15 is poorly affected by β-arrestin desensitization

We next determined whether G15 sensitivity to GPCR desensitization was similar to other G-proteins. β-arrestin opposes prolonged GPCR activity (as described at pag. 11 in "GPCR

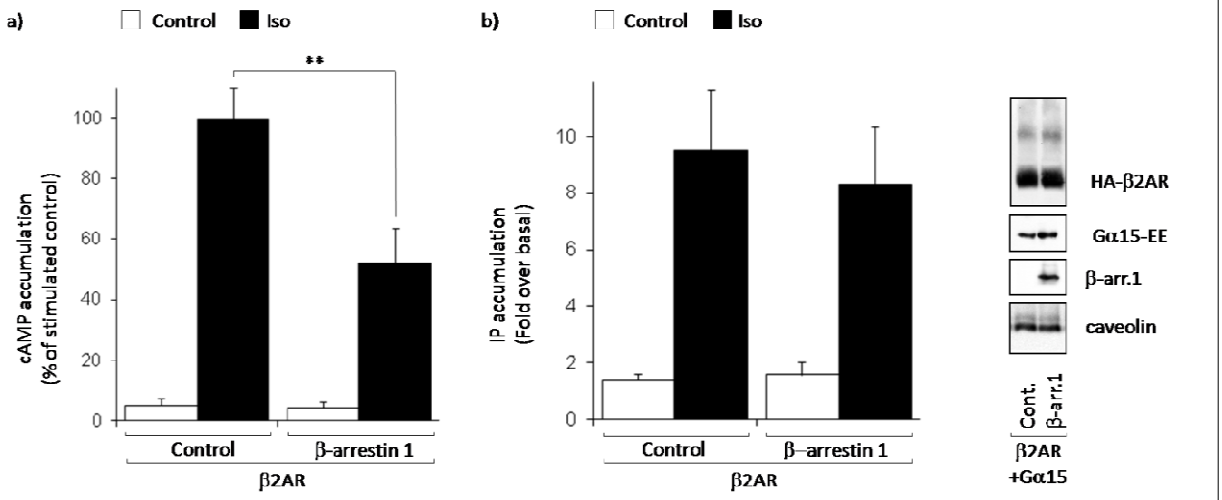
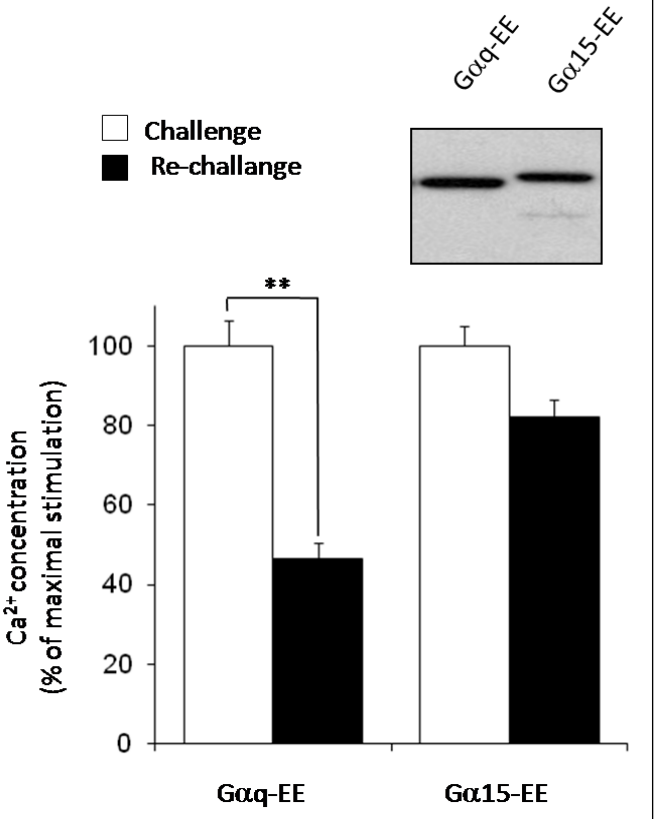


Figure 11 - β2AR signalling through G15 resists β-arrestin dependent desensitization. The β2AR was transfected in COS-7 cells with or without β-arrestin 1. **a)** Stimulation of cAMP accumulation was compared by setting to 100% the maximal stimulation (10 μM Iso) obtained in control transfected cells (with β Gal cDNA instead of β-arrestin 1 cDNA). Stimulated β2AR dependent accumulation of cAMP was significantly dampened in cells co-expressing β-arrestin 1 (**$P < 0.01$). Data represent average ± S.E.M. of three independent experiments performed in duplicate. **b)** The cotransfection of β-arrestin 1 did not produce a statistically significant effect on the stimulated β2AR dependent accumulation of inositol phosphate. Data represent average ± S.E.M of four independent experiments performed in triplicates. In the right side panels, immunoblotting analysis of the expression levels of β2AR (anti-HA antibody), Gα15-EE (anti-EE antibody), β-arrestin 1 and endogenous caveolin 1.

regulation over time") by preventing the stimulatory interaction of the receptor with the G protein. We utilized COS-7 cells, which express low levels of endogenous β-arrestins (Paing et al., 2002), to overexpress β-arrestin 1 and directly assess the efficiency of arrestin-dependent desensitization. As expected, β-arrestin 1 significantly (P<0.01) attenuated β2AR coupling to Gs

reducing the cAMP accumulation induced by 1 h stimulation with 10 μM Iso (Figure 11a). In striking contrast, β2AR coupling to G15 was substantially insensitive to β-arrestin 1 overexpression, leaving unaffected agonist promoted inositol phosphate accumulation (Figure 11b).



β2AR signalling through G15 resists desensitization induced by repeated stimulation. $[Ca^{2+}]_i$ elevation was induced by 10 μM Iso in HEK-293T cells co-expressing β2AR and similar levels of either Gα15 or Gαq carrying the same EE tag (Western blot in the inset panel).

The peaks of $[Ca^{2+}]_i$ transient were measured after the first agonist challenge (set as 100%) and upon re-addition of agonist after 5 minutes washout (re-challenge). The response to the first Iso stimulation in cells transfected with Gα15-EE was 90% ± 13% than in cells transfected with GαqEE. Data represent means of $[Ca^{2+}]_i$ stimulation over basal ± S.E.M. of at least three independent experiments performed in triplicate (**$P < 0.01$).

To confirm that what observed for Gα15 was not the result of some indirect effect of the increased β-arrestin levels unrelated to GPCR desensitization, we monitored intracellular Ca^{2+} mobilization utilizing a protocol of desensitization based on repeated agonist stimuli in HEK-293T cells. In this case HEK-293T cells were chosen because they express higher endogenous levels of β-arrestins as compared to COS-7. In addition, as described by Schmidt et al., in these cells the β2AR can activate Gs signalling directed to Ca^{2+} mobilization via PLCε (Schmidt et al., 2001). Upon transfection with β2AR and either Gα15 or Gαq, we quantified the extent of the transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}_i]$) following Iso stimulation (challenge, **Error! Reference source not found.**); after washing away the ligand, cells were stimulated again and this second $[Ca^{2+}_i]$ increase was also quantified (re-challenge).

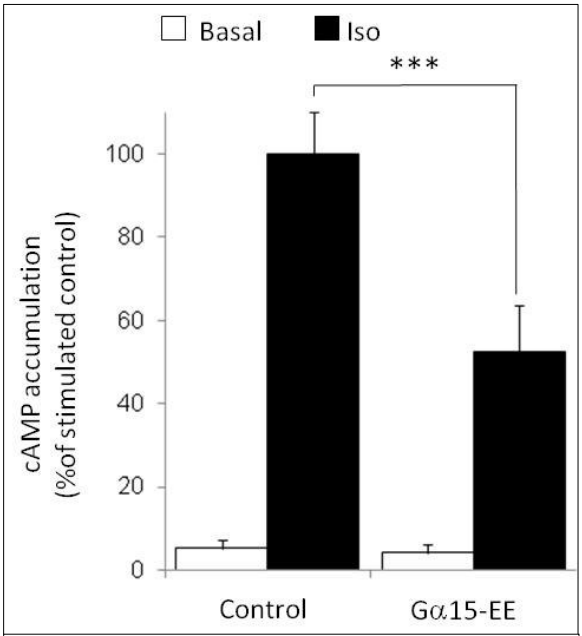


Figure 12 - G15 competes Gs signalling. Co-transfection of Gα15 significantly reduced (**$P < 0.01$) cAMP accumulation stimulated by 10 μM Iso in cells expressing the β2AR. Data represent average ± S.E.M. of three independent experiments performed in duplicates.

The response to the second challenge of 10 μM Iso was significantly attenuated (~50%, $p<0.05$) as compared to the first stimulation in the presence of Gαq but not in the presence of Gα15. It is not clear which G protein subunits participated to produce the $[Ca^{2+}]_i$ transient. Possibly it was a combined effect of the Gαs subunit (indirectly activating PLCε) and of the Gβγ subunits released from Gs and Gi (activating PLCγ) (Schmidt et al., 2001). In any case, by two different approaches, we demonstrated that agonist induced signalling of the β2AR to G15 was resistant to GPCR desensitization.

We hypothesized that Gα15 interacts with the receptor so effectively to compete out not only β-arrestins, but possibly also other G proteins. If this is the case, G15 presence should reduce the ability of GPCRs to couple to other G proteins. Indeed, the effect of Iso on cAMP accumulation was significantly reduced when Gα15-EE was co-transfected (Figure 12d).

G15 resists to β-arrestin-dependent regulation of the DOR and the V2R.

The poor sensitivity to β-arrestin-dependent desensitization was not unique to the signalling triggered by the β2AR. Inositol phosphate production stimulated by DPDPE in COS-7 cells co-transfected with Gα15 and DOR was also unaffected by β-arrestin 1 overexpression (Figure 13). Unlike the β2AR and the DOR, in the case of the V2R the efficiency of β-arrestin dependent desensitization could not be analyzed without keeping into account the coupling toward

endogenous Gq and G11 (Figure 10c). In the absence of Gα15, the overexpression of ubiquitous β-arrestin 1 and 2 isoforms reduced to 35 ± 9% and 36 ± 11% (respectively) the inositol phosphate production induced by 100 nM arginine AVP via endogenous Gq/11 (Katz et al., 1992) (see inset table in Figure 14).

When Gα15 was co-transfected, a β-arrestin dependent reduction was still observed, as shown in the representative experiment in Figure 14. However, a large fraction of the stimulation was left intact. This result appears consistent with β-arrestin-dependent desensitization being effective only on the Gq/11 component leaving the G15 component unaltered.

As an approximation to separate the G15 component from the Gq component, the values obtained in the absence of G15 were subtracted from the value obtained in presence of Gα15 (net

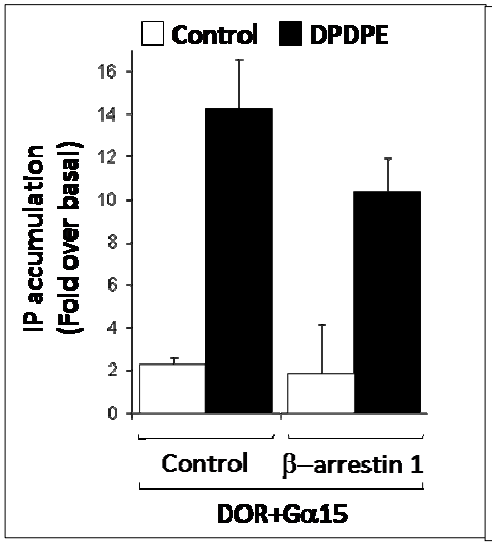
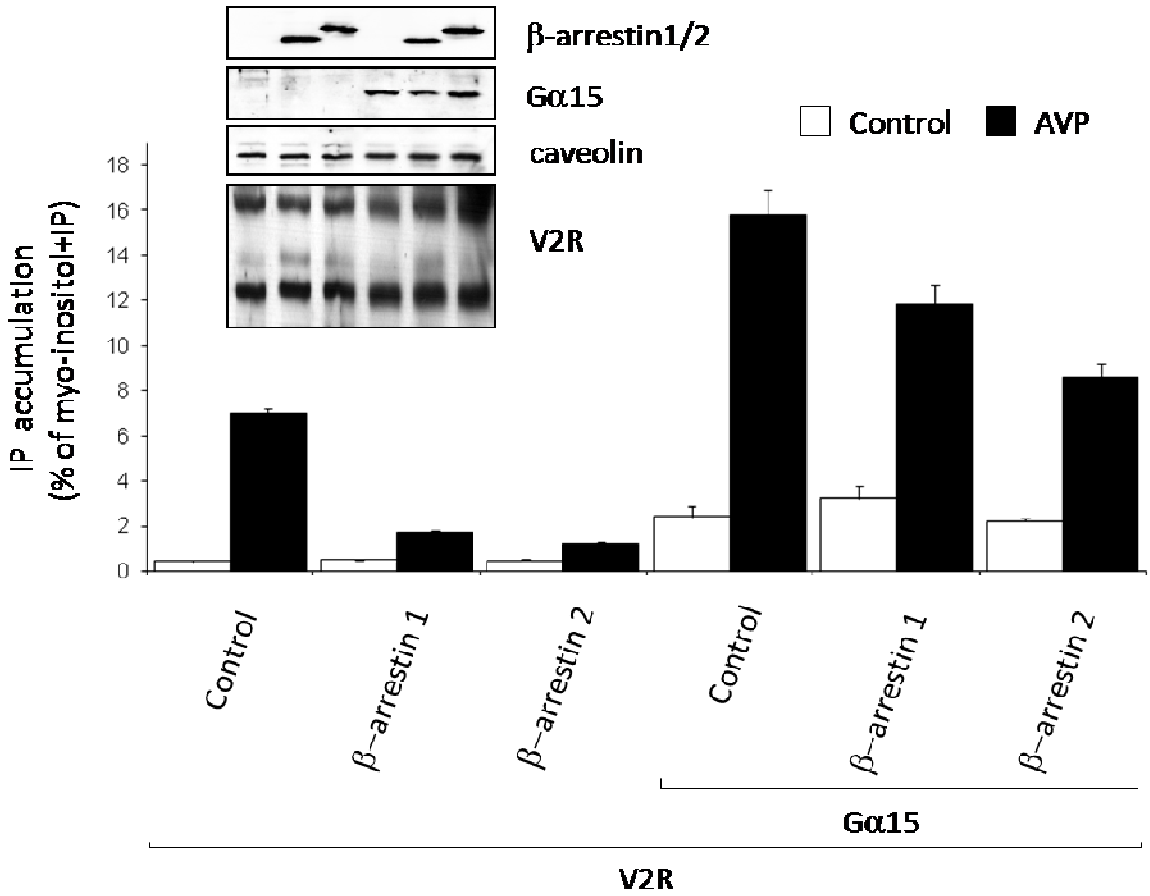


Figure 13 - G15-mediated signaling of DOR and V2R is not affected by β-arrestin overexpression. In COS-7 cells transiently transfected with DOR and Gα15, 10μM of DPDPE stimulated inositol phosphate accumulation to the same extent in the absence or in the presence of overexpressed β-arrestin 1. Values are expressed as fold increase over the basal PLC activity level of cells transfected with an irrelevant plasmid. Data represent average ± S.E.M. of three independent experiments performed in triplicates.

of the basals). This operation was repeated for the control samples and for the two β-arrestins samples. The estimated G15 contribution to V2R signalling was totally unaffected by either β-arrestin 1 or β–arrestin 2 overexpression. (Figure 14, inset table).



	Gq/11		G15	
	average ± S.E.M.	*P* val.	average ± S.E.M.	*P* val.
Control	100%		119% ± 14%	n.s.
β-arrestin1	35% ± 9%	<0.001	114% ± 4%	n.s.
β-arrestin2	36% ± 11%	<0.001	102% ± 14%	n.s.

Figure 14 - G15-mediated signaling of V2R is not affected by β-arrestins overexpression. COS-7 cells expressing V2R with or without Gα15 and β-arrestin 1 or 2 (Western blot in the inset panels illustrate the expression levels of proteins as indicated) were stimulated with 100nM AVP. One representative experiment performed in triplicate is shown (means±S.D.). The experiment described was repeated 4 times and mediated. The resulting values are reported in the inset panel. Gq/11-dependent stimulation in the absence of β-arrestins (control) and Gα15 overexpression was set as 100% in each experiment. β-arrestin-resistant desensitization (defined as the residual IP accumulation following β-arrestin overexpression) was calculated for the G15 component by subtracting the Gq/11 contribution (means±S.E.M.).

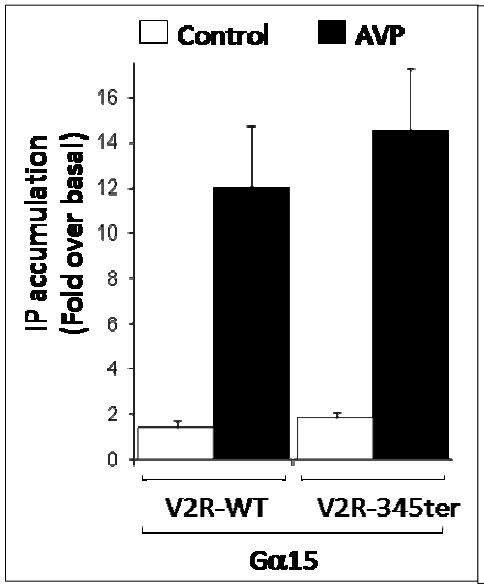


Figure 15 - G15 couples to non phosphorylated V2R - IP accumulation stimulated by 100 nM AVP was not different in COS-7 cells transfected with V2R-WT or with phosphorylation-negative V2R-S345ter mutant. Values are the means±S.E.M. of three independent experiments performed in triplicate.

Phosphorylation stabilizes β-arrestin binding to the receptor (Gurevich and Gurevich, 2003). We sought to rule out the possibility that only the phosphorylated form of the V2R was signalling through G15 by using a phosphorylation-resistant mutant. We previously demonstrated that shortening the V2R carboxyl-terminal tail by insertion of a stop codon at position 345 (V2R-S345ter) eliminates all phosphorylation sites without affecting the affinity for AVP or the coupling to Gs (Innamorati et al., 1997). Inositol phosphate accumulation induced by AVP was similar in cells having Gα15 co-expressed with V2R-S345ter or with V2R WT (Figure 15). This ruled out a preferential interaction between G15 and the phosphorylated state of the receptor, suggesting that the protein complex forms regardless of the presence of the carboxyl-terminus and its multiple phosphorylation sites (Innamorati et al., 1997).

G15 reveals the activity of desensitized GPCR

A mutant form of the V2R (V2R-R137H) has been proposed to be constitutively desensitized even in the absence of ligand. The substitution to histidine of the central arginine of the DRY motif stabilizes in fact the receptor in a conformation that remains stably associated to β-arrestin (Barak et al., 2001). As a consequence, the V2R-R137H is incapable of activating Gs and adenylyl cyclase (Barak et al., 2001; Rosenthal et al., 1993). Accordingly, the V2R-R137H transfected in COS-7 cells did not promote inositol phosphate accumulation, neither in the

presence of endogenous Gαq/11, nor of overexpressed Gq (Figure 16) nor of overexpressed epitope tagged Gαq-EE (Figure 17a).

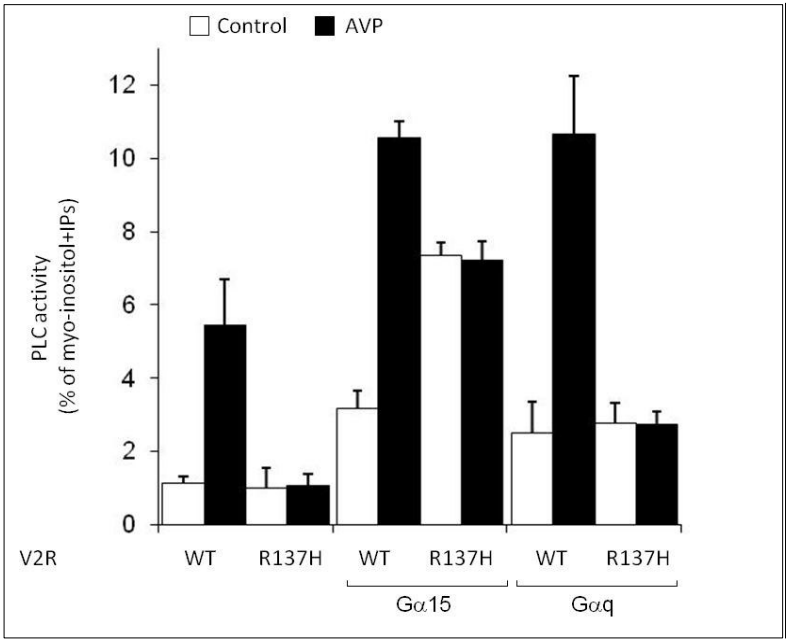


Figure 16 - G15 couples to the constitutively desensitized V2R-R137H mutant. Gαq and Gα15 were individually co-expressed with either the V2R-WT or the V2R-R137H in COS-7 cells utilizing equivalent amount of cDNAs. In the case of the V2R-R137H, receptor dependent IP accumulation is observed in the presence of Gα15 but not Gαq. Values are the means±S.D. obtained in one of three independent experiments.

We hypothesized that G15 would be able to displace β-arrestin and recognize the

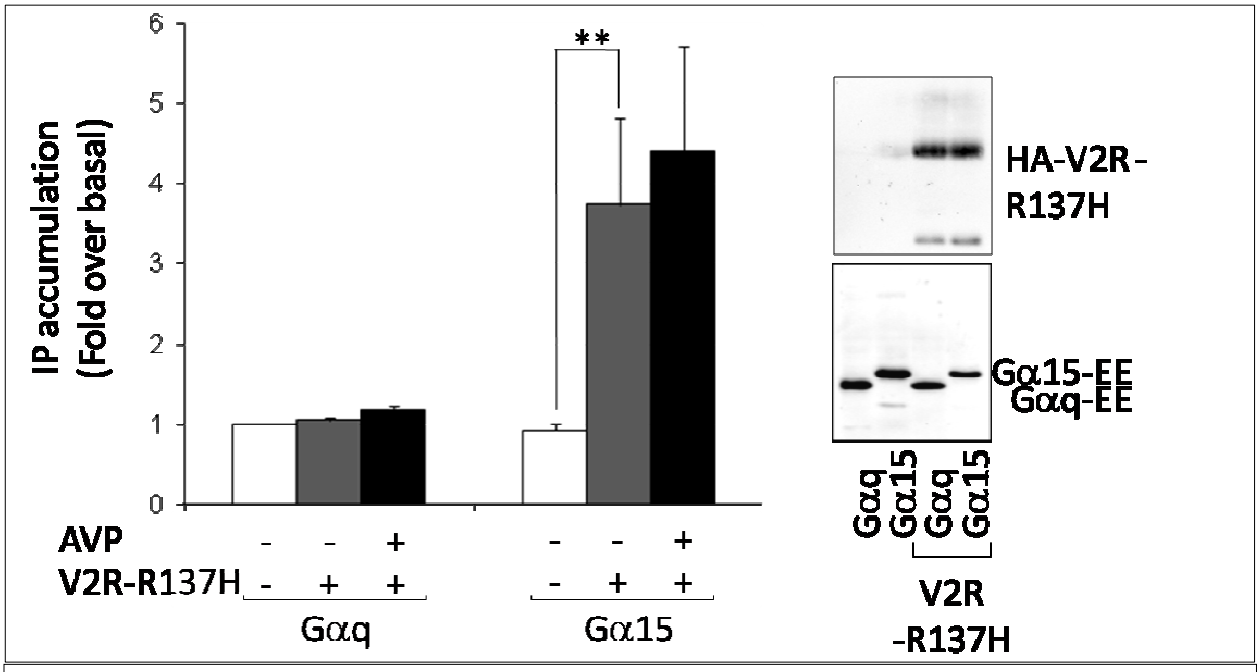


Figure 17 - G15 couples to the constitutively desensitized V2R-R137H mutant. EE tagged Gαq and Gα15 were individually co-expressed with HA tagged V2R-R137H in COS-7 cells. Receptor dependent inositol phosphate accumulation is observed in presence of Gα15-EE (**$P < 0.01$) but not Gαq-EE. Values are expressed as fold increase over the basal PLC activity level of cells transfected with Gq and an irrelevant plasmid instead of V2R-R137H. Data represent average ± S.E.M. of four independent experiments performed in triplicates. In the right side panels, immunoblotting analysis of the expression levels of HA-V2R-R137H (anti-HA antibody), Gαq-EE and Gα15-EE (anti-EE antibody).

hyperphosphorylated mutant. The V2R-R137H was thus co-expressed with Gα15 and inositol phosphate accumulation was measured. Gα15-EE and Gαq-EE as were expressed to comparable levels. The presence of Gα15-EE restored V2R-R137H signalling toward PLC. The effect was not due to an increase in receptor expression, since comparable receptor levels were present regardless of the type of β-subunit present (Figure 17b).

Similar results were obtained utilizing equivalent amounts of cDNAs of the untagged versions of Gαq and Gα15 (Figure 16). Supporting the hypothesis that the V2R-R137H represents a receptor fully locked in an active conformation, AVP did not further increase V2R-R137H activity (Figure 17 and Figure 16).

Molecular mechanism allowing G15 to couple to desensitized GPCR

G15 appears therefore capable of circumventing the stable steric hindrance created by the tight interaction between β-arrestin and V2R-R137H (Barak et al., 2001).

To gain some insight at the molecular mechanism that allows G15 to resist the effects of desensitization, co-immunoprecipitation experiments were set up to analyze the possibility that G15 displays a stronger affinity for the V2R-R137H as compared to other G proteins.

All α subunits were expressed to similar levels (as assessed by direct comparison in Western blot) (Figure 18a). When equal amounts of the constitutively active receptor were precipitated (Figure 18b), only Gα15-EE was found associated to the V2R-R137H (Figure 18c). β-arrestin overexpression did not prevent the interaction of the V2R-137H with Gα15 (Figure 18d).

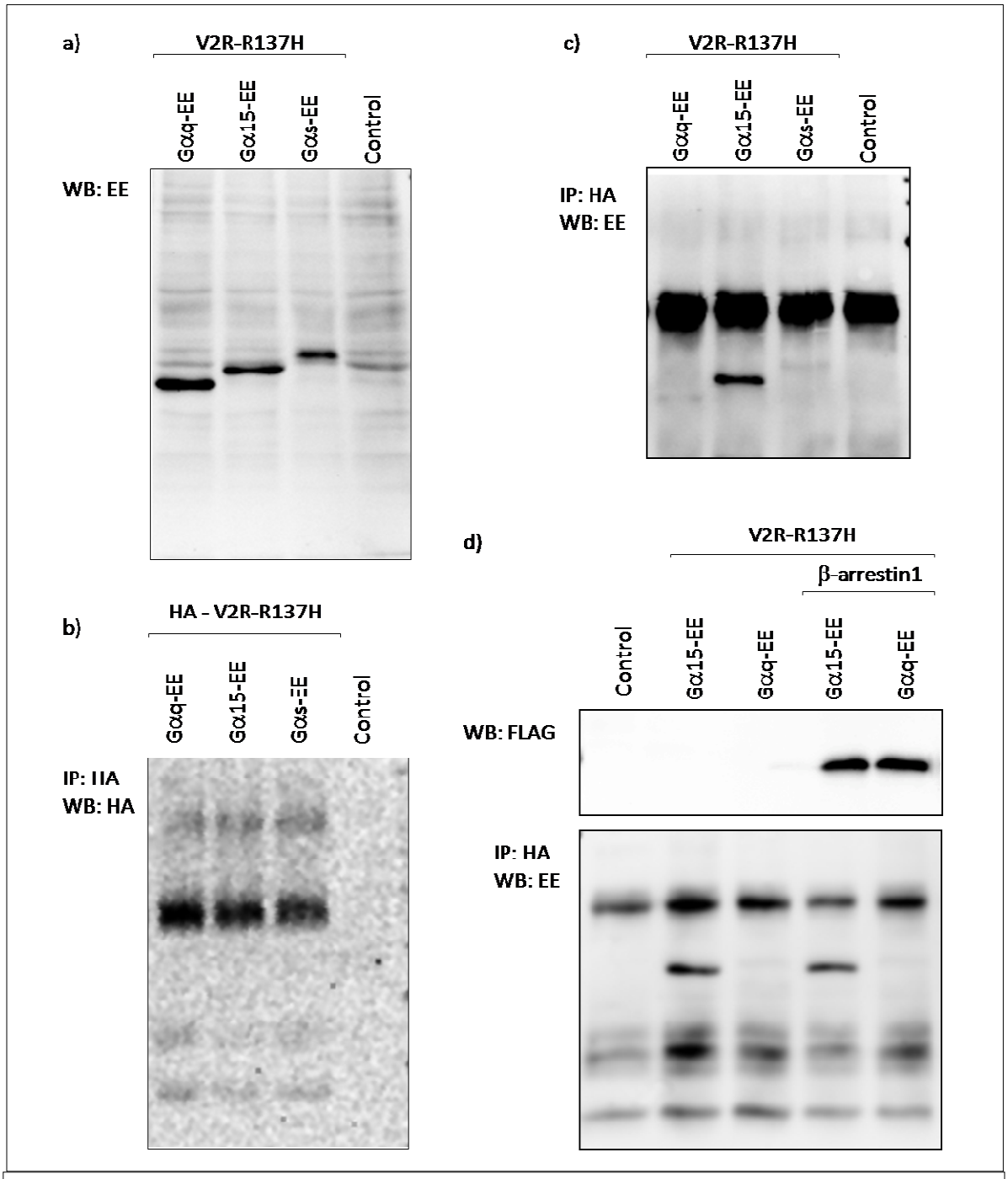


Figure 18 Gα15 stably interacts with constitutively desensitized V2R-R137H. - a) EE tagged Gαq, Gα15 and Gαs were individually co-expressed with the HA tagged V2R-R137H in COS-7 cells as indicated. **a)** Gα proteins total expression levels were compared by Western blot in whole cell lysate. The V2R-R137H was immunoprecipitated utilizing a monoclonal antibody against the HA epitope and immunoblotted with **b)** polyclonal antiHA or **c)** antiEE antibodies. **d)** Gα15 was co-immunoprecipitated with the V2R-R137H as described above. The same amount of Gα15 was recovered (lower panel) in the presence or in the absence of β-arrestin 1 overexpression (upper panel).

One of the consequences of the stable interaction with β-arrestin (Wilbanks et al., 2002; Barak et al., 2003) is that most of the V2R-R137H is sequestered intracellularly (Barak et al., 2001; Rosenthal et al., 1994). We hypothesized that, by interacting with the mutant receptor, G15 could revert the constitutive internalization of the V2R-R137H and rescue it to the cell surface.

The amount of receptor on the plasma membrane was monitored under non permeabilizing conditions by flow cytometry. An HA epitope placed at the amino-terminus of the receptor was utilized for this purpose. Gα15 expression restored a significant fraction of V2R-R137H to the cell surface. We also determined if β-arrestin overexpression could reverse this effect. However, as shown in Figure 19, β-arrestin had no effect on the number of V2R-R137H molecules expressed on the cell surface.

This effect could be explained hypothesizing that the high affinity interaction of Gα15 with the

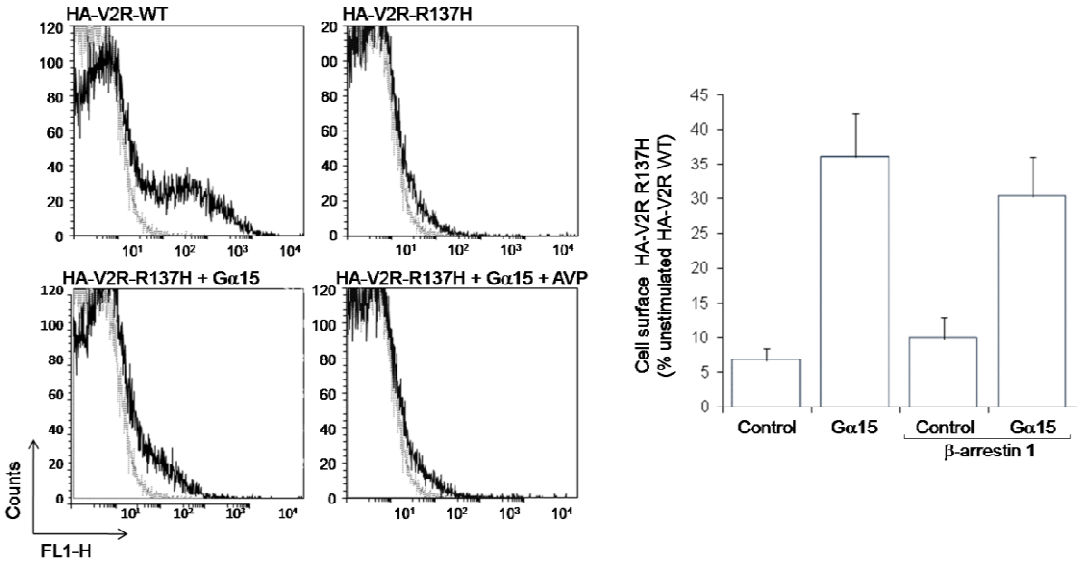


Figure 19 - G15 restores cell surface expression of V2R-R137H. G15 restores cell surface expression of V2R-R137H. Cell surface expression of V2R-R137H was measured by flow-cytometry in COS-7 cells transiently transfected with the indicated combinations of constructs. Fluorescent labeling of the receptor was achieved by an antibody directed to the amino-terminal HA epitope in unpermeabilized cells. **a-d)** FACS profiles representative of four experiments, the black trace represents cells transfected and treated as indicated, the gray trace represents mock transfected cells **e)** Specific surface fluorescence was quantified as percent of the V2R WT transfected in parallel and plotted as means±S.E.M.

receptor hinders the interaction with β-arrestin and thus set at least part of the receptor free to move to the cell surface. Further experiments are required to prove this hypothesis.

Gα15 activates effectors that favor tumor growth

PKCs are among the best characterized effectors of Gq family members. Among PKCs isoforms PKDs have a major role in cell motility, invasion and adhesion (Wang, 2006). PKD also regulates tumor cell invasion and modulates cell motility and adhesion by binding to and

phosphorylating E-cadherin in prostate cancer cells. The pro-proliferative effect of PKD has been demonstrated in many cellular systems, including pancreatic carcinoma cells (Wang, 2006). We sought to assess if PKD is activated by G15 signalling. Gα15 was transfected in COS cells, together with PKD1, and directly stimulated with aluminum fluoride (AlF_3) (Error! Reference source not found.). PKD1 activation level was revealed by Western blot measuring the phosphorylation of serine 744 and 748 with a phosphoaminoacid specific antibody. PKC dependent phosphorylation of these residues induces the activation of the catalytic domain and is thus considered to be indicative of PKD activity state. Very little stimulation was observed in cells overexpressing only PKD1. The presence of G15 potently strengthened the signal demonstrating that PKD1 can be included among downstream effectors of G15 signalling.

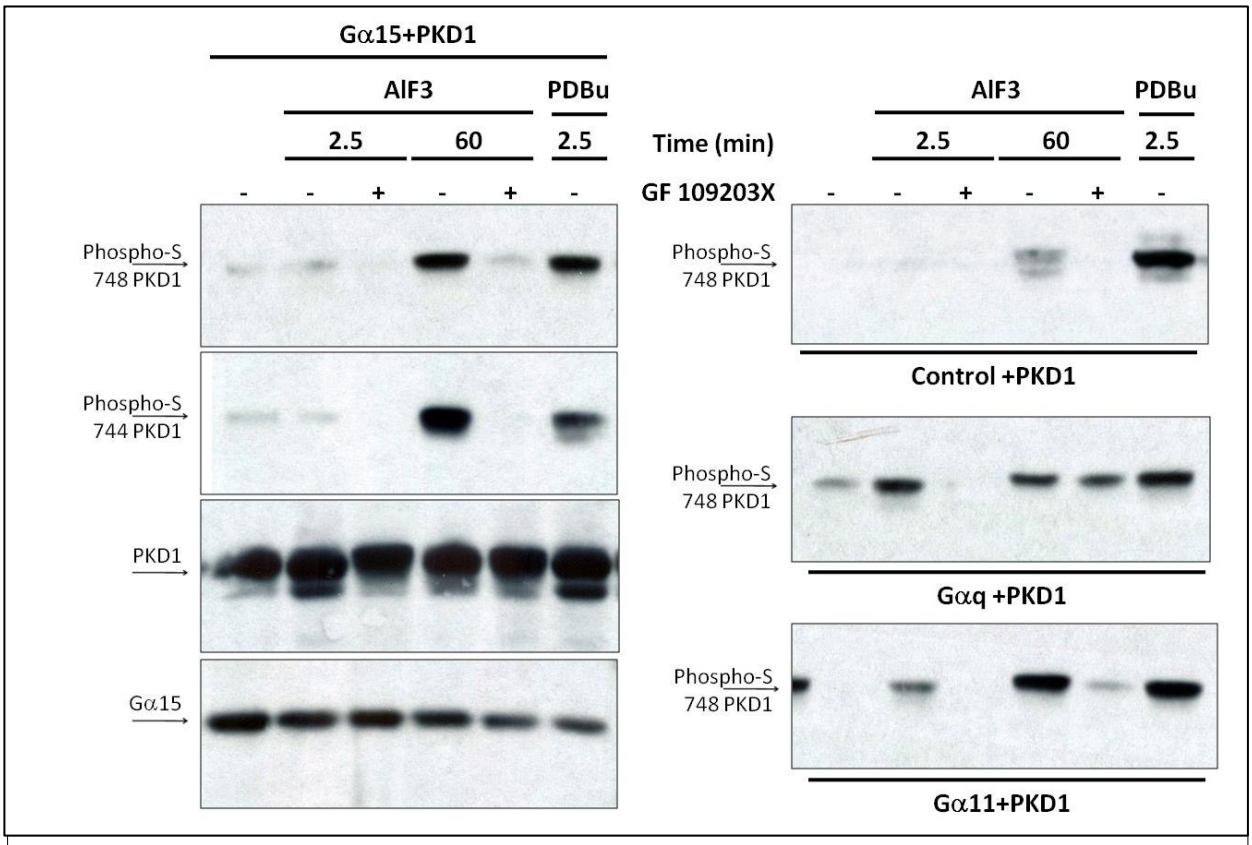


Figure 20 PKD1 activation by G15. - COS-7 cells were transfected as indicated with PKD1 and the α subunits of different G proteins (G15, Gq or G11). The G proteins were directly activated by treatment with AlF_3 for 2.5 or 60 minutes. 100 nM PDBu for 2.5 minutes instead directly activates PKCs. PKD1 activation level was quantified with antiphosphospecific antibodies against S748 or S744. In addition, the total level of PKD1 expression was measured by utilizing an antibody against PKD1 (C20). Gα15 expression level was monitored with specific antibodies. Pretreatment (1 h) with the specific inhibitor GF109203X prevented most of the activation.

The activatory effect was even more evident when G15 was activated by co-transfected β2AR. As shown in Figure 10, the simultaneous presence of the receptor and G15 made COS cells sensitive to Iso by inducing PLC activity. On turn, the stimulation was transmitted downstream by G15, but not Gq, to activate PKD1(Figure 21). In all cases PKD1 activation was reduced by the specific PKC inhibitor GF109203X.

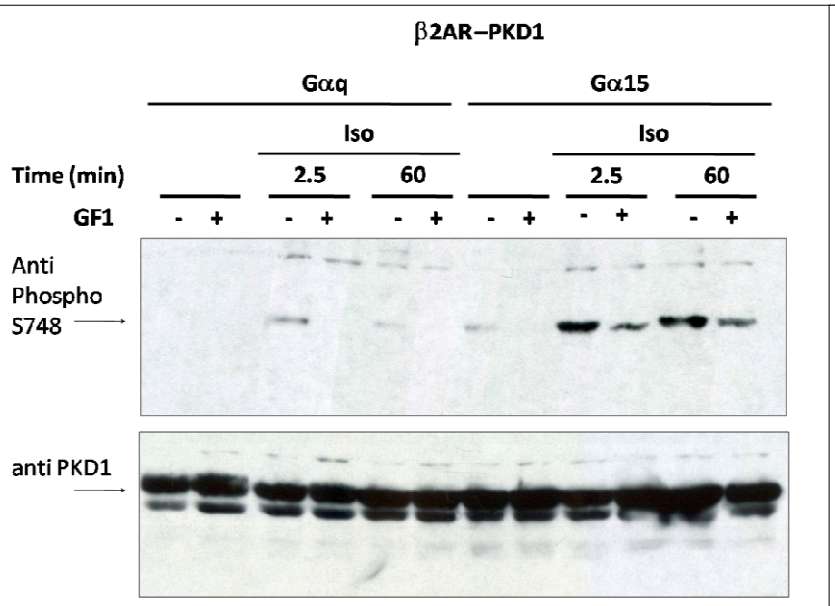


Figure 21 – PKD1 activation by G15. -COS-7 cells were transfected with β2AR and PKD1 in addition to Gq or G15 prior treatment with Iso as indicated. PKD1 activation level was quantified with the anti S748 antibody. Pretreatment (1 h) with the specific inhibitor GF109203X prevented PKD1 activation. Equal amounts of PKD1 were expressed as assessed utilizing an antibody against PKD1 (C20).

PKD1 is upregulated by the V2R-R137H in presence of Gα15

We next asked if G15 would collect the signal produced by the constitutively desensitized V2R-R137H keeping as active an indirect downstream effector like PKD1 (Figure 22). The V2R-R137H was thus compared to the WT after co-expression with Gα15 and PKD1. Similarly to the inositol phosphates accumulation assay, Western blot analysis revealed that even in the absence of the agonist the V2R-R137H kept a sustained phosphorylation of PKD1 (no difference on PKD phosphorylation could be detected if G15 was absent, data not shown). Once again the addition of AVP did not affect the signalling efficiency of the R137H mutant, while the activity of the WT was totally conditional to the presence of the agonist. Similar results were obtained probing S744 or S748. No effect on the total expression level of PKD1 or Gα15 was observed depending on the type of V2R expressed or on the treatment with AVP.

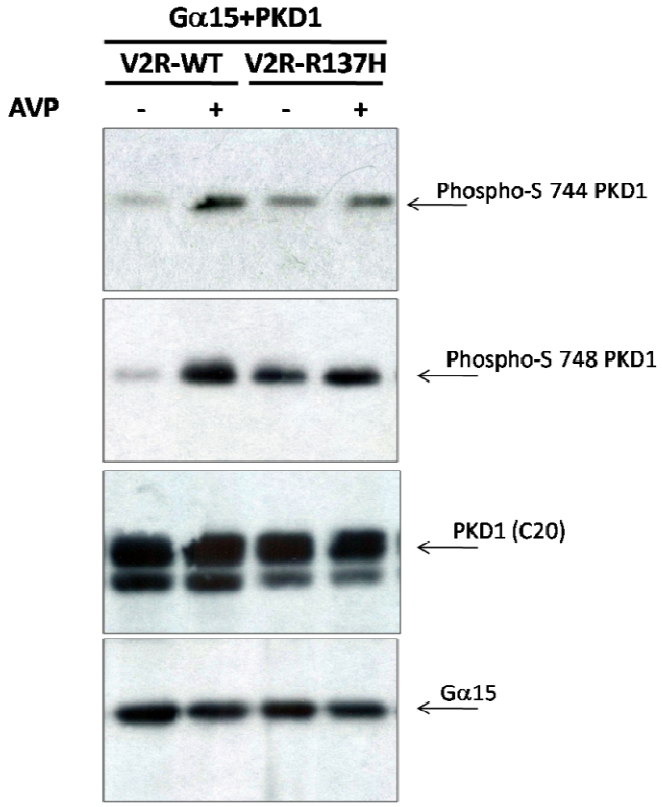


Figure 22 - V2R-R137H sustains G15 dependent PKD1 activation. COS-7 cells were transfected with Gα15 and PKD1in addition to the receptor, either in WT or the R137H form. PKD1 activation level was revealed with the anti phosphorS744 or anti phosphoS748 antibody. Similar amounts of PKD1 and Gα15 were expressed, as assessed by an antibody against PKD1 (C20) and Gα`15 respectively.

Discussion

Heterotrimeric G proteins support cellular signalling in virtually all aspects of cell physiology. G15 is a member of the ubiquitous Gq/11 subfamily that, for the last decade, was described as specific of hematopoietic stem cells (Wilkie et al., 1991; Amatruda, III et al., 1991; Hubbard and Hepler, 2006). Despite such relatively limited pattern of expression, the significance of its presence in our organism remains puzzling and surrounded by many mysteries.

Two Gα15 isoforms

A first conundrum is represented by the observation that two different molecular weight forms were repeatedly described in immunoblot analyzing native Gα15. A 43 kDa form is predominant in normal granulomonocytic cells, whereas erythroid progenitors and platelets mostly express a 46 kDa form. Activated T lymphocytes express both species (Lippert et al., 1997).

Northern blot analysis performed on two cell lines, reports two transcripts (of 2.2 and 2.4 kb), however only one protein specie is detected in the corresponding Western blot (Wilkie et al., 1991). This suggests that the two mRNA forms cannot explain the two protein products. Among other possible explanations, covalent post-translational modifications could be responsible for differential migration. Gα15 is indeed phosphorylated by PKC and palmitoylated. However, PKC phosphorylation does not cause a band shift as reported by Gu et al (Gu et al., 2003). As well, the removal of both the palmitoylated cysteine residues present at Gα15 N-terminus did not significantly affect the migration of the band corresponding to the recombinant protein (Pedone and Hepler, 2007).

However, the downmodulation of Gα15 expression by five different specific shRNA sequences (**Error! Reference source not found.**) proves that both bands are Gα15 and not to another isoforms or another protein recognized by a non specific interaction with the polyclonal antibody.

This result validates the need for further investigations aimed to unravel the molecular details that differentiate these two Gα15 species. Further studies should also be dedicated to assess what differentiates their physiological roles.

Expanding Gα15 expression profile

Gα15 distribution is reported as restricted to tissues that are rich in hematopoietic cells and in cell types of hematopoietic origin (Hubbard and Hepler, 2006; Wilkie et al., 1991), i.e. spleen, bone marrow, embryonic liver and thymus. Consistent with an expression profile limited to hematopoiesis, in adult the signal is lost in liver and thymus. Furthermore, cell lines at different maturation stages (or inducible models, like HL60 cells) maintain Gα15 expression only in the progenitor cells status (Amatruda, III et al., 1991; Grant et al., 1997) to progressively decline it with maturation. This pattern was confirmed by Gα15 presence in CD34positive cells (Pfeilstocker et al., 2000) (Figure 8), erythroid cell lines (Ghose et al., 1999), megakariocytes (den Dekker et al., 2001), B cells progenitors (Mapara et al., 1995). We analyzed other immature adult cells (mesenchimal stem cells) derived from thymus and bone marrow, however, only minimal mRNA levels were present and no protein signal was detected in Western blot. Our data therefore indicate that, despite Gα15 expression overlaps the expression of a stemness marker like CD34, it cannot be generalized to all stem cells.

A more comprehensive analysis of healthy tissues was in good agreement with the literature. However, traces of Gα15 mRNA were present in most tissues analyzed. Possibly, such a small number of transcripts only allows traslation below the detectability levels of the available antibodies (calculated by Krumins et al. to be <1 ng in HeLa cells (Krumins and Gilman, 2006)). Gα15 distribution should therefore be analyzed utilizing a better reagent. Unfortunately, the

similarity with other Gq family members limits the number of epitopes that can be designed to raise a more sensitive antibody.

As described by Rock et al. (Rock et al., 1997a), skin makes an exception to what initially reported. We found that the expression levels in this tissue were comparable to the more homogeneous samples derived from hematopoietic cells and from TEC. Gα15 mRNA was reported in cultured cells from neonatal foreskin (but not in fibroblasts, melanocytes or endothelial cells) (Rock et al., 1997b). *In situ* hybridization revealed a strong expression specifically in epidermis and in hair follicular epithelium but not in other skin appendageal structures.

We also showed that TEC are the major responsible for the signal present in adult thymus. TEC and keratinocytes share a common ectodermal origin while hematopoietic, endothelial and smooth muscle cells (in one report G15 has also been reported in tracheal smooth muscle cells (Bruges et al., 2007)) derive from a specialized subset of the mesoderm represented by blast-like colonies (named blast colony forming cell, BL-CFC) (Mikkola and Orkin, 2006). G15 signalling is therefore exploited by cells derived from at least two different germ layers leaving out tissues derived from the endoderm and healthy organs . In summary, as for G14, G15 expression profile remains much more limited as compared to Gq and G11. However, its expression should not be considered as totally specific of the hemopoietic system and therefore its function should be analyzed within other contexts.

G15 interaction with the V2R-R137H reveals alternative receptor conformations

Evolution diversified the sequence of Gα15 gene (GNA15) from other Gαq family members conferring to this G protein peculiar functional properties, not least the poor sensitivity to desensitization that we describe. Cells exploit GPCR desensitization to finely tune receptor activity and match the cellular responsiveness to the intensity of the stimulation. We observed that the coupling of distinct GPCRs (i.e. β2AR, DOR, V2R) to the promiscuous G15 protein is

remarkably resistant to the desensitization produced either by repeated stimulation, or by emphasizing β-arrestin function with overexpression.

The process of receptor desensitization is not strictly limited to agonist-occupied receptors, rather, homologous phosphorylation and the subsequent interaction with β-arrestin also occur when the receptor becomes active either spontaneously (Innamorati et al., 2006) or because specific mutations lock it in an active conformation (Barak et al., 2001). One example is the V2R-R137H: this mutant V2R is uncoupled by desensitization from both Gs and Gq. Similar to other mutations found in rhodopsin (Robinson et al., 1992), in the α1B adrenergic receptor and in the angiotensin II type 1A receptor (Wilbanks et al., 2002), the constitutive activity promoted by the mutation of a conserved arginine residue is associated to high receptor phosphorylation, constitutive binding to β-arrestin and extensive intracellular translocation (see Introduction at pag. 17). Confirming the anomaly initially observed with the WT receptors, we find that G15 demonstrates a differential sensitivity to V2R-R137H desensitization as compared to other heterotrimeric G protein subtypes, such as its homolog Gq (Figure 16, Figure 17, Figure 18, Figure 22).

Two different substitutions in the same position of the F motif (R→L and R→C) are described for forcing the receptor toward constitutive activity without a correspondent significant initiation of the desensitization process. Consistently, patients carrying R137L or R137C substitutions suffer opposite symptoms to patients carrying the R137H substitution as they report antidiuresis despite absent AVP levels. Another mutation affecting the preceding aspartic residue (D136A) was reported to induce constitutive receptor activity (Morin et al., 1998) but in this case the spontaneous activation leaves large margin for the agonist to further promote receptor activity. All these mutations likely loosen intramolecular ionic forces that normally stabilize the inactive conformation thus facilitating the spontaneous transition to intermediate states of activation (see pag.9). It is possible that each one of these states simulates a receptor conformation, that is physiologically relevant for the activation of specific and alternative functions.

The R137H substitution appears to shift the equilibrium entirely toward such active conformation, since, saturating all V2R-R137H binding sites with the agonist did not further stimulate G15 activity (as measured either by inositol phosphate accumulation or by PKD activation). However, under exactly the same experimental conditions, the addition of AVP

induced the internalization of the receptor rescued to the plasma membrane by the presence of G15 (Figure 19). The conformation stabilized by the R137H mutation therefore does not completely mimic the conformation induced by ligand occupancy. Consistent with the most recent theories on the presence of intermediate GPCRs conformations (see pag.10), a combined expression of G15 with the R137H mutant appears to drive the receptor toward a desensitized state that, although fully active on downstream effectors like PLC and PKD, it is not yet suitable to be recruited by the internalization machinery before the transition is completed by the interaction with the agonist. In other words, to explain why the activation of the effectors and the interaction with the internalization machinery are distinctly modulated by the agonist, we suggest that the presence of G15 modulates the conformation of the V2R-R137H to a state that represents an intermediate toward the full transition provoked by a full agonist.

A similar functional disjunction of two phenomena that for longtime were believed to be inherent to a single activation state, is often referred as "biased agonism" because usually such dissociation is driven by different ligands. However, in this case, it is the results of a modulation driven by the G protein. This is not surprising as highly reminiscent of the so called "GTP effect" on the affinity of the receptor described many years ago (Birnbaumer, 2007).

We foresee that these receptor conformations might exist to fulfill distinct functional effects on specific signalling pathways.

Molecular mechanism supporting G15 coupling to desensitized GPCRs

Adding to the functional evidences offered by G15 effect on inositol phosphate accumulation, Ca^{2+} mobilization and PKD1 function, the ability of G15 to prevail GPCR desensitization better than other G proteins was substantiated by two additional observations. First, Gα15 displays a stronger interaction with the immunoprecipitated "desensitized" receptor (as compared to Gαs or Gαq). Second, there is a functional competition of Gα15 with functions mediated by β-arrestin (i.e. retaining intracellularly internalized V2R).

G15 is the most divergent member of the Gq/11 subfamily and it is characterized by a peculiar poor selectivity that permits coupling to most GPCRs (Offermanns and Simon, 1995). G15 poor sensitivity to β-arrestin dependent desensitization adds a novel feature to its atypical signalling properties.

Alternative interpretations are available to explain the molecular mechanism(s) supporting G15 remarkable resistance to β-arrestin desensitization. G15 could recognize a receptor activation domain left unshielded by β-arrestin docking. However, the simplest hypothesis is that a tight interaction of Gα15 with the receptor displaces β-arrestin and other G proteins from a common binding site. In retina exposed to intense light, rhodopsin desensitization is underlain by an analogous direct competition between the homologs of the G protein (transducin) and arrestin (cone arrestin) with the latter displacing transducin from a common docking region on rhodopsin (Krupnick et al., 1997).

The phosphorylated carboxyl-terminus was not required for V2R coupling to G15 (Figure 15), it can therefore be inferred that G15 does not selectively associate with the phosphorylated form of the receptor. This does not completely exclude that G15 might privilege the "desensitized receptor". In fact, partial desensitization (Innamorati et al., 1997) and β-arrestin binding (Oakley et al., 1999) occur even when the carboxyl-terminus has been artificially removed. However, since β-arrestin overexpression did not reduce G15 signalling, but neither amplified it, a preferential coupling of G15 to the complex formed by the receptor and β-arrestin appears unlikely. Consistently with the hypothesis of a particularly tight interaction of Gα15 with the receptor, it was recently shown that the stimulatory effect on Gq mediated by the viral chemokine receptor homolog pUS28 is markedly sensitive to the inhibition of G15 (Minisini et al., 2003).

Explaining in deeper details the molecular determinants that allow G15 to recognize desensitized receptors as active might not be straightforward. As a matter of fact, G15 coupling promiscuity under 'non-desensitizing' conditions is still largely unexplained (despite the many attempts made by utilizing chimeric GPCRs or chimeric G proteins). As reported in the introduction, the molecular determinants driving the interaction with G15 seem localized in the second intracellular loop of the GPCR but may differ for each receptor type (Xie et al., 1997; Hubbard

and Hepler, 2006). At least for the α1b-adrenergic receptor, only a dramatic deletion of the second intracellular loop effectively prevented the interaction (Wu et al., 1995).

On the β-arrestin side, the solution of the crystal structure of the β-arrestin-receptor complex remains elusive, and few available examples of mutagenesis clearly point to a multi-site interaction (Gurevich and Gurevich, 2006). In conclusion, thus far, not enough information is available to make accurate predictions or design a clear strategy aimed to dissect which domains allow G15 to override the desensitization process.

Physiological significance of G15 resistance to GPCR desensitization

G15 has been acquired relatively late during evolution, likely by gene duplication (Davignon et al., 2000b) and has evolved at an accelerated rate as compared to all other Gα subunits genes (similar to many genes involved in functions that are specifically related to hematopoiesis (Murphy, 1993)). Why have mammalians evolved such peculiar G protein? What is the relevance of its signalling for our organism?

Our data might offer a new perspective to answer these questions. We suggest that future investigations aimed to define Gα15 physiological function should keep into consideration that its effects may become more evident under conditions of intense and prolonged stimulation.

'Sustained' G15 signalling could become particularly relevant to support enduring (and possibly ligand-independent) stimuli generated by GPCRs under exceptional stimulatory conditions, such as inflammation, organogenesis or tissue repair. Under similar circumstances, Gα15 could represent a mean to override the regulation that normally limits GPCR signalling. G15 is expressed within a short time window during early hematopoiesis (Hubbard and Hepler, 2006) or upon lymphocytes activation and cheratinocytes proliferation (Lippert et al., 1997). Gα15 normal expression could therefore be related to the need for producing potent and durable signalling when rapid and intense responses are required, see for instance upon antigen-induced lymphocytes activation and expansion.

Given its promiscuous nature, G15 could amplify signals generated by many GPCRs present in hematopoietic cells and in lymphocytes (including the vasopressin (Yamaguchi et al., 2004), adrenergic (Muthu et al., 2007; Kohm and Sanders, 2001) and opioid receptors (Messmer et al., 2006; Steidl et al., 2004; Sharp, 2006)). S1P4 is a good candidate GPCR for promoting G15 activity; it is part of a family of receptors responding to lysophospholipids or lysosphingolipids and it is involved in cell signalling of many different cell types. EDG6 (endothelial differentiation, G-protein-coupled 6), the gene that encodes for S1P4, is under the control of the same promoter of GNA15 (the gene encoding for Gα15) (Contos et al., 2002). Consistently, according to microarrays data, both genes are simultaneously expressed in mouse fetal liver cells, a model of erythroid differentiation, to become simultaneously silenced during differentiation (Antonella Ronchi, personal communication). An interaction between these two proteins could explain why in presence of serum (which is known to contain S1P (Yatomi et al., 1997)) Gα15 inhibition or downregulation affects erythroid cells growth and differentiation (Ghose et al., 1999).

Other scenarios could be anticipated under pathological circumstances involving protracted GPCR stimulation. G15 enduring signalling could be exploited by the pertussis toxin-resistant constitutive signalling generated by US28. US28 is one of the fourteen GPCRs encoded by the 200kb genome of the human cytomegalovirus (HCMV). During HMCV infection *in vitro*, US28 promiscuously couples to members of the Gi/o, G12/13 families of G-proteins and to G15 (Billstrom et al., 1998; Minisini et al., 2003). Similar to many GPCRs, US28 demonstrates significant activity in the absence of ligands as reported by authors showing that US28 induces the activity of PLC in an agonist independent manner (Sherrill and Miller, 2008). US28 has been suggested to function as a viral oncogene since it can induce tumor formation in fibroblasts injected in nude mice. Such oncogenic effect involves G protein signalling aimed to induce the expression of proangiogenic and cell cycle factors (Sherrill and Miller, 2008).The signalling generated by this viral receptor is reminiscent of what we observed with the V2R-R137H as it occurs despite the carboxyl-terminus of the receptor is constitutively phosphorylated and internalization traps most of the receptors in perinuclear endosomes (Vischer et al., 2006).

G15 poorly regulated signalling could also become relevant to other pathological conditions implying GPCRs hyperstimulation, as for instance in cancer development (Dorsam and Gutkind,

2007). In particular, G15 could create a “signalling shortcut” in tumors that produce large amounts of stimulatory peptides to self-stimulate their growth in an autocrine or paracrine manner (Heasley, 2001).

Conclusions

In conclusion, upon prolonged GPCR activation, and while other G proteins are ineffective, G15 stabilizes a receptor state that remains active towards intracellular effectors. By showing that the promiscuous coupling to G15 makes the V2R, β2AR and DOR more resistant to β-arrestin-dependent desensitization, we suggest a novel mechanism by which GPCRs can generate sustained signalling. Such persistent activation of G15 will supposedly propagate to downstream effectors that include PKD in addition to other signal transducers potentially relevant to physiological conditions associated to intense signalling activity (such as inflammation).

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