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Review

Hormone-induced subcellular redistribution of trimeric G proteins

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Abstract. Trimeric guanine nucleotide-binding proteins (G proteins) function as the key regulatory elements in a number of transmembrane signaling cascades where they convey information from agonist-activated receptors to effector molecules. The subcellular localization of G proteins is directly related to their functional role, i.e., the dominant portion of the cellular pool of G proteins resides in the plasma membrane. An intimate association of G protein subunits with the plasma membrane has been well known for a long time. However, results of a number of independent studies published in the past decade have indicated clearly that exposure of intact target cells to agonists results in subcellular redistribution of the cognate G proteins from plasma membranes to the light-vesicular membrane fractions, in internalization from the cell surface into the cell interior and in transfer from the membrane to the soluble cell fraction (high-speed supernatant), i.e., solubilization. Solubilization of G protein α subunits as a consequence of stimulation of G protein-coupled receptors (GPCRs) with agonists has also been observed in isolated membrane preparations. The membrane-cytosol shift of G proteins was detected even after direct activation of these proteins by non-hydrolyzable analogues of GTP or by cholera toxin-induced ADP-ribosylation. In addition, prolonged stimulation of GPCRs with agonists has been shown to lead to down-regulation of the relevant G proteins. Together, these data suggest that G proteins might potentially participate in a highly complex set of events, which are generally termed desensitization of the hormone response. Internalization, subcellular redistribution, solubilization, and down-regulation of trimeric G proteins may thus provide an additional means (i.e., beside receptor-based mechanisms) to dampen the hormone or neurotransmitter response after sustained (long-term) exposure.

Key words. Trimeric G proteins; subcellular distribution; solubilization; desensitization.

Introduction

Heterotrimeric G proteins composed of α, β, and γ subunits are employed as ubiquitous transducers and regulators of cellular signaling in all eucaryotic cells. They serve as universal switches in regulating transmission of information from membrane-bound G protein-coupled receptors (GPCRs) to various cellular effectors such as adenylyl cyclases, phospholipases, and ion channels [1–6]. Eucaryotic cells are equipped with hundreds of subtypes of GPCRs, which are typical seven-transmembrane helix receptors [7, 8]. GPCRs can be activated by a great number of diverse stimuli, such as light, odorants, hormones, and neurotransmitters. Agonist-activated receptors catalyze the exchange of GDP for GTP on the α subunits of their cognate G proteins; subsequently, the ternary high-affinity complex hormone-receptor-G protein is dissociated to free α_{GTP} and $\beta\gamma$ subunits. Both α_{GTP}

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and $\beta\gamma$ are competent to modulate downstream components of signaling cascades [9, 10]. The activated state lasts until GTP is hydrolyzed to GDP by the intrinsic GTPase activity present in the α subunit. Subsequent reassociation of α with $\beta\gamma$ turns off signal transduction and primes the system to respond to a new stimulus. Recently, a new group of modulatory proteins, known as regulators of G protein signaling (RGS), was identified [11, 12]. Since RGS act as potent GTPase-activating proteins, they might be engaged in timely deactivation of signaling mediated by heterotrimeric G proteins.

Besides switching signal transmission on and off, there is a complicated set of regulatory feedback mechanisms called desensitization, refractility, or tachyphylaxis (used as synonymous expressions). Desensitization protects cells against excessive stimulation and is defined as a decrease in response detected after repeated or long-term agonist exposure. This process is essential from a physiological point of view, because it provides a means to regulate continuous receptor stimulation. The short-term desensitization proceeding within minutes is generally accepted to take place primarily at the receptor level – phosphorylation, sequestration, and internalization of GPCRs [13–15]. Experimental data published during the last 10 years have indicated that prolonged agonist exposure induces transfer of G proteins from plasma membranes to the cytosol and/or other subcellular compartments distinct from plasma membranes. In this way, G proteins might participate in the phenomenon of desensitization, although within a significantly longer time scale than that observed for the receptors. The present review aims to summarize this published evidence and supports the notion that the G proteins need not permanently reside in the plasma membrane but can be released to the cytosol in response to prolonged agonist stimulation.

GPCRs and trimeric G proteins reside in plasma membranes

Plasma membrane localization of hormone-sensitive adenylyl cyclase in liver and white fat cells has been known since 'transducer times,' i.e., long before G proteins were actually discovered [for a review see ref. 1]. Accordingly, trimeric G protein subunits were isolated first from cell membrane preparations [16–18] and hormone-responsive adenylyl cyclase and/or $G_s\alpha$ reconstitutive activity was demonstrated clearly to reside in plasma membrane fractions purified on sucrose density gradients [19–21]. Thus, in naive (hormone-unexposed cells), both GPCRs and the dominant pool of G proteins are localized in plasma membranes.

As trimeric G proteins do not contain hydrophobic transmembrane sequences like their cognate GPCRs, the membrane binding or association of these proteins has to take place in some other way. Contrary to the relatively hydrophilic α subunits, both β and γ turned out to be highly hydrophobic proteins which form tightly packed heterodimers in all conditions except strongly denaturing media [22]. Therefore, the $\beta\gamma$ dimer was initially thought to be crucial for anchoring the α subunit to the plasma membrane [2, 22]. Further experimentation and progress in this field indicated, however, that the interaction with $\beta\gamma$ is not necessarily the only way α subunits bind to the plasma membrane. Independent attachment of G protein α subunits to the plasma membrane was demonstrated first by Simonds and co-workers [23] in COS cells overexpressing most of the G protein α subunits cloned at that time – $G_i\alpha$-1, $G_i\alpha$-2, $G_i\alpha$-3, $G_o\alpha$, and $G_s\alpha$. Despite the huge excess of α over β (an $\alpha:\beta$ ratio greater than 25:1), the α subunits were predominantly membrane bound [23]. Thus, besides $\beta\gamma$, there had to be some additional binding links responsible for association of G protein α subunits with the membrane.

Soon afterwards, G protein α (as well as γ) subunits were discovered to be targets for various types of covalent lipid modifications which represent additional binding links or 'membrane anchors' for attachment of $G\alpha$ to the membrane. G proteins from $G_i\alpha/G_o\alpha/G_t\alpha/G_z\alpha$ families were found to be myristoylated and palmitoylated at glycine 2 and cysteine 3, respectively [24–27], $G_s\alpha$ was found to be palmitoylated at cysteine 3 but not myristoylated [28–30] and $G_q\alpha/G_{11}\alpha$ proteins were found to be modified by two palmitic acid residues at cysteine 9 and 10 [29, 31]. Myristoylation is a stable co-translational modification which is essential for signaling regulation of effector enzymes as well as for membrane attachment and subsequent palmitoylation [32–35]. Myristoylation permits continued membrane association when $G\alpha$ dissociates from $G\beta\gamma$ [36, 37], increases affinity of α subunits for $\beta\gamma$ [38], and facilitates formation of the heterotrimeric forms of G proteins [39, 40]. On the other hand, palmitoylation is an unstable post-translational lipid modification of $G\alpha$, which is readily reversible and thus has the potential to be regulated [41–43]. Palmitate may be embedded in the lipid bilayer and/or may influence protein-protein interactions. Importantly, G protein $\beta\gamma$ subunits were found to enhance palmitoylation and protect $G\alpha$ against the cleavage of palmitate by palmitoyl esterase [42]. A cycle of palmitoylation and depalmitoylation has been proposed to control protein distribution between subdomains of the plasma membrane and/or between membrane and cytoplasm [43].

Hormone stimulation redistributes GPCRs and G proteins from plasma membranes to the light-membrane vesicles

Many investigators in the early 1980s observed that long-term hormone stimulation and desensitization was asso-

ciated with the shift of G protein-coupled receptors from plasma membranes to the light vesicular or low-density membrane fractions distinct from plasma membrane fragments [19–21]. The evidence for agonist-induced intracellular redistribution was based on fractionations of tissue homogenates by differential or sucrose density gradient centrifugation. In addition, redistribution of GPCRs was also manifested as disappearance of hydrophilic radioligand binding sites from the cell surface [44].

The first direct evidence for subcellular redistribution of trimeric G proteins was provided by Haraguchi and Rodbell [45]. These authors reported that isoproterenol stimulation of white fat cells led to translocation of a significant portion of $G_s\alpha$ and $G_i\alpha$ proteins from plasma membranes to light-density vesicles. In parallel, isoproterenol-stimulated adenylyl cyclase and 5′-nucleotidase enzyme activities were shifted as well, though to a smaller extent. Therefore, the non-plasma membrane nature of these low-density membrane fragments could not be unambiguously proven. Even so, these data clearly indicated the hormone-induced change in sedimentation profile of a significant portion of membrane fragments containing G protein α subunits. Similar data were later published by other investigators [46–49].

Cytosolic forms of G proteins

Rodbell [50], in an at that time new and still speculative theory of hormone action, suggested that activated α subunits might be released from the plasma membrane as a consequence of agonist stimulation, and mediate the transfer of hormonal signal (i.e., alongside secondary messengers) into the cell interior. Chabre [51] was the first to characterize the G proteins as peripheral proteins and to present the theoretical background explaining why and how these proteins could be solubilized. However, his ideas were based on the characteristics of membrane association of the rather atypical G protein, transducin (G_t).

The α subunit of transducin, $G\alpha_t$, does indeed behave like a peripheral membrane protein, because light exposure, small changes in ionic strength, or chelation by EDTA release $G_t\alpha$ from membranes to the high-speed supernatant [52]. This is, however, not the case for other types of G proteins. Here, a number of conflicting observations have been published [29, 30, 53–55]. Easy solubilization of transducin is apparently based on the fact that it is the only G protein which is non-palmitoylated. $G\alpha_t$ is heterogeneously modified by myristate and three other less hydrophobic fatty acids at the N terminus, but this modification does not permanently anchor the protein in the membrane [56]. The heterogeneous fatty acylation of $G\alpha_t$ is not dictated by a unique structural feature of this molecule but it is specific for retinal photoreceptor cells [57]. The explanation for such tissue- or cell-specific differences in fatty acid acylation of a G protein N terminus lies in the fact that in different cells, the fatty acyl-CoA pool contains different fatty acids [40].

Solubilization of G protein α subunits by direct activation of G proteins

The very first evidence showing the release of activated $G_s\alpha$ subunits from plasma membranes to cytosol was provided by Lynch and co-workers [58] by measurement of cholera toxin-mediated ADP-ribosylation of rat liver plasma membranes. Following cholera toxin stimulation, a substantial portion of ADP-ribosylated $G_s\alpha$ was no longer membrane associated, but could be recovered in a high-speed supernatant.

Similar results were reported by Milligan's group [59–63]. Incubation of crude membranes from glioma C6 BU1 cells with poorly hydrolyzed analogues of GTP caused the release of $G_s\alpha$ from the membrane to cytosol fraction. This redistribution was already detectable after 5 min [61, 63]. Experiments on intact L6 skeletal myoblasts confirmed that cholera toxin-mediated ADP-ribosylation results in depletion of $G_s\alpha$ from plasma membranes [62].

Hormone-induced solubilization of G protein α subunits

In 1989, Ransnas and co-workers [53] showed for the first time that G protein might be released from plasma membranes as a direct consequence of hormone action. Using ELISA and specific rabbit antipeptide antisera, they were able to detect the increase of free $G_s\alpha$ in a high-speed (200,000 g) supernatant after isoproterenol stimulation of intact S49 lymphoma cells. The agonist-induced solubilization of $G_s\alpha$ was subsequently also demonstrated in in vitro experiments with membranes isolated from S49 lymphoma cells [64, 65]. Under these conditions, isoproterenol-induced release of $G_s\alpha$ from membranes was clearly concentration dependent and could be inhibited by the β-adrenergic blocking agent propranolol, free $\beta\gamma$ subunits, and somatostatin. All data obtained by immunochemical detection of $G_s\alpha$ were convincingly supported by functional analysis using a cyc^--reconstitution assay which clearly indicated a significant increase in G_s-reconstitutive activity in a high-speed supernatant. Solubilization of $G_s\alpha$ was also observed in mastocytoma P-815 cells after stimulation with the prostacyclin analogue iloprost in the presence of GTPγS [66]. In rat pituitary GH4C1 cells, $G_s\alpha$ was transferred from membranes to the cytosol (high-speed supernatant) after stimulation with vasoactive intestinal peptide (VIP) [67]. Recently, Witte and co-workers [68] described isoproterenol-induced solubilization of $G_s\alpha$ from rat cardiac membranes.

Takahashi and co-workers [69–71] detected soluble $G_i\alpha$-2 in mouse mastocytoma P-815 cells, and in subsequent studies they demonstrated that thrombin induces a dramatic release of $G_i\alpha$-2 from the membranes to the high-speed supernatant prepared from these cells. Furthermore, Yajima and co-workers [72] reported that incubation of GH4C1 rat pituitary membranes with somatostatin leads to release of $G_i\alpha$-2, $G_i\alpha$-3 as well as $G_o\alpha$ from membranes and translocation to the soluble (cytosol) fraction.

Besides $G_s\alpha$ and $G_i\alpha/G_o\alpha$, evidence for solubilization of α subunits of G_q/G_{11} proteins has also been provided. In a set of experiments on transfected HEK-293 cells, we were able to demonstrate that prolonged treatment with thyrotropin-releasing hormone induced not only down-regulation of total cellular $G_q\alpha/G_{11}\alpha$ but caused a dramatic shift of these proteins from plasma membranes to the high-speed supernatant [48, 49, 73]. The appearance of $G_q\alpha/G_{11}\alpha$ in the cytosol fraction of GH3 and AtT-20 cells was reported by Ravindra [74]. Arthur and co-workers [75] have shown that even short-term treatment of MDCK cells with bradykinin leads to translocation of a significant portion of $G_q\alpha/G_{11}\alpha$ from plasma membranes to the cytosol.

Soluble forms of G protein α subunits under resting conditions

All the above-mentioned results indicate that G protein α subunits might be solubilized as a result of G protein activation. There are a number of observations indicating that in some cells or tissues, a certain amount of α subunits is present in the cytosol fraction even under resting, i.e., unstimulated conditions. Roth and co-workers [76] quantitated $G_s\alpha$ by both immunoblotting and cholera toxin ADP-ribosylation in atrial and ventricular preparations of porcine heart and detected about 30% of total $G_s\alpha$ in the soluble (cytosol) cell fraction. Interestingly, functional assays utilizing reconstitution of cardiac $G_s\alpha$ with S49 cyc^- membranes revealed that a high-speed supernatant contained about 16% of total cellular $G_s\alpha$ activity [76]. This disproportion suggests that a substantial part of the soluble population of $G_s\alpha$ may not be functional, at least with respect to adenylyl cyclase activation. High cytosolic levels of $G_s\alpha$ have also been demonstrated in rat myocardial preparations. Primary cultures of neonatal rat cardiocytes contained about one-third of total $G_s\alpha$ in a 200,000 g preparation [77]. A similar ratio of cytosolic and membrane-bound $G_s\alpha$ was found in ventricular myocardium of immature as well as adult rats [78]. Intriguingly, high amounts of functional $G_s\alpha$ were also recently detected in cytosol fractions obtained from different regions of rat brain [79]. Cytosolic forms of pertussis toxin-sensitive $G_i\alpha/G_o\alpha$ proteins were also demonstrated for the first time in neutrophils [80]. The presence of soluble α subunits of G_i/G_o proteins in neutrophils was subsequently independently confirmed by two other groups [81–83]. Recently, the cytosolic localization of all three $G_i\alpha$ proteins has been described in MDCK cells and cytosolic $G_i\alpha$-2 was detected in *Dictyostelium discoideum* [84, 85]. Similarly to $G_s\alpha$, a substantial portion of $G_i\alpha$ proteins in cultured hepatocytes (in contrast to cells in tissue) was found in the cytoplasm [86]. Our recent study dealing with the distribution of G proteins in the developing rat myocardium has shown that substantial amounts of α subunits of G_s, G_i/G_o, and G_q proteins occur in the cytosol fraction under control conditions [87]. A cytosolic localization of various G protein α subunits has also been reported in human platelets. Whereas membrane-bound $G_s\alpha$, $G_i\alpha$, and $G_q\alpha$ prevailed over their cytosolic forms, platelet $G_{12}\alpha$ and $G_{16}\alpha$ were predominantly localized in the cytosol [88].

Membrane association and soluble forms of G$\beta\gamma$ subunits

G protein γ subunits undergo post-translational modification by isoprenoid geranylgeranyl (C20) or farnesyl (C15) groups [36, 89, 90]. These covalent modifications certainly contribute to the hydrophobic character of the $\beta\gamma$ subunit dimer and support the firm association of the whole (trimeric) G protein complex with the membrane lipid bilayer [91, 92]. An essential role of prenylation in targeting $\beta\gamma$ to the plasma membrane was demonstrated in experiments with lovastatin. After prolonged treatment of Neuro 2A or PC12 cells with this isoprenylation-blocking drug, a substantial portion of G$\beta\gamma$ was found in the cytoplasm, compared with 100% membrane-bound G$\beta\gamma$ in control cells [91, 93]. Further analysis revealed that beside the isoprenoid moiety, γ subunits bear a methyl group at the C terminus [94]. The carboxylmethylation of the γ subunits does not enhance membrane attachment of G$\beta\gamma$ but is required for interaction with α subunits and optimal G protein-mediated signaling [95].

Based on the results outlined above, G protein $\beta\gamma$ subunits are generally considered as tightly membrane-bound proteins. The only exception is transducin $\beta\gamma_t$, which is soluble [96]. In accordance with this view, G$\beta\gamma$ subunits remained membrane bound in isoproterenol-stimulated S49 lymphoma cells [97] or in receptor-transfected CHO and HEK cell lines which were drastically stimulated by carbachol and thyrotropin-releasing hormone (TRH), respectively [47, 48]. Nevertheless, there are two literature reports claiming the detection of Gβ subunits in the soluble (cytosol) fraction of myocardial cells. A predominant localization of $G\beta_1$ and $G\beta_3$ in the cytosol fraction of rat ventricles was reported by Muramoto and co-workers [98]. A dramatic decrease in cytosolic $G\beta_3$ and concomitant increase in

membrane-associated $G\beta_3$ as a consequence of rat heart perfusion with isoproterenol or epinephrine was detected by Kageyama [99]. The influence of isoproterenol stimulation on the subcellular localization of $G\beta_3$ appeared to be highly selective, because the distribution of $G\beta_1$ and $G\beta_2$ was unchanged under these conditions.

Complex consequences of agonist-induced depletion of the plasma membrane pool of G proteins: internalization, solubilization, and down-regulation

Long-term stimulation of G proteins results in down-regulation [100–103], i.e., a decrease in the total cellular amount of these proteins. Although not examined as widely as the agonist-induced decrease of GPCR levels, down-regulation of G protein α subunits has been observed in a number of systems [for a review see ref. 104]. In general, this phenomenon requires extended periods of cellular exposure to the agonist, it is restricted to the G protein(s) expected to be activated by the agonist-GPCR complex, and it requires cells or tissues which express relatively high levels of the appropriate GPCRs. The last point can be understood on the basis that the cellular levels of G proteins are usually considerably higher (molar excess of several orders) than those of any particular GPCR [105]. Thus, even when considering the capacity of an agonist-occupied GPCR to catalytically activate the G protein population, the fraction of the cellular pool of a specific G protein which becomes activated by a GPCR is likely to be relatively small unless GPCR levels are very high (similar to G proteins). In the studies which have examined the mechanisms responsible for agonist-mediated reduction in cellular G protein levels, an enhanced rate of degradation was routinely observed for the cognate G protein but not for other cellular G proteins which were not activated by that agonist [54, 100, 106, 107]. In contrast, relatively few studies have provided evidence for decreased rates of G protein synthesis or reduction in mRNA levels [104].

As proteolytic degradation proceeds inside the cell, some mechanism must transport, i.e., internalize, proteins into the cell interior. Even in the case of widely studied GPCRs, whether sequestration and internalization is or is not a prerequisite for agonist-induced down-regulation was not clear for a relatively long time. The direct evidence for internalization of GPCRs was missing. The process of sequestration, in which receptors remain detectable by lipophilic ligands but become increasingly inaccessible to hydrophilic, membrane-impermeable ligands was proposed to represent internalization of receptors into an intracellular compartment(s) and to serve as a prerequisite for down-regulation [44, 108, 109]. Another piece of indirect evidence for internalization of GPCRs was based on subcellular fractionation studies. Since the early 1980s prolonged agonist stimulation was known to induce a shift of GPCRs from plasma membranes to the low-density or light-vesicular membrane fractions distinct from plasma membranes [19–21, 44]. This transfer was interpreted as evidence for internalization of GPCRs. The first direct demonstration of internalization of GPCRs, however, was obtained much later by confocal fluorescence microscopy by Zastrow and Kobilka [110, 111] and has been subsequently replicated for a variety of other receptors [112–117].

The same sequence in development of experimental evidence (i.e., from subcellular fractionation studies to confocal fluorescent microscopy) can be traced in the course of demonstrating the internalization of trimeric G proteins. After the pioneering work by Haraguchi and Rodbell [45], isoproterenol stimulation of S49 lymphoma cells was found to transfer $G_s\alpha$ as well as $G\beta$ from plasma membrane to light-vesicular, low-density membrane fractions [46, 97]. Direct evidence for internalization of $G_s\alpha$ was brought by immunofluorescence analysis of doubly transfected HEK-293 cells (β_2-adrenergic receptor and $G_s\alpha$) which indicated internalization of $G_s\alpha$ protein as a consequence of isoproterenol stimulation [118]. Similarly, prolonged agonist (carbachol) stimulation of CHO cells transfected with hm1 receptor resulted in transfer of $G_q\alpha/G_{11}\alpha$ from plasma membrane fractions to low-density vesicles isolated on sucrose density gradients [47]. Subsequent study on HEK-293 cells showed clearly that transfer of G protein from plasma membranes to light vesicles, solubilization, and down-regulation do not represent mutually exclusive phenomena (as originally thought), but may exist simultaneously in a single cell type [48]. Prolonged agonist stimulation of HEK-293 cells doubly transfected with TRH receptor (TRH-R) and $G_{11}\alpha$ resulted in (i) dramatic transfer of both exogenously and endogenously expressed $G_{11}\alpha$ from plasma membrane to low-density membrane fragments, (ii) transfer from plasma membrane fractions to a high-speed (250,000 g) supernatant (solubilization), and (iii) decrease in total cellular amount of these proteins (down-regulation) [48]. Subsequent immunofluorescent analysis of this cell line demonstrated internalization of $G_{11}\alpha$ protein as depletion of surface signal combined with the appearance of distinct patches of fluorescent material inside the cell (endosomes) (fig. 1). Simultaneously, a diffuse signal appeared distributed randomly along the cell interior, which might be correlated with the solubilized pool of G protein. Thus, the results of fluorescence microscopy of intact cells correlated closely with results of subcellular fractionation studies performed with cellular homogenates and fractionation on sucrose density gradients [49]. Agonist-induced internalization of $G_q\alpha/G_{11}\alpha$ proteins has also been demonstrated in MDCK cells after stimulation by bradykinin [75].

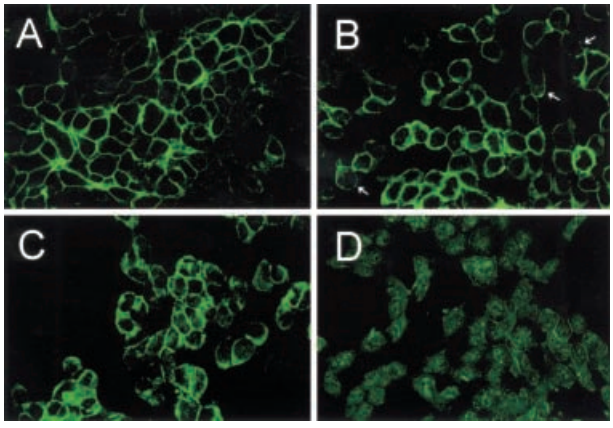

Figure 1. Agonist-induced subcellular redistribution of trimeric G proteins $G_q\alpha/G_{11}\alpha$. HEK cells double transfected with thyrotropin-releasing hormone (TRH) receptor and $G_{11}\alpha$ protein were exposed to TRH and $G_q\alpha/G_{11}\alpha$ proteins detected by indirect immunofluorescence with FITC-labeled secondary antibodies. (*A, B*) Clustering of plasma membrane-associated $G_q\alpha/G_{11}\alpha$ proteins after short-term agonist stimulation. The fluorescence signal distributed uniformly across the cell surface of control cells (*A*) appeared to cluster into discrete patches (arrows) of fluorescent material after 10 min of agonist (TRH) exposure (*B*). (*C, D*) Internalization and down-regulation of $G_q\alpha/G_{11}\alpha$ after prolonged agonist stimulation. Further incubation (for 3 h) of the cells with TRH resulted in transfer of plasma membrane-localized $G_q\alpha/G_{11}\alpha$ immunofluorescence to the cell interior (*C*). Long-term (16 h) treatment with TRH led to a decrease of total immunofluorescence signal, i.e., down-regulation (*D*).

G proteins and their cognate GPCRs are internalized via different pathways

Surprisingly, internalization of G proteins has been found to follow different pathway(s) when compared with their cognate GPCRs [49, 118]. Receptor-activated $G_s\alpha$ did not co-localize with internalized β_2 adrenergic receptor (β_2AR) in endosomes and incubation of cells in hypertonic sucrose to inhibit clathrin-coated pit-mediated endocytosis of β_2AR failed to block isoproterenol-induced internalization of $G_s\alpha$ [118]. In the case of $G_q\alpha/G_{11}\alpha$, internalization was not manifested before 1 h of continuous agonist (TRH) stimulation. Significant transfer to the cell interior was detected between 2 and 4 h and the longer time intervals of TRH stimulation resulted in a decrease of total immunofluorescence signal, i.e. down-regulation [49]. Contrarily, internalization of the cognate receptor (TRH-R) proceeded within minutes, and its characteristics agreed with the clathrin-dependent pathway [119–121].

The difference between TRH-R and $G_{11}\alpha$ internalization pathways was subsequently verified under in vivo conditions in cells expressing VSV-epitope-tagged TRH-R-GFP fusion protein [73]. The fluorescent version of TRH-R was rapidly transferred into the cell interior, while $G_q\alpha/G_{11}\alpha$ proteins, identified by indirect fluorescence, remained at the cell surface. This situation persisted for more than 1 h. At longer time periods, a fraction of plasma membrane $G_q\alpha/G_{11}\alpha$ was also internalized. As before, the results of confocal fluorescent microscopy agreed well with an immunoblot analysis of subcellular fractions. A portion of VSV-TRH-R-GFP fusion protein (detected by GFP- or VSV-directed antibodies) was transferred from plasma membrane to a light-vesicular fraction within 30 min of TRH stimulation, but distribution of $G_q\alpha/G_{11}\alpha$ was unchanged. At longer time intervals, redistribution of G proteins to light-vesicular and soluble fractions occurred as well [73]. The independent nature of GPCR and G protein internalization pathways has also been demonstrated for oxotremorine-induced redistribution of muscarinic receptors and $G_q\alpha/G_{11}\alpha$ [122]. Interestingly, hormone-induced internalization of TRH-R was absent in embryonic fibroblasts prepared from mice lacking $G_q\alpha/G_{11}\alpha$ [123].

Hormone-induced solubilization of G proteins: reality or artifact?

Molecular mechanisms underlying hormonal regulation of membrane attachment of $G_s\alpha$ have recently been proposed to involve reciprocal regulation by palmitate and $\beta\gamma$ subunits [42]. According to this view (fig. 2), hormonal activation promotes dissociation of $G_s\alpha$ from $G\beta\gamma$, accelerates removal of covalently attached palmitate and finally triggers release of $G_s\alpha$ from plasma membranes to the cytosol. Contrarily, $G\beta\gamma$ binding and palmitoylation reciprocally potentiate each other in promoting membrane attachment of $G_s\alpha$. Hormone-induced dissociation of $G_s\alpha$-GTP from $G\beta\gamma$ thus mediates depalmitoylation and translocation of the $G_s\alpha$ protein to the cytoplasm [42].

The membrane-cytosol shift of $G\alpha$ subunits, if proceeding as suggested above [42], should already be detectable after short-term G protein activation. Such a rapid release, however, was not detected in the case of $G_s\alpha$ [46, 124], $G_i\alpha$ [124], or $G_q\alpha/G_{11}\alpha$ proteins [47]. G protein α subunits remained firmly attached to particulate membrane structures after short-term treatment with appropriate agonists. The difficulties in accepting the simple 'activation = solubilization' hypothesis may be further documented by the presence of constitutively active and depalmitoylated α subunits in membrane fractions of MA-104 and HEK-293 cells [124].

When trying to find some rational basis for these discrepancies, it should be stressed that this sort of experiment is difficult to perform without major artificial alterations of the plasma membrane structure, compared with the native, 'hormone-relevant' state of intact cells. This alteration is an unavoidable consequence of the subcellular fractionation procedure. The first potential source of artifacts is time. Hormonal exposure proceeding for a few minutes is much shorter than the 1–2 h of high-speed

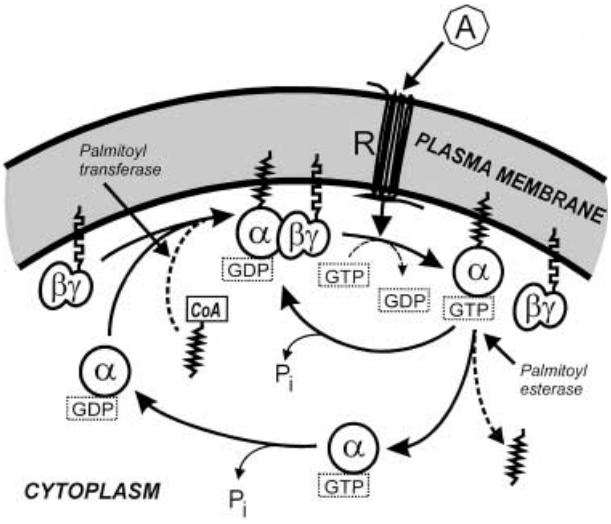

Figure 2. Membrane association of Gα and the palmitoylation-depalmitoylation cycle [modified from ref. 42]. In the resting state, α_{GDP} associates with $\beta\gamma$ and the plasma membrane. Palmitoylation enhances affinity of α for $\beta\gamma$ and strengthens its attachement to the lipid bilayer. $\beta\gamma$ promotes the palmitoylated state by enhancing palmitoylation catalyzed by palmitoyl transferase and by protecting α from attack by palmitoyl esterase. Stimulation of a receptor ('R') by agonist ('A') triggers activation of its cognate G protein, which results in dissociation of activated α_{GTP} from $\beta\gamma$. Free $\beta\gamma$ remains at the membrane by virtue of its isoprenyl group, but free α_{GTP} is rapidly depalmitoylated by palmitoyl esterase and released from the membrane. Intrinsic GTPase activity converts α_{GTP} to α_{GDP} (exhibiting high affinity for $\beta\gamma$), α_{GDP} returns to the plasma membrane by re-association with $\beta\gamma$ and the palmitoylation state is restored.

centrifugation needed for separation of the particulate (membrane) and soluble (cytosol) fractions. The second source of artifacts is homogenization which by itself causes: (i) degradation of intact plasma membrane structure, (ii) changes in association of plasma membrane components with adjacent intracellular structures such as cytoskeleton, (iii) dilution of plasma membrane fragments into the large volumes of homogenization media, and (iv) spontaneous formation of small vesicles originally derived from large plasma membrane sheets.

The third source of difference between native and isolated plasma membrane is a temperature shift from 37 to 0–4 °C and a high hydrostatic pressure affecting the membrane state in the course of isolation and high-speed centrifugation. All these factors, combined and individually, may alter the membrane association of peripheral proteins such as G protein α subunits. One can therefore logically assume that the fast release of $G_s\alpha$ from plasma membranes to a high-speed supernatant (soluble fraction) in intact cells shown by Iiri and co-workers [42], and solubilization of $G_s\alpha$ assessed after homogenization and long-term high-speed centrifugation need not represent the same phenomenon because the experimental systems and conditions differ.

Domain-bound G proteins: a source of plasticity in G protein-plasma membrane interactions?

When considering the above arguments, in full agreement with the ideas of Iiri and co-workers [42] and with experimental findings of other investigators [46, 47, 124], the suggestion can be made that short-term agonist exposure reversibly alters (through the depalmitoylation-palmitoylation cycle) the distribution of G proteins among various plasma membrane compartments which differ in sensitivity toward homogenization and/or subsequent steps involved in separation of the particulate (membrane) and cytosol (soluble) fractions. Depending on the cell type, characteristics of hormone action (such as receptor number, agonist concentration, and time of exposure), and any details of subcellular fractionation procedure, the less firmly associated G protein pools may be solubilized and recovered in a high-speed supernatant.

A heterogeneous distribution of G proteins in plasma membranes has indeed been demonstrated both in intact cells [49], detergent-resistant [125], and sonicated plasma membrane fragments [126]. In intact doubly transfected HEK-293 cells (TRH-R and $G_{11}\alpha$), localization of $G_{11}\alpha$ was restricted exclusively to plasma membranes within 10–60 min of agonist stimulation [49]. Nevertheless, a fraction of $G_{11}\alpha$ fluorescence signal appeared to cluster to discrete segregated patches of the plasma membrane (compared with the largely homogeneous plasma membrane distribution prior to addition of TRH) after 10 min of TRH exposure (fig. 1). Accordingly, in detergent extracts of these cells, $G_q\alpha/G_{11}\alpha$ proteins were distributed in a wide range of low-density membrane domains isolated by flotation in sucrose density gradients as well as in the 'bulk membrane phase' located in the non-floating, high-density area of the gradient [125]. Sustained hormone stimulation of these cells induced a significant shift of G proteins from detergent-insensitive membrane domains to the bulk membrane phase. In S49 lymphoma and MDCK cells, $G\alpha_i$, $G\alpha_o$, and $G\beta$ were distributed over a wide range of sonicated membrane fragments isolated by flotation on OptiPrep density gradients [126].

Over the past few years, targeting of different GPCRs, G proteins, and other signaling molecules to plasma membrane microdomains has been described for a number of cell lines and tissues [127–133]. A critical role of lipid modifications of G proteins for their recruitment and partitioning into these microdomains has recently been evaluated [134]. At least two distinct types of microdomains can be distiguished morphologically, biochemically, and functionally – lipid rafts and caveolae [135, 136]. These membrane structures may serve to compartmentalize signals, thereby optimizing signal transduction between an agonist and specific effectors [137]. In addition, such a compartmentation of G protein-mediated transmembrane signaling might play an impor-

tant role in desensitization of the hormone response [125, 138].

In conclusion

All the above-gathered literature data and views dealing with the hormone-induced subcellular redistribution of trimeric G proteins along with some other speculative ideas for future research have inspired us to formulate the following model.

In the resting state, the entire population of plasma membrane-bound G protein α subunits is palmitoylated, associated with $\beta\gamma$, and localized both in the bulk membrane phase and membrane microdomains. Under these conditions, membrane microdomains are small and not visible by light microscopy. G proteins in microdomains are restricted in their lateral motion by protein-protein interactions with other proteins present in these structures (as suggested by Neubig [139]), while G proteins in the bulk membrane phase are freely mobile, as assumed by the collisional coupling hypothesis [140].

Under physiological conditions of G protein activation (low agonist concentration, low receptor number, short-term exposure), signal transduction is preferentially realized through the G proteins localized in plasma membrane microdomains ('signalosomes') packed with signaling molecules. In this case, a relatively small portion of Gα is depalmitoylated and freed from $\beta\gamma$. The depalmitoylated α subunits, however, are not released from the plasma membrane and remain in the same place (microdomain) as in the resting state. Caveolin (a major structural protein of caveolae) might directly bind and stabilize Gα within caveolae [136, 141]. Thus, not only the specific lipid composition of membrane microdomains [134], but also protein-protein interactions can protect the free, depalmitoylated Gα from being solubilized. This rather labile arrangement may be affected by homogenization and/or membrane isolation procedures and, therefore, diverse results might be obtained under different experimental conditions (see discussion above). The freely mobile pool of G proteins in the bulk membrane phase is not activated under physiological conditions because of the low probability of efficient collisional coupling to activated receptors (spare receptors?), and there is no overall change in the cellular distribution of G proteins under these conditions.

In contrast, drastic stimulation (high agonist concentration, high receptor number, long-term exposure) alters the cellular distribution of G proteins. Under these conditions, plasma membrane microdomains associate into larger clusters visible by light microscopy [49]. Subsequently, these clustered formations are separated from the membrane in the form of small (light) vesicles and internalized. In parallel, the randomly distributed G proteins in the bulk membrane phase also become activated by collisional coupling to activated receptors. The activated Gα from this pool, however, is not stabilized by protein-protein interactions and the specific lipid environment of the microdomain. In this case, depalmityolated G protein α subunits are released from the plasma membrane and may be recovered in the soluble cell fraction. Some of these speculations are outlined in figure 3.

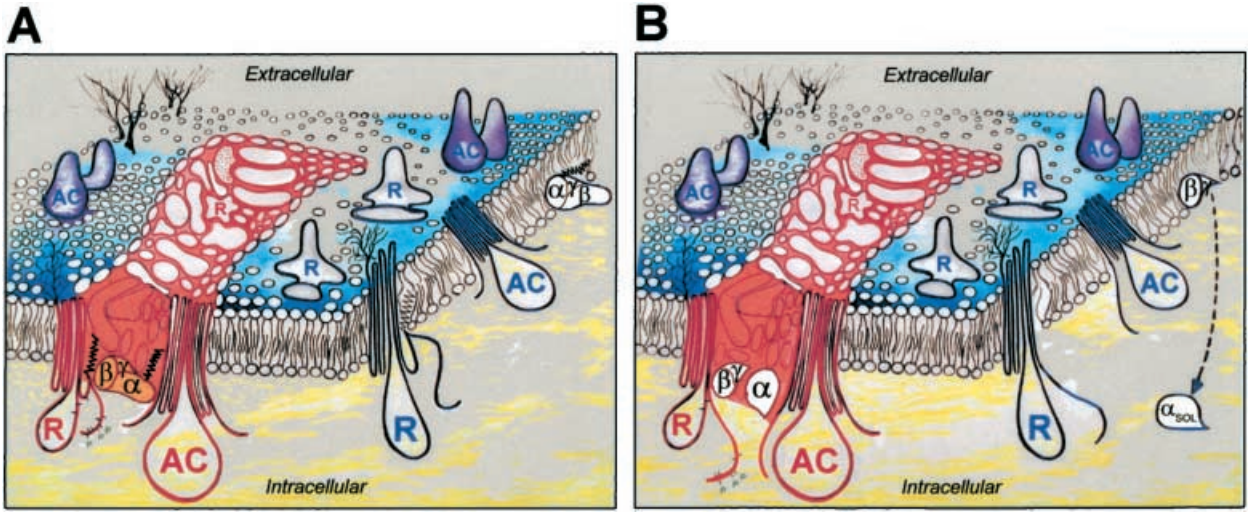

Figure 3. A simplified model of membrane organization of trimeric G proteins and agonist-induced solubilization. In the resting state (*A*), G proteins as well as GPCRs are distributed both in the bulk membrane phase (depicted in blue) and membrane microdomains (depicted in red). Upon mild stimulation by an agonist, activated α and $\beta\gamma$ are separated but still remain in plasma membrane microdomain (*B, left*). Contrarily, drastic stimulation may cause release of a substantial portion of G protein α subunits into the cytosol (*B, right*). R, G protein-coupled receptor; α, β, and γ, subunits of a G protein; AC, adenylyl cyclase; α_{SOL}, solubilized (cytosolic) form of Gα; /\/\/\/\/\, palmitic acid; P, sites of potential phosphorylation.

Taken together, experimental evidence demonstrating the subcellular redistribution of G protein subunits as a consequence of agonist stimulation might well support the notion for a potential role of G proteins in the process of desensitization. Regulation of cellular G protein levels and localization can obviously provide an appropriate locus to generate heterologous desensitization and thus contribute to the control of cellular sensitivity.

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1 Rodbell M. (1980) The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature **284:** 17–22
2 Gilman A. G. (1987) G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. **56:** 615–649
3 Birnbaumer L., Abramowitz J. and Brown A. M. (1990) Receptor-effector coupling by G proteins. Biochim. Biophys. Acta **1031:** 163–224
4 Neer E. J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. Cell **80:** 249–257
5 Helmreich J. M. and Hofmann K. P. (1996) Structure and function of poteins in G-protein coupled signal transfer. Biochim. Biophys. Acta **1286:** 285–322
6 Hamm H. E. (1998) The many faces of G protein signaling. J. Biol. Chem. **273:** 669–672
7 Baldwin J. M. (1994) Structure and function of receptors coupled to G proteins. Curr. Opin. Cell Biol. **6:** 180–190
8 Ji T. H., Grossmann M. and Ji I. (1998) G protein-coupled receptors. I. Diversity of receptor-ligand interactions. J. Biol. Chem. **273:** 17299–17302
9 Birnbaumer L. (1992) Receptor-to-effector signaling through G proteins: roles for $\beta\gamma$ dimers as well as α subunits. Cell **71:** 1069–1072
10 Hildebrant J. D. (1997) Role of subunit diversity in signalling by heterotrimeric G proteins. Biochem. Pharmacol. **54:** 325–339
11 Wieland T. and Chen C. K. (1999) Regulators of G-protein signalling: a novel protein family involved in timely deactivation and desensitization of signalling via heterotrimeric G proteins. Naunyn Schmiedebergs Arch. Pharmacol. **360:** 14–26
12 Burchett S. A. (2000) Regulators of G protein signaling: a bestiary of modular protein binding domains. J. Neurochem. **75:** 1335–1351
13 Grady E. F., Bohm S. K. and Bunnett N. W. (1997) Turning off the signal: mechanisms that attenuate signaling by G protein-coupled receptors. Am. J. Physiol. **273:** G586–G601
14 Ferguson S. S., Zhang J., Barak L. S. and Caron M. G. (1998) Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. Life Sci. **62:** 1561–1565
15 Clark R. B., Knoll B. J. and Barber R. (1999) Partial agonists and G protein-coupled receptor desensitization. Trends Pharmacol. Sci. **20:** 279–286
16 Northup J. K., Sternweis P. C., Smigel M. D., Schleifer L. S., Ross E. M. and Gilman A. G. (1980) Purification of the regulatory component of adenylate cyclase. Proc. Natl. Acad. Sci. USA **77:** 6516–6520
17 Hanski E., Sternweis P. C., Northup J. K., Dromerick A. W. and Gilman A. G. (1981) The regulatory component of adenylate cyclase: purification and properties of the turkey erythrocyte protein. J. Biol. Chem. **256:** 12911–12919
18 Bokoch G. M., Katada T., Northup J. K., Ui M. and Gilman A. G. (1984) Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. J. Biol. Chem. **259:** 3560–3567
19 Waldo G. L., Northup J. K., Perkins J. P. and Harden T. K. (1983) Characterization of an altered membrane form of the β-adrenergic receptor produced during agonist-induced desensitization. J. Biol. Chem. **258:** 13900–13908
20 Strader C. D., Sibley D. R. and Lefkowitz R. J. (1984) Association of sequestered β-adrenergic receptors with the plasma membrane: a novel mechanism for receptor down regulation. Life Sci. **35:** 1601–1610
21 Clark R. B., Friedman J., Prashad N. and Ruoho A. E. (1985) Epinephrine-induced sequestration of the β-adrenergic receptor in cultured S49 WT and cyc$^-$ lymphoma cells. J. Cyclic Nucleotide Protein Phosphor. Res. **10:** 97–119
22 Sternweis P. C. (1986) The purified α subunits of G_o and G_i from bovine brain require $\beta\gamma$ for association with phospholipid vesicles. J. Biol. Chem. **261:** 631–637
23 Simonds W. F., Collins R. M., Spiegel A. M. and Brann M. R. (1989) Membrane attachment of recombinant G-protein α-subunits in excess of $\beta\gamma$ subunits in a eukaryotic expression system. Biochem. Biophys. Res. Commun. **164:** 46–53
24 Buss J. E., Mumby S. M., Casey P. J., Gilman A. G. and Sefton B. M. (1987) Myristoylated α subunits of guanine nucleotide-binding regulatory proteins. Proc. Natl. Acad. Sci. USA **84:** 7493–7497
25 Mumby S. M., Heukeroth R. O., Gordon J. I. and Gilman A. G. (1990) G-protein α-subunit expression, myristoylation, and membrane association in COS cells. Proc. Natl. Acad. Sci. USA **87:** 728–732
26 Spiegel A. M., Backlund P. S. Jr, Butrynski J. E., Jones T. L. and Simonds W. F. (1991) The G protein connection: molecular basis of membrane association. Trends Biochem. Sci. **16:** 338–341
27 Grassie M. A., McCallum J. F., Guzzi F., Magee A. I., Milligan G. and Parenti M. (1994) The palmitoylation status of the G-protein Go1α regulates its activity of interaction with the plasma membrane. Biochem. J. **302:** 913–920
28 Degtyarev M. Y., Spiegel A. M. and Jones T. L. (1993) The G protein α_s subunit incorporates [^{3}H]palmitic acid and mutation of cysteine-3 prevents this modification. Biochemistry **32:** 8057–8061
29 Wedegaertner P. B., Chu D. H., Wilson P. T., Levis M. J. and Bourne H. R. (1993) Palmitoylation is required for signaling functions and membrane attachment of Gqα and Gsα. J. Biol. Chem. **268:** 25001–25008
30 Mumby S. M., Kleuss C. and Gilman A. G. (1994) Receptor regulation of G-protein palmitoylation. Proc. Natl. Acad. Sci. USA **91:** 2800–2804
31 Hepler J. R., Biddlecome G. H., Kleuss C., Camp L. A., Hofmann S. L., Ross E. M. et al. (1996) Functional importance of the amino terminus of Gqα. J. Biol. Chem. **271:** 496–504
32 Gallego C., Gupta S. K., Winitz S., Eisfelder B. J. and Johnson G. L. (1992) Myristoylation of the Gαi2 polypeptide, a G protein α subunit, is required for its signaling and transformation functions. Proc. Natl. Acad. Sci. USA **89:** 9695–9699
33 Galbiati F., Guzzi F., Magee A. I., Milligan G. and Parenti M. (1994) N-terminal fatty acylation of the α-subunit of the G-protein Gi1: only the myristoylated protein is a substrate for palmitoylation. Biochem. J. **303:** 697–700
34 Galbiati F., Guzzi F., Magee A. I., Milligan G. and Parenti M. (1996) Chemical inhibition of myristoylation of the G-protein Gi1α by 2-hydroxymyristate does not interfere with its palmitoylation or membrane association: evidence that palmitoylation, but not myristoylation, regulates membrane attachment. Biochem. J. **313:** 717–720
35 Wilson P. T. and Bourne H. R. (1995) Fatty acylation of α_z: effects of palmitoylation and myristoylation on α_z signaling. J. Biol. Chem. **270:** 9667–9675

36 Mumby S. M., Casey P. J., Gilman A. G., Gutowski S. and Sternweis P. C. (1990) G protein γ subunits contain a 20-carbon isoprenoid. Proc. Natl. Acad. Sci. USA **87:** 5873–5877

37 Jones T. L., Simonds W. F., Merendino J. J. Jr, Brann M. R. and Spiegel A. M. (1990) Myristoylation of an inhibitory GTP-binding protein α subunit is essential for its membrane attachment. Proc. Natl. Acad. Sci. USA **87:** 568–572

38 Linder M. E., Pang I. H., Duronio R. J., Gordon J. I., Sternweis P. C. and Gilman A. G. (1991) Lipid modifications of G protein subunits: myristoylation of Goα increases its affinity for $\beta\gamma$. J. Biol. Chem. **266:** 4654–4659

39 Hallak H., Brass L. F. and Manning D. R. (1994) Failure to myristoylate the α subunit of Gz is correlated with an inhibition of palmitoylation and membrane attachment, but has no effect on phosphorylation by protein kinase C. J. Biol. Chem. **269:** 4571–4576

40 Wedegaertner P. B., Wilson P. T. and Bourne H. R. (1995) Lipid modifications of trimeric G proteins. J. Biol. Chem. **270:** 503–506

41 Ross E. M. (1995) Protein modification: palmitoylation in G-protein signaling pathways. Curr. Biol. **5:** 107–109

42 Iiri T., Backlund P. S. Jr, Jones T. L., Wedegaertner P. B. and Bourne H. R. (1996) Reciprocal regulation of Gsα by palmitate and the $\beta\gamma$ subunit. Proc. Natl. Acad. Sci. USA **93:** 14592–14597

43 Mumby S. M. (1997) Reversible palmitoylation of signaling proteins. Curr. Opin. Cell Biol. **9:** 148–154

44 Hertel C., Coulter S. J. and Perkins J. P. (1985) A comparison of catecholamine-induced internalization of β-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells: inhibition by phenylarsine oxide. J. Biol. Chem. **260:** 12547–12553

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

46 Kvapil P., Novotny J., Svoboda P. and Ransnas L. A. (1994) The short and long forms of the α subunit of the stimulatory guanine nucleotide-binding protein are unequally redistributed during (–)-isoproterenol-mediated desensitization of intact S49 lymphoma cells. Eur. J. Biochem. **226:** 193–199

47 Svoboda P. and Milligan G. (1994) Agonist-induced transfer of the α subunits of the guanine-nucleotide-binding regulatory proteins Gq and G11 and of muscarinic m1 acetylcholine receptors from plasma membranes to a light-vesicular membrane fraction. Eur. J. Biochem. **224:** 455–462

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

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

50 Rodbell M. (1985) Programmable messengers: a new theory of hormone action. Trends Biochem. **10:** 461–464

51 Chabre M. (1987) The G protein connection: is it the membrane or the cytoplasm? Trends Biochem. **12:** 213–215

52 Chabre M. and Deterre P. (1989) Molecular mechanism of visual transduction. Eur. J. Biochem. **179:** 255–266

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

54 Levis M. J. and Bourne H. R. (1992) Activation of the α subunit of Gs in intact cells alters its abundance, rate of degradation, and membrane avidity. J. Cell Biol. **119:** 1297–1307

55 Wedegaertner P. B. and Bourne H. R. (1994) Activation and depalmitoylation of Gsα. Cell **77:** 1063–1070

56 Neubert T. A., Johnson R. S., Hurley J. B. and Walsh K. A. (1992) The rod transducin α subunit amino terminus is heterogeneously fatty acylated. J. Biol. Chem. **267:** 18274–18277

57 Johnson R. S., Ohguro H., Palczewski K., Hurley J. B., Walsh K. A. and Neubert T. A. (1994) Heterogeneous N-acylation is a tissue- and species-specific posttranslational modification. J. Biol. Chem. **269:** 21067–21071

58 Lynch C. J., Morbach L., Blackmore P. F. and Exton J. H. (1986) Alpha-subunits of Ns are released from the plasma membrane following cholera toxin activation. FEBS Lett. **200:** 333–336

59 McArdle H., Mullaney I., Magee A., Unson C. and Milligan G. (1988) GTP analogues cause release of the α subunit of the GTP binding protein, GO, from the plasma membrane of NG108-15 cells. Biochem. Biophys. Res. Commun. **152:** 243–251

60 Milligan G., Mullaney I., Unson C. G., Marshall L., Spiegel A. M. and McArdle H. (1988) GTP analogues promote release of the α subunit of the guanine nucleotide binding protein, Gi2, from membranes of rat glioma C6 BU1 cells. Biochem. J. **254:** 391–396

61 Milligan G. and Unson C. G. (1989) Persistent activation of the α subunit of Gs promotes its removal from the plasma membrane. Biochem. J. **260:** 837–841

62 Milligan G., Unson C. G. and Wakelam M. J. (1989) Cholera toxin treatment produces down-regulation of the α-subunit of the stimulatory guanine-nucleotide-binding protein (Gs). Biochem. J. **262:** 643–649

63 Carr C., Loney C., Unson C., Knowler J. and Milligan G. (1990) Chronic exposure of rat glioma C6 cells to cholera toxin induces loss of the α-subunit of the stimulatory guanine nucleotide-binding protein (Gs). Eur. J. Pharmacol. **188:** 203–209

64 Ransnas L. A., Leiber D. and Insel P. A. (1991) Inhibition of subunit dissociation and release of the stimulatory G-protein, Gs, by $\beta\gamma$-subunits and somatostatin in S49 lymphoma cell membranes. Biochem. J. **280:** 303–307

65 Ransnas L. A., Jasper J. R., Leiber D. and Insel P. A. (1992) β-Adrenergic-receptor-mediated dissociation and membrane release of the Gs protein in S49 lymphoma-cell membranes: dependence on Mg^{2+} and GTP. Biochem. J. **283:** 519–524

66 Negishi M., Hashimoto H. and Ichikawa A. (1992) Translocation of α subunits of stimulatory guanine nucleotide-binding proteins through stimulation of the prostacyclin receptor in mouse mastocytoma cells. J. Biol. Chem. **267:** 2364–2369

67 Yajima Y., Akita Y., Saito T. and Kawashima S. (1998) VIP induces the translocation and degradation of the α subunit of Gs protein in rat pituitary GH4C1 cells. J. Biochem. (Tokyo) **123:** 1024–1030

68 Witte K., Schnecko A. and Lemmer B. (1999) Agonist-induced release of splice variants of the α subunit of the stimulatory G-protein from rat cardiac membranes. Biochem. Pharmacol. **57:** 539–543

69 Takahashi S., Hashida K., Yatsunami K., Fukui T., Negishi M., Katada T. et al. (1991) Characterization of cytosolic pertussis toxin-sensitive GTP-binding protein in mastocytoma P-815 cells. Biochim. Biophys. Acta **1093:** 207–215

70 Takahashi S., Negishi M. and Ichikawa A. (1991) Cytosol promotes the guanine nucleotide-induced release of the α subunit of Gi2 from the membrane of mouse mastocytoma P-815 cells. J. Biol. Chem. **266:** 5367–5370

71 Takahashi S., Negishi M., Ohnishi M. and Ichikawa A. (1992) Involvement of protein kinase C in thrombin-induced translo-

cation of Gi2α from the membrane to the cytosol in mouse mastocytoma P-815 cells. Biochim. Biophys. Acta **1134:** 17–24

72 Yajima Y., Akita Y., Katada T. and Saito T. (1993) Somatostatin induces release of the α subunits of pertussis toxin-sensitive G proteins in native membranes and in intact GH4C1 rat pituitary cells. Mol. Cell. Endocrinol. **92:** 143–152

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

74 Ravindra R. (1997) Is signal transduction modulated by an interaction between heterotrimeric G-proteins and tubulin? Endocrine **7:** 127–143

75 Arthur J. M., Collinsworth G. P., Gettys T. W. and Raymond J. R. (1999) Agonist-induced translocation of $G_q/_{11}\alpha$ immunoreactivity directly from plasma membrane in MDCK cells. Am. J. Physiol. **276:** F528–F534

76 Roth D. A., Urasawa K., Leiber D., Insel P. A. and Hammond H. K. (1992) A substantial proportion of cardiac Gs is not associated with the plasma membrane. FEBS Lett. **296:** 46–50

77 Novotny J., Gustafson B., Kvapil P. and Ransnas L. A. (1994) Adenovirus infection of myocardial cells induces an enhanced sensitivity to β-adrenergic agonists by increasing the concentration of the stimulatory G-protein. Biochem. Mol. Biol. Int. **34:** 993–1001

78 Novotny J., Bourova L., Malkova O., Svoboda P. and Kolar F. (1999) G proteins, β-adrenoreceptors and β-adrenergic responsiveness in immature and adult rat ventricular myocardium: influence of neonatal hypo- and hyperthyroidism. J. Mol. Cell. Cardiol. **31:** 761–772

79 Ihnatovych I., Hejnova L., Kostrnova A., Mares P., Svoboda P. and Novotny J. (2001) Maturation of rat brain is accompanied by differential expression of the long and short splice variants of Gsα protein: identification of cytosolic forms of Gsα. J. Neurochem. **79:** 88–97

80 Bokoch G. M., Parkos C. A. and Mumby S. M. (1988) Purification and characterization of the 22,000-dalton GTP-binding protein substrate for ADP-ribosylation by botulinum toxin, G22K. J. Biol. Chem. **263:** 16744–16749

81 Volpp B. D., Nauseef W. M. and Clark R. A. (1989) Subcellular distribution and membrane association of human neutrophil substrates for ADP-ribosylation by pertussis toxin and cholera toxin. J. Immunol. **142:** 3206–3212

82 Rudolph U., Koesling D., Hinsch K. D., Seifert R., Bigalke M., Schultz G. et al. (1989) G-protein α-subunits in cytosolic and membranous fractions of human neutrophils. Mol. Cell. Endocrinol. **63:** 143–153

83 Rudolph U., Schultz G. and Rosenthal W. (1989) The cytosolic G-protein α-subunit in human neutrophils responds to treatment with guanine nucleotides and magnesium. FEBS Lett. **251:** 137–142

84 Hamilton S. E. and Nathanson N. M. (1997) Differential localization of G-proteins, Gαo and Gαi-1, -2, and -3, in polarized epithelial MDCK cells. Biochem. Biophys. Res. Commun. **234:** 1–7

85 Root P. A., Prince A. and Gundersen R. E. (1999) Aggregation of *Dictyostelium discoideum* is dependent on myristoylation and membrane localization of the G protein α-subunit, Gα2. J. Cell. Biochem. **74:** 301–311

86 Cadrin M., McFarlane-Anderson N., Harper M. E., Gaffield J. and Begin-Heick N. (1996) Comparison of the subcellular distribution of G-proteins in hepatocytes in situ and in primary cultures. J. Cell. Biochem. **62:** 334–341

87 Novotny J., Bourova L., Kolar F. and Svoboda P. (2001) Membrane-bound and cytosolic forms of heterotrimeric G proteins in young and adult rat myocardium: influence of neonatal hypo- and hyperthyroidism. J. Cell. Biochem. **82:** 215–224

88 Giesberts A. N., Ginneken M. van, Gorter G., Lapetina E. G., Akkerman J. W. and Willigen G. van (1997) Subcellular localization of α-subunits of trimeric G-proteins in human platelets. Biochem. Biophys. Res. Commun. **234:** 439–444

89 Yamane H. K., Farnsworth C. C., Xie H. Y., Howald W., Fung B. K., Clarke S. et al. (1990) Brain G protein γ subunits contain an all-trans- geranylgeranylcysteine methyl ester at their carboxyl termini. Proc. Natl. Acad. Sci. USA **87:** 5868–5872

90 Iniguez-Lluhi J. A., Simon M. I., Robishaw J. D. and Gilman A. G. (1992) G protein $\beta\gamma$ subunits synthesized in Sf9 cells: functional characterization and the significance of prenylation of γ. J. Biol. Chem. **267:** 23409–23417

91 Muntz K. H., Sternweis P. C., Gilman A. G. and Mumby S. M. (1992) Influence of γ subunit prenylation on association of guanine nucleotide-binding regulatory proteins with membranes. Mol. Biol. Cell **3:** 49–61

92 Higgins J. B. and Casey P. J. (1996) The role of prenylation in G-protein assembly and function. Cell. Signal. **8:** 433–437

93 Nusse O. and Neer E. J. (1996) Localization of Gαo to growth cones in PC12 cells: role of Gαo association with receptors and G$\beta\gamma$. J. Cell Sci. **109:** 221–228

94 Fung B. K., Yamane H. K., Ota I. M. and Clarke S. (1990) The γ subunit of brain G-proteins is methyl esterified at a C-terminal cysteine. FEBS Lett. **260:** 313–317

95 Rosenberg S. J., Rane M. J., Dean W. L., Corpier C. L., Hoffman J. L. and McLeish K. R. (1998) Effect of γ subunit carboxyl methylation on the interaction of G protein γ subunits with $\beta\gamma$ subunits of defined composition. Cell. Signal. **10:** 131–136

96 Fung B. K., Hurley J. B. and Stryer L. (1981) Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc. Natl. Acad. Sci. USA **78:** 152–156

97 Novotny J., Kvapil P., Bokoch G. M. and Ransnas L. A. (1995) Isoproterenol-induced subcellular redistribution of G-protein β subunits in S49 lymphoma cells demonstrated by a novel competitive ELISA. Arch. Physiol. Biochem. **103:** 202–210

98 Muramoto S., Murakami T., Urasawa K., Kawaguchi H. and Kitabatake A. (1995) Cytosolic localization of β_3-subunit of heterotrimeric GTP binding protein in rat hearts. Biochem. Biophys. Res. Commun. **206:** 799–804

99 Kageyama K. (1995) β-Adrenergic receptor-mediated changes in subcellular localization of G protein β subunits in perfused rat hearts. Hokkaido Igaku Zasshi **70:** 497–506

100 Mitchell F. M., Buckley N. J. and Milligan G. (1993) Enhanced degradation of the phosphoinositidase C-linked guanine-nucleotide-binding protein $G_q\alpha/G_{11}\alpha$ following activation of the human M1 muscarinic acetylcholine receptor expressed in CHO cells. Biochem. J. **293:** 495–499

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

102 Mullaney I., Shah B. H., Wise A. and Milligan G. (1995) Expression of the human β_2-adrenoceptor in NCB20 cells results in agonist activation of adenylyl cyclase and agonist-mediated selective down-regulation of Gsα. J. Neurochem. **65:** 545–553

103 Jasper J. R. (1998) Stepping back and looking forward: down-regulation of G proteins as a mechanism of desensitization in tissues. Focus on 'Carbachol-induced desensitization of PLC-β pathway in rat myometrium: downregulation of $G_q\alpha/G_{11}\alpha$'. Am. J. Physiol. **275:** C634–C635

104 Milligan G. (1993) Agonist regulation of cellular G protein levels and distribution: mechanisms and functional implications. Trends Pharmacol. Sci. **14:** 413–418

105 Milligan G. (1996) The stoichiometry of expression of protein components of the stimulatory adenylyl cyclase cascade and the regulation of information transfer. Cell. Signal. **8:** 87–95

106 Shah B. H., MacEwan D. J. and Milligan G. (1995) Gonadotrophin-releasing hormone receptor agonist-mediated down-regulation of $G_q\alpha/G_{11}\alpha$ (pertussis toxin-insensitive) G proteins in α T3-1 gonadotroph cells reflects increased G protein turnover but not alterations in mRNA levels. Proc. Natl. Acad. Sci. USA **92:** 1886–1890
107 Westerlo E. van de, Yang J., Logsdon C. and Williams J. A. (1995) Down-regulation of the G-proteins $G_q\alpha$ and $G_{11}\alpha$ by transfected human M3 muscarinic acetylcholine receptors in Chinese hamster ovary cells is independent of receptor down-regulation. Biochem. J. **310:** 559–563
108 Yu S. S., Lefkowitz R. J. and Hausdorff W. P. (1993) β-Adrenergic receptor sequestration: a potential mechanism of receptor resensitization. J. Biol. Chem. **268:** 337–341
109 Pippig S., Andexinger S. and Lohse M. J. (1995) Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization. Mol. Pharmacol. **47:** 666–676
110 Zastrow M. von and Kobilka B. K. (1992) Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. J. Biol. Chem. **267:** 3530–3538
111 Zastrow M. von and Kobilka B. K. (1994) Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. J. Biol. Chem. **269:** 18448–18452
112 Hein L., Ishii K., Coughlin S. R. and Kobilka B. K. (1994) Intracellular targeting and trafficking of thrombin receptors: a novel mechanism for resensitization of a G protein-coupled receptor. J. Biol. Chem. **269:** 27719–27726
113 Moore R. H., Sadovnikoff N., Hoffenberg S., Liu S., Woodford P., Angelides K. et al. (1995) Ligand-stimulated β_2-adrenergic receptor internalization via the constitutive endocytic pathway into rab5-containing endosomes. J. Cell Sci. **108:** 2983–2991
114 Zhang J., Ferguson S. S., Barak L. S., Menard L. and Caron M. G. (1996) Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. J. Biol. Chem. **271:** 18302–18305
115 Holtmann M. H., Roettger B. F., Pinon D. I. and Miller L. J. (1996) Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. J. Biol. Chem. **271:** 23566–23571
116 Barak L. S., Ferguson S. S., Zhang J., Martenson C., Meyer T. and Caron M. G. (1997) Internal trafficking and surface mobility of a functionally intact β_2-adrenergic receptor-green fluorescent protein conjugate. Mol. Pharmacol. **51:** 177–184
117 Gaudriault G., Nouel D., Dal Farra C., Beaudet A. and Vincent J. P. (1997) Receptor-induced internalization of selective peptidic mu and delta opioid ligands. J. Biol. Chem. **272:** 2880– 2888
118 Wedegaertner P. B., Bourne H. R. and Zastrow M. von (1996) Activation-induced subcellular redistribution of Gsα. Mol. Biol. Cell **7:** 1225–1233
119 Nussenzveig D. R., Heinflink M. and Gershengorn M. C. (1993) Agonist-stimulated internalization of the thyrotropin-releasing hormone receptor is dependent on two domains in the receptor carboxyl terminus. J. Biol. Chem. **268:** 2389– 2392
120 Ashworth R., Yu R., Nelson E. J., Dermer S., Gershengorn M. C. and Hinkle P. M. (1995) Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling. Proc. Natl. Acad. Sci. USA **92:** 512–516
121 Petrou C. P. and Tashjian A. H. Jr (1995) Evidence that the thyrotropin-releasing hormone receptor and its ligand are recycled dissociated from each other. Biochem. J. **306:**107–113
122 Sorensen S. D., Linseman D. A. and Fisher S. K. (1999) Distinct mechanisms for the endocytosis of muscarinic receptors and $G_{q/11}$. Eur. J. Pharmacol. **372:** 325–328
123 Yu R. and Hinkle P. M. (1999) Signal transduction and hormone-dependent internalization of the thyrotropin-releasing hormone receptor in cells lacking G_q and G_{11}. J. Biol. Chem. **274:** 15745–15750
124 Huang C., Duncan J. A., Gilman A. G. and Mumby S. M. (1999) Persistent membrane association of activated and depalmitoylated G protein α subunits. Proc. Natl. Acad. Sci. USA **96:** 412–417
125 Pesanova Z., Novotny J., Cerny J., Milligan G. and Svoboda P. (1999) Thyrotropin-releasing hormone-induced depletion of $G_q\alpha/G_{11}\alpha$ proteins from detergent-insensitive membrane domains. FEBS Lett. **464:** 35–40
126 Huang C., Hepler J. R., Chen L. T., Gilman A. G., Anderson R. G. and Mumby S. M. (1997) Organization of G proteins and adenylyl cyclase at the plasma membrane. Mol. Biol. Cell **8:** 2365–2378
127 Nomura R., Inuo C., Takahashi Y., Asano T. and Fujimoto T. (1997) Two-dimensional distribution of Gi2α in the plasma membrane: a critical evaluation by immunocytochemistry. FEBS Lett. **415:** 139–144
128 De Weerd W. F. and Leeb-Lundberg L. M. (1997) Bradikinin sequesters B2 bradikinin receptors-coupled Gα subunits and Gα i in caveolae in DDT1 MF-2 smooth muscle cells. Biol. Chem. **272:** 17858–17866
129 Schwencke C., Okumura S., Yamamoto M., Geng Y. J. and Ishikawa Y. (1999) Colocalization of β-adrenergic receptors and caveolin within the plasma membrane. J. Cell. Biochem. **75:** 64–72
130 De Luca A., Sargiacomo M., Puca A., Sgaramella G., De Paolis P., Frati G. et al. (2000) Characterization of caveolae from rat heart: localization of postreceptor signal transduction molecules and their rearrangement after norepinephrine stimulation. J. Cell. Biochem. **77:** 529–539
131 Miotti S., Bagnoli M., Tomassetti A., Colnaghi M. I. and Canevari S. (2000) Interaction of folate receptor with signaling molecules lyn and Gαi-3 in detergent-resistant complexes from the ovary carcinoma cell line IGROV1. J. Cell Sci. **113:** 349–357
132 Rybin V. O., Xu X., Lisanti M. P. and Steinberg S. F. (2000) Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae: a mechanism to functionally regulate the cAMP signaling pathway. J. Biol. Chem. **275:** 41447–41457
133 Gaborik Z., Szaszak M., Szidonya L., Balla B., Paku S., Catt K. J. et al. (2001) β-Arrestin- and dynamin-dependent endocytosis of the AT1 angiotensin receptor. Mol. Pharmacol. **59:** 239–247
134 Moffett S., Brown D. A. and Linder M. E. (2000) Lipid-dependent targeting of G proteins into rafts. J. Biol. Chem. **275:** 2191–2198
135 Simons K. and Toomre D. (2000) Lipid rafts and signal transduction. Nat. Rev. Mol. Cell. Biol. **1:** 31–39
136 Oh P. and Schnitzer J. E. (2001) Segregation of heterotrimeric G proteins in cell surface microdomains: Gq binds caveolin to concentrate in caveolae, whereas Gi and Gs target lipid rafts by default. Mol. Biol. Cell **12:** 685–698
137 Ostrom R. S., Post S. R. and Insel P. A. (2000) Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving Gs. J. Pharmacol. Exp. Ther. **294:** 407–412
138 Murthy K. S., Grider J. R. and Makhlouf G. M. (2000) Heterologous desensitization of response mediated by selective PKC-dependent phosphorylation of Gi-1 and Gi-2. Am. J. Physiol. **279:** C925–C934
139 Neubig R. R. (1994) Membrane organization in G-protein mechanisms. FASEB J. **8:** 939–946
140 Tolkovsky A. M. and Levitzki A. (1978) Mode of coupling between the β-adrenergic receptor and adenylate cyclase in turkey erythrocytes. Biochemistry **17:** 3795–3810
141 Li S., Okamoto T., Chun M., Sargiacomo M., Casanova J. E., Hansen S. H. et al. (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. J. Biol. Chem. **270:** 15693–1570