CellPress

Review

Emerging Paradigms of G Protein-Coupled Receptor Dephosphorylation

Andrea Kliewer,[1] Rainer K. Reinscheid,[1,2] and Stefan Schulz[1,*]

Elucidation of the molecular mechanisms underlying G protein-coupled receptor (GPCR) dephosphorylation remains a major challenge. While specific GPCR phosphatases (GRPs) have eluded identification, prevailing models propose that receptors must first internalize into acidic endosomes to become dephosphorylated in a housekeeping-like process. Recently, phosphosite-specific antibodies, combined with siRNAs targeting specific phosphatase transcripts, have facilitated the identification of distinct protein phosphatase 1 (PP1) and PP2 catalytic subunits as *bona fide* GRPs. Similar to phosphorylation, GPCR dephosphorylation is temporally and spatially regulated, starting immediately after receptor activation at the plasma membrane and continuing along the endocytic pathway. Dephosphorylation disrupts receptor–arrestin complexes, thus terminating arrestin-dependent signaling. Partially dephosphorylated GPCRs may remain membrane bound for renewed agonist activation while others undergo endocytosis. After internalization, further dephosphorylation facilitates the transition into the recycling pathway, leading to either plasma membrane repopulation or lysosomal degradation. These findings reveal unappreciated cellular sites and regulatory functions of receptor dephosphorylation and call for revised models of the GPCR activation/deactivation cycle.

Current View of the GPCR Phosphorylation/Dephosphorylation Cycle

GPCRs are the largest family of about 800 cell-surface receptors that regulate almost every physiological function. Agonist activation of a GPCR triggers binding of the associated heterotrimeric G protein, which in turn activates a second-messenger system. For many GPCRs, quenching of this signal involves phosphorylation of the receptor by **GPCR kinases (GRKs)** (see Glossary) or second messenger-dependent protein kinases such as PKA or PKC. Phosphorylation by GRKs increases the affinity for arrestins, which uncouple the receptor from the G protein and target the receptor to clathrin-coated pits for internalization, thus **desensitizing** the primary signaling. The internalized GPCR is either trafficked to lysosomes for degradation or recycled back through an endosomal pathway to the plasma membrane. Once a GPCR is activated, phosphorylated, and bound to arrestin, return to its resting state requires dissociation or degradation of the agonist, dissociation of the arrestin, and dephosphorylation. This model predicted an essential role for receptor endocytosis: a GPCR had to cycle through endosomal compartments to be dephosphorylated and eventually **resensitized** (Figure 1).

The large number of GPCRs is in contrast to only six GRKs and 13 catalytic phosphatase subunits that have been identified in the human genome, implying broad enzymatic promiscuity towards their GPCR substrates. However, recently a model of 'phosphorylation barcoding' has been proposed in which distinct phosphorylation patterns of various serine (S) and threonine (T)

Trends

Dephosphorylation is an integral part of the G protein-coupled receptor (GPCR) activation/deactivation cycle and essential for receptor resensitization. However, comparably little is known about the specific protein phosphatases (PPs), kinetic and structural parameters, subcellular localization, and functional contributions of accessory proteins involved in GPCR dephosphorylation.

Recent advances using phosphosite-specific antibodies combined with systematic siRNA knockdown have matched specific phosphatase catalytic subunits from the PP1 and PP2 families with several GPCRs. The GPCR–arrestin complex recruits a specific phosphatase immediately after agonist activation and dephosphorylation continues along the endocytic pathway. GPCR dephosphorylation is also necessary for termination of arrestin-dependent signaling.

Early *in vivo* studies have linked GPCR dephosphorylation with physiological functions such as analgesic tolerance (opioid receptors), thyroid function (thyrotropin-releasing hormone receptor), and diminished drug responsiveness of endocrine tumors (somatostatin receptors).

[1]Institute of Pharmacology and Toxicology, Jena University Hospital, Friedrich-Schiller-Universität, 07747 Jena, Germany
[2]Institute of Physiology I, University Hospital Münster, Westfälische Wilhelms-Universität, 48149 Münster, Germany

*Correspondence:
Stefan.Schulz@med.uni-jena.de (S. Schulz).

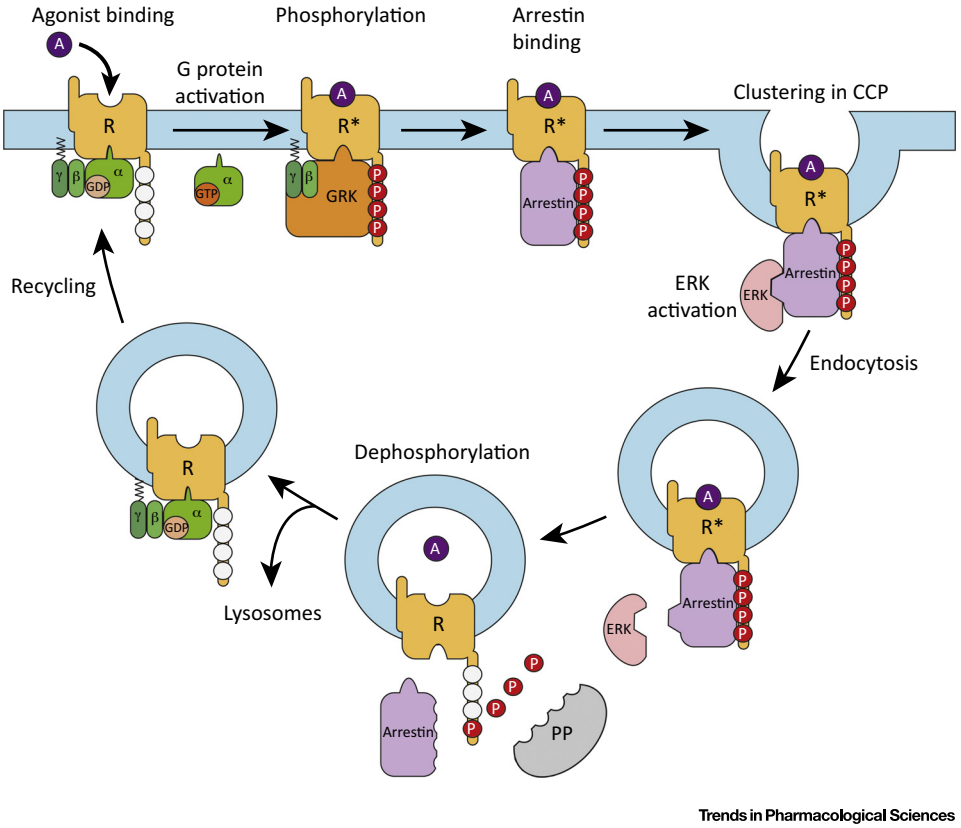

Figure 1. Current View of the G Protein-Coupled Receptor (GPCR) Phosphorylation/Dephosphorylation Cycle. Agonist activation of a GPCR triggers activation of the associated heterotrimeric G protein, which in turn stimulates a second-messenger system. For many GPCRs quenching of this signal involves phosphorylation of the receptor by GPCR kinases (GRKs). Phosphorylation by GRKs increases the affinity for arrestins, which uncouple the receptor from the G protein and target the receptor to clathrin-coated pits (CCPs) for internalization. Once a GPCR is internalized, return to its resting state requires dissociation or degradation of the agonist, dephosphorylation by an endosomal protein phosphatase (PP), and dissociation of the arrestin. Subsequently, the GPCR is either trafficked to lysosomes for degradation or recycled back through an endosomal pathway to the plasma membrane. Thus, the current model predicts an essential role for receptor endocytosis: a GPCR has to cycle through endosomal compartments to be dephosphorylated and eventually resensitized. ERK, extracellular signal-regulated kinase.

residues on the intracellular C-terminal tails of most GPCRs are read by arrestins and direct further signaling [1–4]. It is reasonable to assume that this phosphoserine/threonine barcode may also influence interactions with **PPs**.

Over the past two decades, GPCR phosphorylation has been studied in great detail. Surprisingly little attention has been paid to GPCR dephosphorylation, although these biochemical processes represent two sides of the same coin. Study of PPs interacting with GPCRs has also been hampered by the inherent complexity of phosphatase biochemistry: phosphatases are multimeric holoenzymes in which a catalytic subunit associates with one or more regulatory and/or structural subunits in a cell type-specific manner. As a result, and to date, not one phosphatase holoenzyme has been identified to dephosphorylate a particular GPCR, but merely individual catalytic subunits.

However, the critical importance of dephosphorylation in the GPCR activation/inactivation cycle appears obvious. This review aims to summarize current progress in the identification of

GRPs and their mechanisms of influencing GPCR recycling and thus terminating desensitization and proposes some caveats and strategies to identify specific phosphatase holoenzymes involved in GPCR recycling.

Identification of GRPs

GRKs, as well as second messenger-dependent kinases such as PKA and PKC, phosphorylate GPCRs on intracellular serine and threonine residues, the majority of which are then dephosphorylated by PP1 or PP2A [5]. These PPs are multisubunit enzymes that achieve specificity by interacting with a wide array of scaffolding and targeting subunits. The PP1 phosphatase family contains three catalytic subunits (PP1α, PP1β, and PP1γ) that can bind directly to substrates through numerous conserved motifs or via targeting and scaffolding subunits. Over 150 proteins have been shown to interact with the three PP1 catalytic subunits and this list is certainly incomplete [6]. PP2As are heterotrimers comprising a catalytic subunit, a scaffolding or structural subunit, and a targeting subunit. In addition, mammalian cells express PP2 B (calcineurin), PP3, PP4, and PP5 isoforms, all of which form multimeric enzyme complexes. Rather than the catalytic subunits, the regulatory and structural subunits provide the essential determinants for the subcellular localization, substrate specificity, and fine-tuning of phosphatase activity. In addition, an increasing number of PP inhibitory proteins control the cell's dephosphorylation capacity [7–10].

The first GPCR-specific PP identified was the gene product of *retinal degeneration C* (RDGC), required for rhodopsin dephosphorylation in *Drosophila melanogaster*. The catalytic domain of RDGC exhibits high homology with PP1, PP2, and PP3 [11–13]. Loss of RDGC causes disturbance of light signal transduction and leads to light-dependent retinal degeneration [12]. RDGC binds to calmodulin and a mutation in an IQ motif that eliminates the calmodulin–RDGC interaction prevents dephosphorylation of rhodopsin *in vivo* and disrupts termination of the photoresponse, indicating that RDGC is a calmodulin-dependent PP [14].

At the same time, a PP2A-related phosphatase activity that dephosphorylates β2-adrenoceptors (β2ARs) was identified [15,16]. It was proposed that this phosphatase is tethered to vesicular membranes and that receptors have to internalize into an acidic endosomal compartment to become dephosphorylated. However, subsequent work has shown that inhibition of β2AR internalization with dominant-negative dynamin or hypertonic sucrose did not affect the rate of receptor dephosphorylation [17]. Indeed, dephosphorylation of PKA and GRK sites of β2ARs can occur upon the plasma membrane and in the cytosol [18,19]. Similarly, dopamine D_1 receptor dephosphorylation was not blocked in the presence of concanavalin A, which also inhibits receptor internalization [20]. More recent studies have shown that phosphatase inhibitors such as okadaic acid and calyculin A can block the dephosphorylation of several GPCRs, including β2ARs, the dopamine D_1 receptor, parathyroid hormone receptor 1 (PTH1), the thromboxane A receptor (TXA), and vasopressin receptor 1 (V_1) [17,21–23]. However, all available chemical PP inhibitors lack selectivity for specific catalytic PP subunits. Consequently, the specific phosphatases responsible for the dephosphorylation of these and many other GPCRs eluded identification. Progress had been hampered by two main factors: (i) the mandatory use of radioactive dephosphorylation assays with their inherent lack of spatial and temporal resolution; and (ii) the limited availability and selectivity of chemical phosphatase inhibitors.

Two major technical improvements have recently helped to significantly advance our knowledge: first, the development of phosphosite-specific antibodies, and second, siRNA knockdown of individual GRKs and phosphatase catalytic subunits have produced detailed insights into GPCR phosphorylation and dephosphorylation cycles. A growing list of GPCRs has been investigated closely employing these new experimental tools (Table 1). For the first time, several

Glossary

Desensitization: a physiological/cellular process describing the effect of repeated agonist activation producing diminished subsequent responses of a receptor; at the cellular level, desensitization involves GPCR phosphorylation, arrestin binding, and internalization, effectively disrupting second-messenger signaling.
G protein-coupled receptor (GPCR) kinase (GRK): a family of enzymes that specifically phosphorylate activated GPCRs.
Protein phosphatase (PP): a family of ubiquitous enzymes involved in dephosphorylation of proteins.
Resensitization: a physiological/cellular process describing the recycling and reactivation of desensitized receptors, involving dephosphorylation, dissociation of arrestin, and reinsertion of receptors into the plasma membrane.

Table 1. GPCR Dephosphorylation

GPCR	Phosphoamino acid site	Phosphoamino acid detection method	Phosphatase	PP identification	Cell/tissue model	Refs
β2AR	$S^{262/355/356}$	Phosphorylation site-specific AB	PP1 type	Pharmacological PP inhibitors, including PP1 inhibitor 2	HEK293, A431, heterologous	[14]
β2AR	$S^{355/356}$	Phosphorylation site-specific AB	PP2A	siRNA knockdown of I2PP2A	Neonatal ventricular myocytes from TG mice, heterologous GPCR expression	[25]
5-HT_{2A}	S^{291}, T^{386}	No detailed analysis (internalization as a measure of phosphorylation)	PP2A	Pharmacological PP inhibitors	HEK293, heterologous	[26]
D_1	n.a.	No detailed analysis	PP2B	Coimmunoprecipitation	Mouse neocortical tissue, endogenous	[58]
D_2	n.a.	No detailed analysis	PP2A	Coimmunoprecipitation	Mouse renal proximal tubule cells, endogenous	[59]
$mGlu_1$	n.a.	No detailed analysis (internalization as a measure of phosphorylation)	PP2A	siRNA knockdown (only against PP2A), overexpression of dominant-negative PP, pharmacological PP inhibitors	HEK293, Neuro2A, heterologous	[27]
$mGlu_5$	n.a.	No detailed analysis (internalization as a measure of phosphorylation)	PP2A (PP2B partially)	siRNA (preselection of PP2 family by pharmacological PP inhibitors)	HEK293, Neuro2A, heterologous	[28]
$mGlu_7$	S^{862}	Phosphorylation site-specific AB	PP1γ	Overexpression of dominant-negative PP, direct binding of PP to C-terminal tail	HEK293, transfected primary rat hippocampal neurons, heterologous	[29]
MOR	S^{375}, T^{370}	Phosphorylation site-specific AB	PP1γ	siRNA	HEK293, heterologous	[35]
NK_1	n.a.	No detailed analysis	PP2A	Pharmacological PP inhibitors, agonist-dependent coimmunoprecipitation with β-arrestin1	HEK293, KNRK, heterologous	[43]
sst_2	$T^{353/354/356/359}$	Phosphorylation site-specific AB	PP1β	siRNA, agonist-dependent coimmunoprecipitation with β-arrestin1	HEK293, heterologous	[32,51]
	$S^{341/343}$	Phosphorylation site-specific AB	Unknown	siRNA		
sst_2	$T^{353/354/356/359}$	Phosphorylation site-specific AB	PP2A	Pharmacological PP inhibitors	CHO K1, heterologous	[31]
	$S^{341/343}$	Phosphorylation site-specific AB	Unknown	Pharmacological PP inhibitors		
sst_3	$S^{337/361}$, $T^{341/348}$	Phosphorylation site-specific AB	PP1α, PP1β	siRNA	HEK293, AtT-20, heterologous	[34]
sst_5	T^{333}	Phosphorylation site-specific AB	PP1γ	siRNA	HEK293, heterologous	[33]
TRH	T^{365} ($S^{355/360/364}$)	Phosphorylation site-specific AB	PP1α (PP1β, PP1γ partially)	siRNA, overexpression of dominant-negative PP	HEK293, heterologous GH3, endogenous	[41]

AB, antibody; n.a., not applicable.

critical questions surrounding the GPCR phosphorylation–dephosphorylation cycle can be experimentally addressed. First, individual modified serine/threonine residues can be identified; second, hierarchical orders and kinetics of phosphorylation/dephosphorylation can be studied, together with the functional role of individual phosphoserine/threonine residues in GPCR recycling. In addition, subcellular compartments and the temporal regulation of enzymatic modification may be identified, the identity and regulation of the GRKs and PPs involved in the 'GPCR cycle' can be studied, the role of internalization in GPCR resensitization may be resolved, and, finally, *in vitro* and *in vivo* GPCR phosphorylation/dephosphorylation patterns may be correlated.

Examples of GRPs

The GPCR phosphorylation cycle is probably best studied for β2ARs. Using chemical PP inhibitors, PP1-type phosphatases were postulated to dephosphorylate β2ARs [17]. However, previous studies had also suggested members of the PP2 family of phosphatases [24–27]. As Table 1 illustrates, phosphosite-specific antibodies were first used on β2ARs to elucidate the functional significance of S^{355} and S^{356} for receptor desensitization and internalization. The same study also demonstrated the importance of PP2A for β2AR dephosphorylation by siRNA knockdown of an endogenous PP2A inhibitor protein [28]. These conflicting results illustrate the limitations of earlier experimental approaches and underscore the critical importance of precision tools such as phosphosite-specific antibodies and siRNA knockdown of individual PP catalytic subunits, although the latter experimental approach has not yet been applied to β2ARs.

PP2A was also postulated to dephosphorylate 5-HT_{2A} serotonin receptors using pharmacological PP inhibitors, although confirmation of these results by siRNA knockdown has not yet been published [29]. Combinations of chemical PP inhibitors were also used to narrow the field of phosphatases for the metabotropic glutamate receptors $mGlu_1$ and $mGlu_5$. In both cases subsequent siRNA knockdown confirmed PP2A as the enzyme required for receptor recycling back to the plasma membrane [30,31]. These two studies did not directly investigate receptor phosphorylation status or individual phosphoamino acids but used receptor recycling between the plasma membrane and endosomal compartments as readouts. Nevertheless, it appears that reduced expression of PP2A is sufficient to prevent resensitization of $mGlu_1$ and $mGlu_5$.

Agonist-dependent recycling of $mGlu_7$ was recently linked to PP1γ, as overexpression of a dominant-negative mutant of PP1γ prevented the reinsertion of internalized receptors into the plasma membrane [32]. Moreover, PP1γ coimmunoprecipitated with $mGlu_7$ and colocalizes with the receptor at the plasma membrane on agonist stimulation [32,33]. However, the functional role of phosphorylation/dephosphorylation of $mGlu_7$ on S^{862} is the opposite of other GPCRs in that constitutive or agonist-induced S^{862} phosphorylation stabilizes membrane insertion whereas its dephosphorylation triggers internalization. Therefore, $mGlu_7$ undergoes agonist-induced endocytosis by activating a phosphatase rather than a kinase like most other GPCRs.

The most profound progress on mechanisms of GPCR dephosphorylation has been made in the somatostatin receptor family. For the somatostatin sst_2 receptor, Ghosh and Schonbrunn reported different spatial and temporal patterns of receptor dephosphorylation [34]. Specifically, reversal of receptor phosphorylation was determined by the duration of prior agonist exposure. They showed that dephosphorylation of T^{353}/T^{354} was not affected by either hypertonic sucrose or dynasore, which prevent receptor internalization, whereas dephosphorylation of S^{341}/S^{343} was completely blocked. Dephosphorylation of T^{353}/T^{354}, but not of S^{341}/S^{343}, was sensitive to okadaic acid treatment. These results suggested that receptor dephosphorylation is determined by the duration of agonist stimulation and compartment-specific

enzymatic activity and that hierarchical patterns of phosphorylation and dephosphorylation might exist. However, these studies did not identify a specific phosphatase responsible for sst_2 dephosphorylation.

More recently, Pöll *et al*. used a combination of phosphosite-specific antibodies, chemical PP inhibitors, and unbiased siRNA knockdown screening of all catalytic PP subunits to identify the GRP that catalyzes the rapid dephosphorylation of T^{353}, T^{354}, T^{356}, and T^{359} of the sst_2 receptor [35]. The phosphatase activity required for rapid dephosphorylation of sst_2 was inhibited in a dose-dependent manner only by calyculin A and not by okadaic acid. Both calyculin A and okadaic acid can effectively block PP2, PP4, and PP5 activity. In contrast to okadaic acid, calyculin A is also a potent inhibitor of PP1 activity, suggesting that members of the PP1 family might dephosphorylate the ^{353}TTETQRT^{359} motif of the sst_2 receptor. Simultaneous knockdown of all three catalytic PP1 subunits confirmed that PP1 activity was required for efficient sst_2 dephosphorylation. Selective inhibition of PP1β expression resulted in enhanced ^{353}TTETQRT^{359} phosphorylation in the presence of agonist and clearly delayed receptor dephosphorylation after agonist removal. Inhibition of PP2A, PP2B, PP4, or PP5 expression did not alter the time course of sst_2 dephosphorylation. Thus, these findings identified PP1β as a *bona fide* GRP for the β-arrestin acceptor site of the sst_2 receptor.

Inhibition of PP1β expression facilitates the detection of phosphorylated sst_2 receptors at the plasma membrane only 5 min after agonist exposure and throughout extended treatment periods. These results strongly suggest that sst_2 receptor dephosphorylation is initiated directly after receptor activation at or near the plasma membrane and confirm earlier findings showing that T^{353}/T^{354} dephosphorylation did not require receptor internalization [34] (Figure 3). Interestingly, S^{341}/S^{343} dephosphorylation occurs with a delayed time course, suggesting that sst_2 dephosphorylation may be initiated at the plasma membrane and continues along the endocytic pathway although not precluding a second, as-yet-unidentified enzyme activity for sst_2 dephosphorylation within the cytosol.

Comparative examination of sst_5 and sst_2 somatostatin receptors revealed strikingly different patterns of dephosphorylation and recycling. Whereas fast sst_5 trafficking correlates with rapid T^{333} phosphorylation and dephosphorylation, sst_2 recycling appears to be delayed due to slower dephosphorylation (Figures 2 and 3). Analysis of sst_5 using chemical PP inhibitors and siRNA knockdown screening revealed that T^{333} dephosphorylation is inhibited in a dose-dependent manner only by calyculin A and not by okadaic acid, suggesting that PP1 activity was required. siRNA knockdown experiments confirmed that only PP1γ knockdown results in robust inhibition of sst_5 dephosphorylation [36]. These results suggest PP1γ to be the predominant GRP responsible for rapid T^{333} dephosphorylation of sst_5.

Using the same experimental strategy, the phosphorylation and dephosphorylation of sst_3 was recently deciphered, showing that S^{337}/S^{361} and T^{341}/T^{348} are critical for receptor recycling and are likely a substrate for PP1α and PP1β [37]. The study also revealed significantly different kinetic rates for receptor phosphorylation (occurring within seconds) and dephosphorylation (on the order of minutes).

For the mu-opioid receptor (MOR), another member of the family of peptide-ligand GPCRs, PP1γ was recently identified to catalyze T^{370} and S^{375} dephosphorylation at or near the plasma membrane within minutes after agonist removal, thus facilitating receptor recycling [38] (Figure 2). Knockdown of PP1γ expression by siRNA significantly attenuated MOR recycling to the plasma membrane. The study also demonstrated unexpected agonist-dependent phosphorylation patterns for MOR, including agonist-selective recruitment of GRKs, suggesting a distinct phosphorylation hierarchy and biased signaling of different types of agonist.

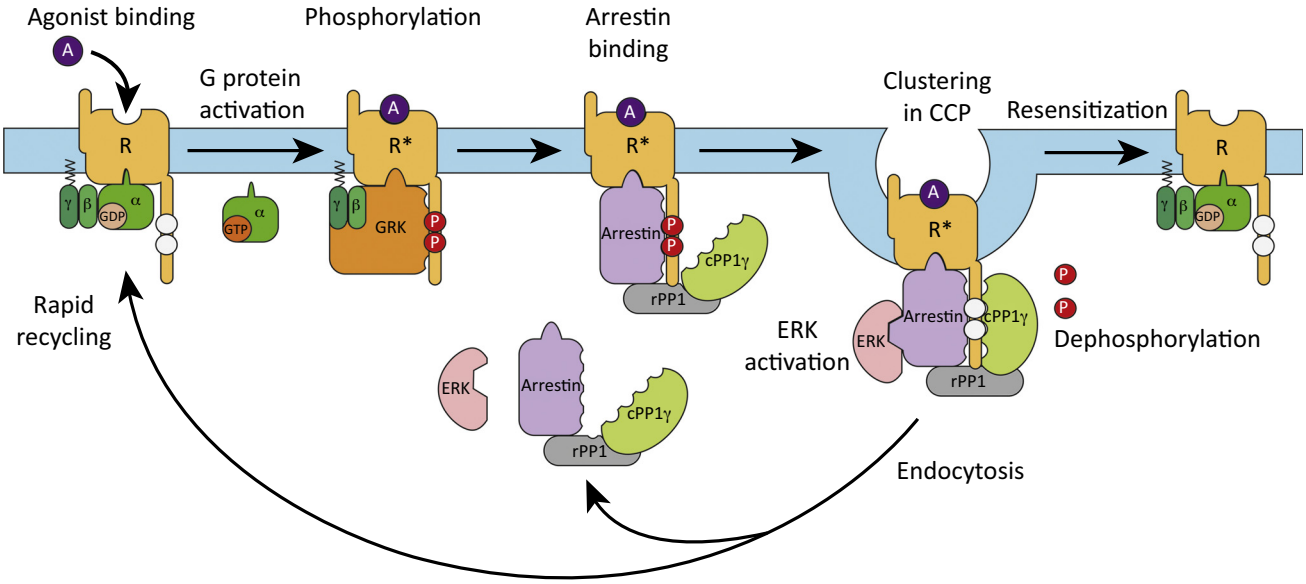

Figure 2. Proposed Model of Fast G Protein-Coupled Receptor (GPCR) Dephosphorylation. For GPCRs undergoing fast dephosphorylation, including the mu-opioid receptor (MOR) and the sst_5 receptor, the catalytic protein phosphatase 1 gamma (cPP1γ) subunit was identified to catalyze serine/threonine dephosphorylation at the plasma membrane within seconds to minutes after agonist removal. Typically, these GPCRs form unstable complexes with arrestins that are rapidly disrupted. After dephosphorylation the receptor is either resensitized at the plasma membrane or recycled back through an endosomal pathway. Regulatory PP1 (rPP1) subunits involved in this process have not yet been identified. ERK, extracellular signal-regulated kinase.

Specifically, the peptidic full agonist DAMGO was found to recruit GRK2 and GRK3 to promote phosphorylation of T^{370} and S^{375} while morphine selectively recruited GRK5 to the receptor complex, producing only S^{375} phosphorylation. This concept was later extended by showing that high-efficacy MOR agonists promote higher-order phosphorylation via GRK2/3 activation whereas morphine-induced GRK5-dependent phosphorylation of S^{375} alone results in attenuated β-arrestin mobilization and limited receptor internalization [39–41]. Together, these observations were hypothesized to contribute to morphine-induced tolerance and dependence [40,42].

In mouse embryonic fibroblasts from β-arrestin2 knockout mice, the G_q-coupled thyrotropin-releasing hormone (TRH) receptor is rapidly dephosphorylated at the cell membrane and unable to undergo significant internalization on agonist stimulation, consistent with the role of β-arrestin in GPCR recycling [43]. Using an unbiased siRNA library against all PP catalytic subunits and chemical PP inhibitors, Gehret and Hinkle (2013) recently linked PP1α to TRH receptor dephosphorylation. They showed that inhibition of PP1α synthesis and overexpression of dominant-negative PP1α preserved receptor phosphorylation whereas overexpression of PP1α accelerated dephosphorylation. However, knockdown of all three PP1 catalytic subunits suppresses TRH receptor dephosphorylation much more powerfully than knockdown of PP1α alone, suggesting that different PP1 isoforms could function redundantly [44]. Importantly, the TRH receptor phosphorylation cycle was also confirmed in GH3 cells, which were derived from a pituitary tumor and express the TRH receptor endogenously [45].

Agonist-induced and β-arrestin-dependent association of PP2A with neurokinin receptor 1 (NK_1) was demonstrated by coimmunoprecipitation [46]. The study also showed that this process occurred predominantly at the plasma membrane where a majority of NK_1 resides after agonist-induced desensitization. The authors concluded that recruitment of PP2A to a pool of

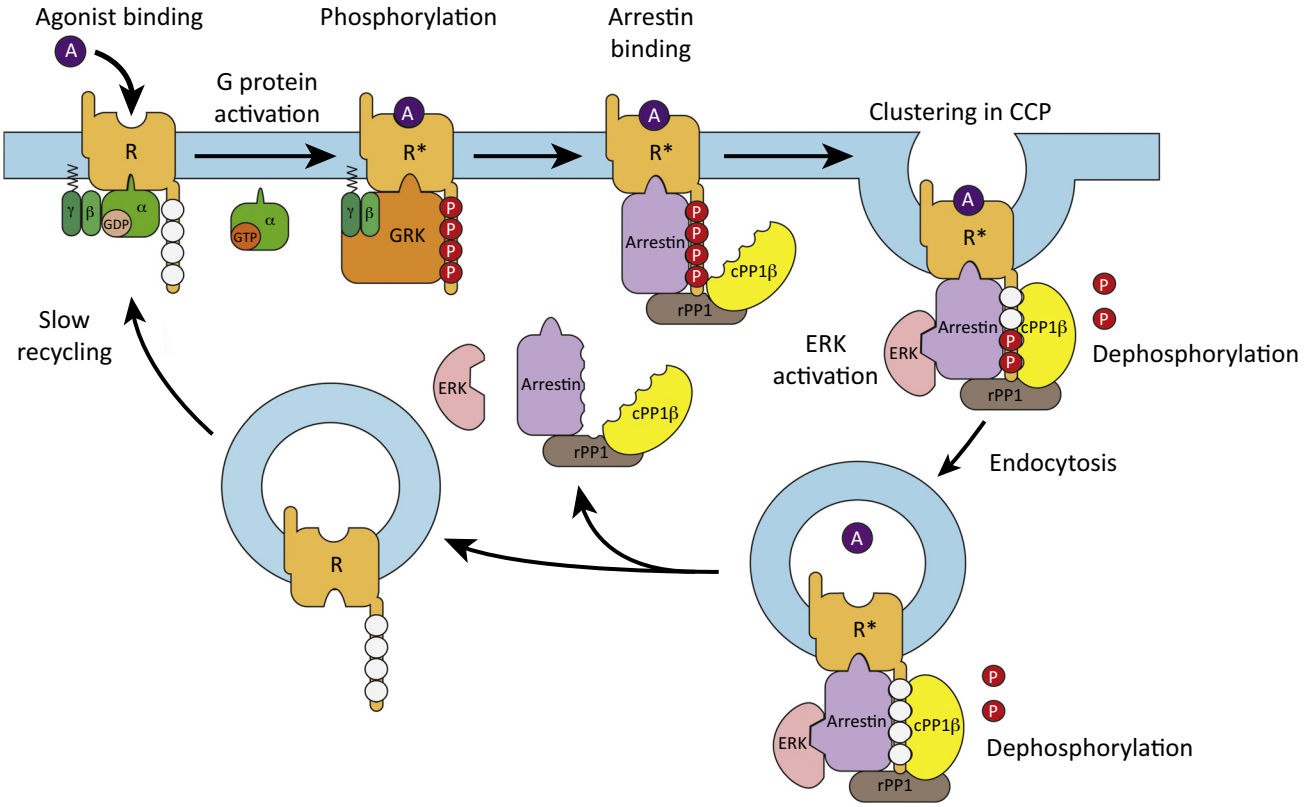

Figure 3. Proposed Model of Slow G Protein-Coupled Receptor (GPCR) Dephosphorylation. For GPCRs undergoing slow dephosphorylation, such as the sst_2 receptor, the catalytic protein phosphatase 1 beta (cPP1β) subunit was identified to catalyze serine/threonine dephosphorylation. Typically, these GPCRs form stable complexes with arrestins that co-internalize into the same endocytic vesicles. This dephosphorylation process is initiated at the plasma membrane and continues along the endosomal pathway. PP1β-mediated dephosphorylation promotes dissociation of arrestins and hence facilitates the quenching of arrestin-dependent signaling. Subsequently, the GPCR is recycled back through an endosomal pathway to the plasma membrane. Regulatory PP1 (rPP1) subunits involved in this process have not yet been identified. GRK, GPCR kinase; ERK, extracellular signal-regulated kinase.

desensitized, phosphorylated, and β-arrestin-bound NK_1 residing in the plasma membrane might be a key step in rapid resensitization of the receptor, in contrast to the slow endosomal recycling pathway. However, treatment with the chemical PP inhibitors okadaic acid and fostriecin attenuated only a relatively small fraction of NK_1 resensitization, indicating that additional enzymatic activities are required for rapid NK_1 dephosphorylation. Unbiased siRNA knockdown of individual PP catalytic subunits might reveal additional phosphatase activities involved in the rapid recovery of NK_1. Also, the phosphorylation patterns and contributions of individual serine/threonine residues of NK_1 have not yet been mapped. It is therefore too early to conclude whether PP2A is the predominant PP involved in NK_1 recycling [47].

The currently available data using the more specific siRNA knockdown screening of phosphatase activities suggest an intriguing picture: PP1-like phosphatases appear to interact predominantly with peptide GPCRs, whereas GPCRs with small-molecule ligands such as adrenoceptors and dopaminergic and metabotropic glutamate receptors might be preferred substrates for PP2-type PPs (Table 1). Clearly, future studies are required to substantiate this early hypothesis and delineate structural motifs or specific accessory protein mechanisms mediating substrate selectivity.

Subcellular Location of GPCR Dephosphorylation

Early work on β2ARs suggested that dephosphorylation was critically dependent on internalization and would thus occur almost exclusively in endosomal compartments [48]. As mentioned above, these studies were limited by the low spatial resolution of the ^{32}P radioactive labels used in the experiments, which could not differentiate between individual phosphoserine/threonine residues. Only since the introduction of phosphosite-specific antibodies, combined with confocal laser microscopy, has it been revealed that phosphorylated GPCRs may reside in the plasma membrane and undergo rapid resensitization. Almost every recent study of GPCR dephosphorylation has now concluded that phosphatase activity is recruited immediately to the GPCR–arrestin complex at or near the plasma membrane. A significant fraction of phosphorylated GPCRs then undergoes at least partial dephosphorylation and is resensitized without entering intracellular compartments. Such a mechanism has now been shown for β2ARs, three somatostatin receptor subtypes (sst_2, sst_3, and sst_5), MOR, the TRH receptor, $mGlu_1$ and $mGlu_5$, and NK_1, albeit using different methodologies and experimental tools.

The exact contribution of GPCR dephosphorylation to receptor resensitization has recently been questioned after an in-depth analysis of the β2AR phosphorylation cycle [17]. The authors concluded that although dephosphorylation of β2ARs started immediately at the plasma membrane, it may not be required for receptor resensitization; rather, dissociation of β-arrestin on agonist removal appeared to be most critical. The most compelling evidence for this hypothesis was the observation that GRK-site-phosphorylated β2ARs resided in the plasma membrane but were able to induce renewed adenylate cyclase stimulation if previous agonist had been removed; thus, they were fully resensitized yet still phosphorylated. Only under conditions of persistent agonist presence do β2ARs undergo internalization, where both agonist dissociation and dephosphorylation occur in acidified endosomal compartments. These observations challenge the meaning of dephosphorylation as a mandatory requirement for receptor resensitization. Nevertheless, they also confirmed that GPCR dephosphorylation starts at the plasma membrane, probably by recruitment of PPs to the GPCR–arrestin complex. Further dephosphorylation may then continue along the endocytic pathway. It is currently unknown whether, once recruited, phosphatases co-internalize together with their GPCR substrates or whether different endosomal phosphatases newly engage their substrates once they have entered endosomal structures. All experiments using chemical PP blockers or siRNA knockdown indicate that more than one phosphatase activity contributes to complete GPCR dephosphorylation, which may also reside in different cellular compartments. Future studies capable of resolving PP isoforms at the subcellular level may help to answer this question. It is noteworthy that all recent studies agree on the conclusion that internalization is not required for GPCR dephosphorylation and resensitization, that dephosphorylation occurs at the plasma membrane, and that complete GPCR dephosphorylation is not required for receptor resensitization.

Specificity and Selectivity of PP Recruitment

An important question that remained unanswered in the aforementioned studies on dephosphorylation is what determines the activity and localization of phosphatases at the plasma membrane and how is substrate specificity ensured?

GPCR dephosphorylation has long been viewed as an unregulated process of limited functional significance. Initial evidence suggests that PP1β-mediated ^{353}TTETQRT^{359} dephosphorylation of the sst_2 receptor may play a role in fine-tuning unconventional β-arrestin-dependent signaling. GRK2/3-driven phosphorylation of the ^{353}TTETQRT^{359} motif is essential for β-arrestin binding that facilitates G_i protein-independent, but β-arrestin-dependent, extracellular signal-regulated kinase (ERK) activation [35,49]. Inhibition of PP1β expression results in a robust increase in β-arrestin-dependent ERK activation in somatostatin-treated human

embryonic kidney 293 (HEK293) cells that stably express the sst_2 receptor [35]. This effect was not observed after exposure to epidermal growth factor (EGF) (inducing heterologous sst_2 phosphorylation) or after inhibition of PP1α or PP1γ expression under otherwise identical conditions, suggesting that diminished PP1 activity does not directly lead to a general enhancement of ERK excitability [35]. These findings suggest a model in which engagement of PP1β facilitates GPCR dephosphorylation, which in turn leads to disruption of the β-arrestin–GPCR complex and thereby limits β-arrestin-dependent ERK signaling. The importance of dephosphorylation for disruption of the GPCR–arrestin complex has been verified for several GPCRs, including β2ARs, NK_1, and the TRH receptor, and could therefore suggest a common mechanism.

β-Arrestins serve as scaffolds to facilitate receptor internalization and initiate a second wave of signaling [50–53]. However, it is unknown how PP-targeting subunits can bring the phosphatase holoenzyme complex into proximity with phosphorylated GPCRs. Kliewer *et al.* have shown that siRNA knockdown of β-arrestin1 strongly inhibits sst_2 receptor dephosphorylation. Coimmunoprecipitation experiments demonstrate that β-arrestin1 and PP1β exist as a constitutive complex that mediates rapid dephosphorylation of sst_2 receptors at or near the plasma membrane. By contrast, β-arrestin2 is not essential for rapid sst_2 receptor dephosphorylation. Together these findings reveal a novel scaffolding function of β-arrestin1 that facilitates efficient targeting of PP1β to phosphorylated GPCRs [54]. Similar observations were made for β2AR, NK_1, and the TRH receptor, suggesting a model in which a particular β-arrestin isoform in complex with the phosphorylated carboxyl-terminal GPCR tail provides a structural framework for selective recruitment of particular PP isoforms.

At the level of GPCR carboxyl-terminal sequences and their phosphorylated serine/threonine residues, it is unclear which mechanisms regulate PP selectivity and specificity. It is possible that carboxyl-terminal phosphorylation motifs, specific sequences within the intracellular loops of the receptor, or β-arrestin trafficking patterns may contribute to phosphatase selection.

We have recently observed that closely related somatostatin receptor subtypes can be dephosphorylated by distinct PP1 isoforms. However, it was unknown which GPCR domain directs the engagement of specific PP1 isoforms with the receptor. Transplantation of the sst_2 tail to the sst_5 receptor led to predominant dephosphorylation by PP1β whereas the reverse transplantation resulted in predominant dephosphorylation by PP1γ [55]. Moreover, swapping the cytoplasmic tails led to complete reversal of the trafficking profiles of these two receptors.

The remarkable selectivity in the recruitment of specific PP1 catalytic subunits to individual somatostatin receptor subtypes is surprising. PP1 catalytic subunits bind to their regulatory subunits and some substrates in a mutually exclusive manner through a conserved RVxF motif. The three isoforms of the PP1 catalytic subunit share greater than 90% sequence identity, including the regions that interact with the RVxF sequence. However, neither the human sst_2 nor the sst_5 receptor contains a potential PP1-binding motif in its carboxyl-terminal tail, suggesting that somatostatin receptors do not bind to PP1 exclusively by the canonical RVxF motif. Instead, the association with PP1 may occur directly through a noncanonical interaction or multiple weak interactions or indirectly via one or more regulatory subunits of PP1. Such PP-targeting subunits are prime candidates for bringing phosphatases into proximity with phosphorylated GPCRs. Nevertheless, the identity of such PP1-targeting subunits remains to be elucidated for both sst_2 and sst_5, although the existing data clearly indicate that the carboxyl-terminal regions of different somatostatin receptor subtypes contain important structural determinants for their PP1 selectivity [55].

Further increasing the complex picture of PP recruitment was the observation that various scaffolding A-kinase-anchoring proteins (AKAPs) are involved in targeting phosphatases to the βAR complex [56]. One of the AKAPs, gravin, has been shown to associate with βARs and its interaction increases with agonist stimulation, recruiting PP2A to the receptor complex [24,25,56]. Another AKAP, AKAP 79, associates with βARs as a multiprotein complex containing PP2b (calcineurin) and PKA [26,27]. These studies suggest that dephosphorylation could occur due to factors present constitutively at the plasma membrane or that are recruited following agonist stimulation. Once these components are present in the complex, they may promote βAR dephosphorylation at the plasma membrane and/or in endosomal compartments.

Finally, there is also an emerging role for endogenous PP inhibitors in the regulation of GRP activity. The observation that phosphoinositide 3-kinase gamma (PI3Kγ) increases βAR desensitization and its absence leads to preservation of receptor function laid the foundation for the hypothesis that PI3Kγ may inhibit βAR resensitization [57–59]. Inhibition of PI3Kγ results in significant activation of PP2A, because endogenous PI3Kγ inhibits PP2A by phosphorylating the intracellular inhibitor of PP2A (I2PP2A), resulting in enhanced binding of I2PP2A to PP2A [28]. The observation that βAR internalization is significantly attenuated by inhibition of PI3Kγ, whereas dephosphorylation of βARs continues under these conditions, indicates that receptors can effectively undergo dephosphorylation and resensitization at the plasma membrane [60].

Evidence for Direct Interaction of PPs and GPCRs

Direct interaction of PPs and GPCRs has been demonstrated by coimmunoprecipitation for only a few receptors. sst_2 was found in a constitutive complex with β-arrestin1 and PP1β [54]. Likewise, NK_1 coimmunoprecipitated with β-arrestin1 and PP2A [46]. A yeast two-hybrid screen with the carboxyl-terminal tail of $mGlu_7$ identified the catalytic subunits of PP1γ1 and PP1γ2 as binding partners [33]. Dopamine D_1 receptors were shown in complex with PP2B, while dopamine D_2 receptors pulled down the PP2A catalytic subunit and regulatory subunit PPP2R2C in renal tissues [61,62]. These examples illustrate how little evidence of such GPCR–PP interaction is currently available yet how critical these types of experiments will be for further understanding of the multiprotein complexes that are involved in the GPCR phosphorylation/dephosphorylation cycle.

In vivo Correlation

Traditionally, GPCR phosphorylation/dephosphorylation cycles have been studied exclusively *in vitro* in heterologous cellular environments by overexpressing the respective GPCR. In a few cases, cell lines expressing a particular GPCR endogenously have also been used. Thus far, *in vivo* agonist-dependent GPCR phosphorylation has been investigated only for MOR, sst_2, and TRH, while *in vivo* dephosphorylation data are available only for MOR. Importantly, no specific PP has been identified to dephosphorylate a particular GPCR *in vivo*. The scarcity of data probably reflects the difficulty of finding tissue preparations that express sufficient quantities of a particular GPCR *in vivo*. A certain enrichment of GPCR protein is sometimes present in highly specialized and rather homogeneous endocrine glands, which were used in the case of sst_2 and TRH.

sst_2 is an important drug target in the treatment of neuroendocrine tumors [63]. However, the responsiveness of the tumor tissue diminishes after prolonged (> 1 year) treatment with the stable somatostatin analog octreotide [64]. This high clinical relevance prompted investigations into sst_2 phosphorylation *in vivo*. In rat pituitary and pancreas and in agreement with the *in vitro* data, octreotide was found to stimulate complete phosphorylation of all four threonine residues

in the ^{353}TTETQRT^{359} cluster of the carboxyl terminal tail, followed by receptor internalization, when rats were treated with the agonist [49]. Interestingly, another stable somatostatin analog, pasireotide or SOM230, failed to stimulate sst_2 phosphorylation and internalization *in vivo*, suggesting biased signaling. Direct investigation of sst_2 in human neuroendocrine tumor samples revealed that sst_2 phosphorylation is observed only in octreotide-treated patients, where most sst_2 receptors are found internalized, whereas in octreotide-naïve biopsy samples all sst_2 protein remained unphosphorylated and on the cell surface [65]. Furthermore, levels of internalization correlated with octreotide dose and treatment duration. Although Waser *et al.* used phosphosite-specific antibodies directed against S^{341}/S^{343} to detect phosphorylated sst_2 and the data are thus not directly comparable to the *in vivo* studies in rats, these results highlight the clinical importance of GPCR phosphorylation as they might help to explain therapeutic responses and the consequences of long-term treatment.

Regarding the TRH receptor, staining of rat pituitary tissue with a phosphosite-specific antiserum demonstrated the presence of phosphorylated TRH receptors in cells secreting thyroid-stimulating hormone (TSH) or prolactin, as expected. In addition, staining increased significantly after treatment of the animals with TRH, showing that agonist-dependent phosphorylation of the native TRH receptor occurs *in vivo* [66].

Due to its prominent role in opioid addiction, MOR *in vivo* phosphorylation was studied in great detail and distinct differences to the *in vitro* data were observed [42]. Most importantly, hierarchical and agonist-specific MOR phosphorylation was replicated in mouse brain tissue, including the first-ever direct quantification of phosphorylated MOR peptide fragments by mass spectrometry [40]. All MOR agonists, including morphine, were found to promote S^{375} phosphorylation *in vivo*. However, a phosphorylation-deficient mutant mouse (S375A) revealed that the development of analgesic tolerance to repeated administration of high-efficacy agonists critically depends on S^{375}. Paradoxically, morphine-induced tolerance appears to involve a mechanism independent of S^{375} phosphorylation [42]. In addition, the importance of GRK3 and GRK5 for MOR phosphorylation and its relation to morphine reward and dependence was demonstrated *in vivo* [40]. Dephosphorylation of MOR at S^{375} occurs shortly after agonist clearance and MOR is fully dephosphorylated after 8 h, whereas analgesic tolerance to morphine persists even after 12 h [42]. The *in vivo* data on MOR phosphorylation and dephosphorylation provide a cautionary example when extrapolating *in vitro* observations to complex *in vivo* functions such as analgesic tolerance. However, at the biochemical level a widespread overlap between heterologous *in vitro* expression systems and agonist-induced MOR phosphorylation in mouse brain is encouraging, supporting the translational power of such models.

Some Caveats

The power of phosphosite-specific antibodies and siRNA knockdown of gene products in the study of GPCR phosphorylation/dephosphorylation cycles is obvious and the combination of the two tool sets will greatly improve the interpretability and comparability of data in future studies. Conversely, our knowledge regarding GPCR phosphorylation and GRPs remains incomplete since not all available studies have employed the same high-quality methods. For example, the identity of any phosphorylated serine/threonine residues is unknown for $mGlu_1$, $mGlu_5$, and $mGlu_7$, and for NK_1. Likewise, many earlier studies used chemical PP inhibitors to 'prescreen' potential phosphatase candidates that were then confirmed by a targeted siRNA knockdown approach (e.g., $mGlu_1$, $mGlu_5$). Also, no siRNA knockdown of PP2A has unequivocally demonstrated the importance of this enzyme for β2AR dephosphorylation. Unbiased siRNA knockdowns of all possible PP catalytic subunits have been performed for only a few GPCRs. It is therefore conceivable that additional PPs contributing to GPCR

dephosphorylation have been missed. Furthermore, all matches between GPCRs and PPs obtained with chemical PP inhibitors should be viewed as preliminary.

A caveat persists in the siRNA approach itself: knockdowns are never complete but only produce a relative low abundance of transcripts and derived proteins. Depending on the stability of both the siRNA and the previously translated protein, the effect size of RNA knockdowns can be limited. This is illustrated by the often incomplete inhibition of GPCR dephosphorylation after siRNA knockdown of specific PP catalytic subunits. Given the multitude of GPCRs and the limited number of PPs, it is most likely that a certain level of PP promiscuity exists, although preferential enzyme–substrate interaction has been demonstrated for all GPCRs under investigation. Ultimately, the demonstration of direct GPCR–phosphatase interaction by unbiased pull-down assays and proteomic analysis will be necessary to identify all phosphatases participating in GPCR dephosphorylation. Such pull-down assays will also be crucial in identifying PP holoenzymes, including all elusive regulatory and structural subunits, which may finally help to explain substrate and targeting specificity. It should, however, be mentioned that available kinetic data indicate a rather short interaction period between the phosphatase and the GPCR substrate, which would certainly impede their identification by pull-down assays.

Beyond linguistics, careful attention should be paid to the terms 'desensitization', 'internalization', and 'receptor resensitization'. Much experimental evidence suggests that each represents a specific stage in the GPCR life cycle and can be functionally separated. As we know now, for instance, GPCRs can be desensitized '*in situ*' at the plasma membrane without undergoing internalization. Likewise, GPCR phosphorylation does not mandate internalization, nor is full dephosphorylation required for resensitization. Similarly, internalized GPCRs may continue unconventional signaling. Thus, internalization should not be viewed as equivalent to desensitization.

Finally, caution should prevail about the generalization of data obtained from heterologous expression systems. Thus far, agonist-induced *in vivo* phosphorylation of only three GPCRs has been investigated in some detail. Good correlation between *in vitro* and *in vivo* data was found for biochemical measures such as agonist-dependent phosphorylation patterns, agonist-induced internalization, and recycling of internalized receptors after agonist removal. However, no specific PP–GPCR interaction has yet been identified *in vivo*. Obviously, further studies on other GPCR phosphorylation/dephosphorylation cycles are required in suitable *in vivo* models to strengthen the significance of the *in vitro* data and possibly link them with complex physiological functions.

Concluding Remarks

GPCR dephosphorylation has long been the stepchild of the much better studied events underlying GPCR phosphorylation, although both events are intimately linked. Recent progress and the introduction of novel experimental tools such as phosphosite-specific antibodies and siRNA knockdown have benefited both fields and helped especially to advance our understanding of GPCR dephosphorylation. Distinct phosphorylation and dephosphorylation patterns of the carboxyl-terminal receptor domains have been elucidated for several GPCRs with the help of phosphosite-specific antibodies. The functional significance of individual phosphoserine/threonine residues for arrestin recruitment, for internalization, or as a determinant of phosphatase selectivity has been mapped. Domain swaps in somatostatin receptors have demonstrated that GRP specificity is primarily determined by the carboxyl-terminal tail. GPCR dephosphorylation is initiated at the plasma membrane and continues along the endocytic pathway. Alternatively, other, as-yet-unidentified enzyme activities may be responsible for dephosphorylation within the cytosol.

Outstanding Questions

What are the identities and functional roles of phosphorylated serine/threonine residues in the GPCR carboxyl-terminal domain in dephosphorylation and GPCR recycling?

What is the hierarchical order and kinetics of phosphorylation/dephosphorylation at specific serine/threonine residues?

In which subcellular compartments does GPCR phosphorylation/dephosphorylation occur and how are enzymatic modifications spatially and temporally regulated?

What are the kinases and phosphatases for each GPCR? What are the identities of structural/targeting subunits in phosphatase holoenzymes and how do they confer substrate specificity?

Is dephosphorylation required for receptor internalization and how important is internalization for GPCR resensitization?

How well are *in vitro* and *in vivo* GPCR phosphorylation/dephosphorylation patterns correlated and is dephosphorylation linked to specific physiological processes in the organism?

GPCRs have been classified based on their β-arrestin interaction. Class A GPCRs, such as sst_5, form relatively unstable β-arrestin complexes, internalize without β-arrestin, and recycle rapidly, while their endocytosis is driven by phosphorylation [67]. Class B receptors, such as sst_2, form stable β-arrestin complexes that co-internalize and recycle slowly. It is currently too early to say whether such differences in arrestin interaction also affect phosphatase recruitment. Clearly, they do not constitute a specificity signature that may assist in selecting distinct PP families. By contrast, the few currently available data suggest that ligand size rather than intracellularly associated proteins may determine preferential PP interaction. More precisely, it appears that PP1-family phosphatases act predominantly on peptide–ligand GPCRs whereas PP2-type phosphatases may prefer GPCRs with small-molecule ligands.

Rapid dephosphorylation of plasma membrane-bound GPCRs has emerged as an important mechanism in receptor resensitization. The observation that β-arrestin1 and PP1β exist as constitutive complexes mediating rapid dephosphorylation of sst_2 receptors at or near the plasma membrane may be the biochemical equivalent. We propose that rapid GPCR dephosphorylation by PPs is a crucial step in a variety of distinct, interdependent cellular functions including receptor resensitization, termination of β-arrestin signaling, and initiation of endosomal G protein signaling.

Future studies investigating the phosphorylation/dephosphorylation cycles of GPCRs should adhere to more standardized protocols using phosphosite-specific antibodies and siRNA knockdown to increase comparability when matching a certain phosphatase to a GPCR (see Outstanding Questions). Unbiased siRNA screens are especially useful in this respect. For the more distant future, it is hoped that proteomic analyses will lead to the identification of PP holoenzyme complexes. Knowledge of the targeting and regulatory subunits associated with the PP catalytic domain will be essential for better understanding of the molecular basis of GPCR–PP substrate specificity.

Additional *in vivo* studies are clearly needed to validate existing *in vitro* data. Finally, suitable mouse models carrying point mutations of GPCR phosphorylation sites may be employed more commonly to investigate complex physiological functions that are hypothesized to be modulated by GPCR phosphorylation/dephosphorylation cycles (see Outstanding Questions).

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft grants SCHU924/10-3, SCHU924/11-2, SCHU924/14-1, SCHU924/15-1, and SFB/TR166-TPC5 and Deutsche Krebshilfe grant 109952.

References

1. Nobles, K.N. *et al.* (2011) Distinct phosphorylation sites on the β_2-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci. Signal.* 4, ra51
2. Prihandoko, R. *et al.* (2016) Distinct phosphorylation clusters determine the signaling outcome of free fatty acid receptor 4/G protein-coupled receptor 120. *Mol. Pharmacol.* 89, 505–520
3. Butcher, A.J. *et al.* (2011) Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. *J. Biol. Chem.* 286, 11506–11518
4. Tobin, A.B. *et al.* (2008) Location, location, location . . . site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol. Sci.* 29, 413–420
5. Barford, D. *et al.* (1998) The structure and mechanism of protein phosphatases: insights into catalysis and regulation. *Annu. Rev. Biophys. Biomol. Struct.* 27, 133–164
6. Bollen, M. *et al.* (2010) The extended PP1 toolkit: designed to create specificity. *Trends Biochem. Sci.* 35, 450–458
7. Ceulemans, H. and Bollen, M. (2004) Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev.* 84, 1–39
8. Eichhorn, P.J. *et al.* (2009) Protein phosphatase 2A regulatory subunits and cancer. *Biochim. Biophys. Acta* 1795, 1–15
9. Janssens, V. *et al.* (2008) PP2A holoenzyme assembly: *in cauda venenum* (the sting is in the tail). *Trends Biochem. Sci.* 33, 113–121
10. Perrotti, D. and Neviani, P. (2008) Protein phosphatase 2A (PP2A), a drugable tumor suppressor in $Ph1^+$ leukemias. *Cancer Metastasis Rev.* 27, 159–168
11. Byk, T. *et al.* (1993) Regulatory arrestin cycle secures the fidelity and maintenance of the fly photoreceptor cell. *Proc. Natl. Acad. Sci. U. S. A.* 90, 1907–1911
12. Vinos, J. *et al.* (1997) A G protein-coupled receptor phosphatase required for rhodopsin function. *Science* 277, 687–690
13. Steele, F.R. *et al.* (1992) *Drosophila* retinal degeneration C (*rdgC*) encodes a novel serine/threonine protein phosphatase. *Cell* 69, 669–676

14. Lee, S.J. and Montell, C. (2001) Regulation of the rhodopsin protein phosphatase, RDGC, through interaction with calmodulin. *Neuron* 32, 1097–1106
15. Pitcher, J.A. *et al.* (1995) The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc. Natl. Acad. Sci. U. S. A.* 92, 8343–8347
16. Krueger, K.M. *et al.* (1997) The role of sequestration in G protein-coupled receptor resensitization. Regulation of β2-adrenergic receptor dephosphorylation by vesicular acidification. *J. Biol. Chem.* 272, 5–8
17. Tran, T.M. *et al.* (2007) Characterization of β2-adrenergic receptor dephosphorylation: comparison with the rate of resensitization. *Mol. Pharmacol.* 71, 47–60
18. Iyer, V. *et al.* (2006) Differential phosphorylation and dephosphorylation of β2-adrenoceptor sites Ser262 and Ser355, 356. *Br. J. Pharmacol.* 147, 249–259
19. Kelly, E. (2006) G-protein-coupled receptor dephosphorylation at the cell surface. *Br. J. Pharmacol.* 147, 235–236
20. Gardner, B. *et al.* (2001) The role of phosphorylation/dephosphorylation in agonist-induced desensitization of D_1 dopamine receptor function: evidence for a novel pathway for receptor dephosphorylation. *Mol. Pharmacol.* 59, 310–321
21. Spurney, R.F. (2001) Regulation of thromboxane receptor (TP) phosphorylation by protein phosphatase 1 (PP1) and PP2A. *J. Pharmacol. Exp. Ther.* 296, 592–599
22. Innamorati, G. *et al.* (1998) A serine cluster prevents recycling of the V_2 vasopressin receptor. *Proc. Natl. Acad. Sci. U. S. A.* 95, 2222–2226
23. Chauvin, S. *et al.* (2002) Parathyroid hormone receptor recycling: role of receptor dephosphorylation and β-arrestin. *Mol. Endocrinol.* 16, 2720–2732
24. Lin, F. *et al.* (2000) Gravin-mediated formation of signaling complexes in beta 2-adrenergic receptor desensitization and resensitization. *J. Biol. Chem.* 275, 19025–19034
25. Tao, J. *et al.* (2003) Protein kinase A regulates AKAP250 (gravin) scaffold binding to the β2-adrenergic receptor. *EMBO J.* 22, 6419–6429
26. Fraser, I.D. *et al.* (2000) Assembly of an A kinase-anchoring protein?–β_2-adrenergic receptor complex facilitates receptor phosphorylation and signaling. *Curr. Biol.* 10, 409–412
27. Cong, M. *et al.* (2001) Regulation of membrane targeting of the G protein-coupled receptor kinase 2 by protein kinase A and its anchoring protein AKAP79. *J. Biol. Chem.* 276, 15192–15199
28. Vasudevan, N.T. *et al.* (2011) Inhibition of protein phosphatase 2A activity by PI3Kγ regulates β-adrenergic receptor function. *Mol. Cell* 41, 636–648
29. Raote, I. *et al.* (2013) Functional selectivity in serotonin receptor 2A ($5\text{-}HT_{2A}$) endocytosis, recycling, and phosphorylation. *Mol. Pharmacol.* 83, 42–50
30. Pandey, S. *et al.* (2014) Metabotropic glutamate receptor 1 recycles to the cell surface in protein phosphatase 2A-dependent manner in non-neuronal and neuronal cell lines. *J. Neurochem.* 131, 602–614
31. Mahato, P.K. *et al.* (2015) Differential effects of protein phosphatases in the recycling of metabotropic glutamate receptor 5. *Neuroscience* 306, 138–150
32. Suh, Y.H. *et al.* (2013) Regulation of metabotropic glutamate receptor 7 (mGluR7) internalization and surface expression by Ser/Thr protein phosphatase 1. *J. Biol. Chem.* 288, 17544–17551
33. Enz, R. (2002) The metabotropic glutamate receptor mGluR7b binds to the catalytic γ-subunit of protein phosphatase 1. *J. Neurochem.* 81, 1130–1140
34. Ghosh, M. and Schonbrunn, A. (2011) Differential temporal and spatial regulation of somatostatin receptor phosphorylation and dephosphorylation. *J. Biol. Chem.* 286, 13561–13573
35. Poll, F. *et al.* (2011) Rapid dephosphorylation of G protein-coupled receptors by protein phosphatase 1β is required for termination of β-arrestin-dependent signaling. *J. Biol. Chem.* 286, 32931–32936
36. Petrich, A. *et al.* (2013) Phosphorylation of threonine 333 regulates trafficking of the human sst_5 somatostatin receptor. *Mol. Endocrinol.* 27, 671–682
37. Lehmann, A. *et al.* (2016) Identification of phosphorylation sites regulating sst_3 somatostatin receptor trafficking. *Mol. Endocrinol.* 30, 645–659
38. Doll, C. *et al.* (2012) Deciphering μ-opioid receptor phosphorylation and dephosphorylation in HEK293 cells. *Br. J. Pharmacol.* 167, 1259–1270
39. Just, S. *et al.* (2013) Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol. Pharmacol.* 83, 633–639
40. Gluck, L. *et al.* (2014) Loss of morphine reward and dependence in mice lacking G protein-coupled receptor kinase 5. *Biol. Psychiatry* 76, 767–774
41. McPherson, J. *et al.* (2010) Mu-opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol. Pharmacol.* 78, 756–766
42. Grecksch, G. *et al.* (2011) Analgesic tolerance to high-efficacy agonists but not to morphine is diminished in phosphorylation-deficient S375A mu-opioid receptor knock-in mice. *J. Neurosci.* 31, 13890–13896
43. Jones, B.W. and Hinkle, P.M. (2005) β-Arrestin mediates desensitization and internalization but does not affect dephosphorylation of the thyrotropin-releasing hormone receptor. *J. Biol. Chem.* 280, 38346–38354
44. Gehret, A.U. and Hinkle, P.M. (2013) siRNA screen identifies the phosphatase acting on the G protein-coupled thyrotropin-releasing hormone receptor. *ACS Chem. Biol.* 8, 588–598
45. Hinkle, P.M. *et al.* (2012) Desensitization, trafficking, and resensitization of the pituitary thyrotropin-releasing hormone receptor. *Front. Neurosci.* 6, 180
46. Murphy, J.E. *et al.* (2011) Protein phosphatase 2A mediates resensitization of the neurokinin 1 receptor. *Am. J. Physiol. Cell Physiol.* 301, C780–C791
47. Rozengurt, E. (2011) Neurokinin 1 receptor desensitization and resensitization: is it all happening at the membrane? Focus on "Protein phosphatase 2A mediates resensitization of the neurokinin 1 receptor". *Am. J. Physiol. Cell Physiol.* 301, C772–C774
48. Sibley, D.R. *et al.* (1987) Regulation of transmembrane signaling by receptor phosphorylation. *Cell* 48, 913–922
49. Poll, F. *et al.* (2010) Pasireotide and octreotide stimulate distinct patterns of sst_{2A} somatostatin receptor phosphorylation. *Mol. Endocrinol.* 24, 436–446
50. DeFea, K.A. (2011) Beta-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? *Cell. Signal.* 23, 621–629
51. Lefkowitz, R.J. and Shenoy, S.K. (2005) Transduction of receptor signals by β-arrestins. *Science* 308, 512–517
52. DeWire, S.M. *et al.* (2007) β-Arrestins and cell signaling. *Annu. Rev. Physiol.* 69, 483–510
53. Luttrell, L.M. and Gesty-Palmer, D. (2010) Beyond desensitization: physiological relevance of arrestin-dependent signaling. *Pharmacol. Rev.* 62, 305–330
54. Kliewer, A. and Schulz, S. (2014) Differential regulation of somatostatin receptor dephosphorylation by β-arrestin1 and β-arrestin2. *Naunyn Schmiedebergs Arch. Pharmacol.* 387, 263–269
55. Lehmann, A. *et al.* (2014) Carboxyl-terminal receptor domains control the differential dephosphorylation of somatostatin receptors by protein phosphatase 1 isoforms. *PLoS One* 9, e91526
56. Shih, M. *et al.* (1999) Dynamic complexes of β2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. *J. Biol. Chem.* 274, 1588–1595
57. Nienaber, J.J. *et al.* (2003) Inhibition of receptor-localized PI3K preserves cardiac β-adrenergic receptor function and ameliorates pressure overload heart failure. *J. Clin. Invest.* 112, 1067–1079
58. Oudit, G.Y. *et al.* (2003) Phosphoinositide 3-kinase gamma-deficient mice are protected from isoproterenol-induced heart failure. *Circulation* 108, 2147–2152

59. Patrucco, E. *et al.* (2004) PI3Kγ modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. *Cell* 118, 375–387

60. Vasudevan, N.T. *et al.* (2011) Regulation of β-adrenergic receptor function: an emphasis on receptor resensitization. *Cell Cycle* 10, 3684–3691

61. Adlersberg, M. *et al.* (2004) Regulation of dopamine D-receptor activation *in vivo* by protein phosphatase 2B (calcineurin). *J. Neurochem.* 90, 865–873

62. Zhang, Y. *et al.* (2016) Dopamine D2 receptors' effects on renal inflammation are mediated by regulation of PP2A function. *Am. J. Physiol. Renal Physiol.* 310, F128–F134

63. Oberg, K. (2005) Neuroendocrine tumors of the gastrointestinal tract: recent advances in molecular genetics, diagnosis, and treatment. *Curr. Opin. Oncol.* 17, 386–391

64. Plockinger, U. *et al.* (2008) Selective loss of somatostatin receptor 2 in octreotide-resistant growth hormone-secreting adenomas. *J. Clin. Endocrinol. Metab.* 93, 1203–1210

65. Waser, B. *et al.* (2012) Phosphorylation of sst_2 receptors in neuroendocrine tumors after octreotide treatment of patients. *Am. J. Pathol.* 180, 1942–1949

66. Jones, B.W. *et al.* (2007) Phosphorylation of the endogenous thyrotropin-releasing hormone receptor in pituitary GH3 cells and pituitary tissue revealed by phosphosite-specific antibodies. *J. Biol. Chem.* 282, 12893–12906

67. Oakley, R.H. *et al.* (2000) Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J. Biol. Chem.* 275, 17201–17210