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Fine-tuning somatostatin receptor signalling by agonist-selective phosphorylation and dephosphorylation: IUPHAR Review 5

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This article, written by members of the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) subcommittee for the somatostatin receptors, confirms the existing nomenclature for these receptors and reviews our current understanding of their structure, pharmacology and functions and their likely physiological roles in health and disease. More information on this receptor family can be found in the Concise Guide to PHARMACOLOGY (http://onlinelibrary.wiley.com/doi/10.1111/bph.12445/abstract) and for each member of the family in the corresponding database http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=61&familyType=GPCR

The biological actions of somatostatin are mediated by a family of five GPCRs, named sst_1 to sst_5. Somatostatin receptors exhibit equally high-binding affinities to their natural ligand somatostatin-14 and largely overlapping distributions. The overexpression of somatostatin receptors in human tumours is the molecular basis for diagnostic and therapeutic application of the stable somatostatin analogues octreotide, lanreotide and pasireotide. The efficiency of somatostatin receptor signalling is tightly regulated and ultimately limited by the coordinated phosphorylation and dephosphorylation of intracellular carboxyl-terminal serine and threonine residues. Here, we review and discuss recent progress in the generation and application of phosphosite-specific antibodies for human sst_2 and sst_5 receptors. These phosphosite-specific antibodies are unique tools to monitor the spatial and temporal dynamics of receptors phosphorylation and dephosphorylation. Using a combined approach of phosphosite-specific antibodies and siRNA knock-down screening, relevant kinases and phosphatases were identified. Emerging evidence suggests distinct mechanisms of agonist-selective fine-tuning for individual somatostatin receptors. The recently uncovered differences in phosphorylation and dephosphorylation of these receptors may hence be of physiological significance in mediating responses to acute, persistent or repeated stimuli in a variety of target tissues.

Abbreviations

ACTH, adrenocorticotropic hormone; GH, growth hormone; GRP, G-protein coupled receptor phosphatase; GRK, G-protein coupled receptor kinase; PP, protein phosphatase; RDGC, retinal degeneration c; SS-14, somatostatin-14; sst, somatostatin receptor; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone

Links to online information in the IUPHAR/BPS Guide to PHARMACOLOGY

Targets	Ligands
β_2-adrenoceptor (β2AR, β2-adrenergic receptor)	adrenocorticotrophin (ACTH)
D_1 receptor (D1R dopamine receptor 1)	BIM 23268
D_2 receptor (D2R dopamine receptor 2)	epidermal growth factor (EGF)
extracellular-signal regulated kinase (ERK)	gastrin-17
beta adrenergic receptor kinase 1 (GRK2, G protein-coupled receptor kinase 2)	growth hormone 1
beta adrenergic receptor kinase 2 (GRK3, G protein-coupled receptor kinase 3)	growth hormone 2
G protein-coupled receptor kinase 5 (GRK5)	ghrelin
G protein-coupled receptor kinase 6 (GRK6)	glucagon
PTH1 receptor (parathyroid hormone receptor 1)	insulin
protein kinase C (PKC)	lanreotide
somatostatin receptors	octreotide
sst_1 receptor (somatostatin receptor 1)	pasireotide
sst_2 receptor (somatostatin receptor 2)	SRIF-14 (SS-14, somatostatin-14)
sst_3 receptor (somatostatin receptor 3)	thyrotropin-releasing hormone (TRH)
sst_4 receptor (somatostatin receptor 4)	thyroid-stimulating hormone (TSH)
sst_5 receptor (somatostatin receptor 5)	
TP receptor (thromboxane A2 receptor)	
V_{1A} receptor (vasopressin receptor 1)	

This table lists protein targets and ligands which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, Alexander *et al.*, 2013b, Alexander *et al.*, 2013c).

Somatostatin and somatostatin analogues

The peptide hormone somatostatin is widely distributed throughout the brain and periphery where it regulates the release of a variety of hormones including growth hormone (GH), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), glucagon, insulin, gastrin and ghrelin (Weckbecker *et al.*, 2003; Park *et al.*, 2012). Natural somatostatin binds with high affinity to all five somatostatin receptor subtypes (Alexander *et al.*, 2013b). However, the clinical utility of somatostatin is limited due to its rapid degradation in human plasma. Consequently, a number of metabolically stable somatostatin analogues including octreotide and lanreotide have been synthesized. Octreotide and lanreotide bind with high sub-nanomolar affinity to sst_2. In clinical practice, octreotide and lanreotide are used as first-choice medical treatment of neuroendocrine tumours such as GH-secreting adenomas and carcinoids (Donangelo and Melmed, 2005; Oberg *et al.*, 2010; Gatto *et al.*, 2013). Recently, the novel multireceptor somatostatin analogue, pasireotide (SOM230), has been approved for the treatment of Cushing's disease, a condition with high sst_5 expression (Ben-Shlomo *et al.*, 2009a; Colao *et al.*, 2012; Feelders and Hofland, 2013). In contrast to octreotide, pasireotide exhibits particular high sub-nanomolar affinity to sst_5 (Ma *et al.*, 2005). Compounds currently under clinical and preclinical evaluation include somatoprim (DG3173) (Plockinger *et al.*, 2012) and dopastatin (BIM23A760) (Jaquet *et al.*, 2005; Ferone *et al.*, 2007). Somatoprim exhibits a unique binding profile with high affinity to sst_2, sst_4 and sst_5. Dopastatin is a chimeric molecule that is directed towards sst_2 somatostatin and D_2 dopamine receptors. Thus, of the five somatostatin receptor subtypes, only sst_2 and sst_5 are proven drug targets for clinically available somatostatin analogues.

Localization of somatostatin receptors in normal and neoplastic human tissues

Unequivocal detection of endogenous GPCRs in human tissues is notoriously difficult. Although early studies succeeded in detecting somatostatin receptors in human neuroendocrine tumours using polyclonal antibodies, the recent generation of rabbit monoclonal antibodies strongly facilitated the immunohistochemical identification of somatostatin receptors in human normal tissues (Fischer *et al.*, 2008; Lupp *et al.*, 2011). In the anterior pituitary, sst_2 immunoreactivity is present at the plasma membrane of GH- and TSH-producing but not ACTH-producing cells. Whereas GH-producing cells express both sst_2 and sst_5, ACTH-

producing cells selectively express sst_5 (Ben-Shlomo *et al.*, 2009b; Ben-Shlomo and Melmed, 2010). In pancreatic islets, both sst_5 and sst_2 immunoreactivity are present at the plasma membrane of all insulin- and glucagon-producing cells. Along the gastrointestinal tract, both sst_2 and sst_5 are abundantly expressed in neuroendocrine cells (Kaemmerer *et al.*, 2011). The majority of sst_2-expressing neuroendocrine cells do not contain somatostatin but exist in close proximity to somatostatin-containing cells. Within the gastric mucosa, virtually all gastrin-containing as well as all ghrelin-containing cells express sst_2 receptors at their plasma membrane. In addition, sst_2 is highly expressed in myenteric neurons that receive dense innervation from somatostatin-containing fibres and terminals. The recent generation of rabbit monoclonal antibodies for sst_1 and sst_3 receptors revealed a largely overlapping expression in the pituitary, pancreatic islets and enteric ganglion cells (Lupp *et al.*, 2012; 2013). However, little is known about the biological significance of this overlapping expression of several somatostatin receptor subtypes in target tissues.

Somatostatin receptors are expressed at high levels in neuroendocrine tumours and endocrine-related malignancies. Pronounced sst_5 expression is found in all GH adenomas and the majority of ACTH-producing pituitary adenomas (Lupp *et al.*, 2011). In contrast, sst_2 is present in only 85% of GH adenomas and not found in ACTH adenomas. Inactive adenomas selectively express sst_3 receptors (Lupp *et al.*, 2012). The majority of neuroendocrine tumours of the gastrointestinal tract express sst_2 and sst_5 receptors with generally higher expression of sst_2 (Oberg *et al.*, 2010). In addition, the majority of prostate carcinomas are sst_5 positive. Likewise, sst_5 is present in nearly all mammary carcinomas whereas sst_2 is present only in a minor fraction of these cases. Recently, two novel splice variants of sst_5 were identified in normal tissues and pituitary tumours (Duran-Prado *et al.*, 2009).

The high expression of somatostatin receptors in neuroendocrine tumours is the molecular basis for diagnostic and therapeutic application of stable somatostatin analogues. The recent generation of rabbit monoclonal antibodies has also provided evidence for the clinical relevance of immunohistochemical somatostatin receptor determination. In fact, several recent studies have shown that the immunocytochemical evaluation of the sst_2 receptor status using the rabbit monoclonal antibody UMB-1 is of predictive value (Fischer *et al.*, 2008; Korner *et al.*, 2012). In fact, UMB-1 immunohistochemistry strongly predicts a biochemical response to adjuvant treatment with octreotide or lanreotide in acromegalic patients (Casarini *et al.*, 2009; Reubi *et al.*, 2010). UMB-1 immunohistochemistry is also a valid tool to select patients suitable for *in vivo* somatostatin receptor targeting (Kaemmerer *et al.*, 2011). These studies clearly show that the sst_2 receptor is the drug target for octreotide and that the sst_2 receptor status should be evaluated by UMB-1 immunohistochemistry before octreotide-based diagnostic or therapeutic intervention.

Whereas UMB-1 immunohistochemistry does not detect sst_2 receptors in ACTH-producing pituitary adenomas, UMB-4 immunohistochemistry reveals sst_5 receptors in nearly all of these cases (Lupp *et al.*, 2011). Conversely, the majority of ACTH adenomas respond to pasireotide but not to octreotide (Ben-Shlomo *et al.*, 2009a). However, it remains to be seen whether the evaluation of the sst_5 receptor status using UMB-4 immunohistochemistry in ACTH adenomas is also of predictive value.

Agonist-selective somatostatin receptor phosphorylation

Signalling and trafficking of somatostatin receptors were recently reviewed in detail (Liu *et al.*, 2005; Cescato *et al.*, 2006; Jacobs and Schulz, 2008; Csaba *et al.*, 2012). We will therefore focus on recent progress in the analysis of their phosphorylation and dephosphorylation. Earlier studies used whole cell phosphorylation assays to elucidate agonist-induced phosphorylation of somatostatin receptors. From these studies, it became evident that the sst_2 receptor can undergo heterologous PKC-mediated and homologous agonist-mediated phosphorylation (Hipkin *et al.*, 2000; Elberg *et al.*, 2002). Agonist-induced sst_2 phosphorylation is rapid and robust and occurs at carboxyl-terminal serine and threonine residues (Liu *et al.*, 2008). Analysis of serial truncation and site-directed mutants identified a cluster of four threonine residues, namely T353, T354, T356 and T359, within the cytoplasmic ^{353}TTETQRT^{359} motif as major sites of agonist-driven phosphorylation (Poll *et al.*, 2010). Phosphorylation of this cluster of threonine residues is required for the formation of stable β-arrestin complexes and subsequent co-internalization of the sst_2 receptor and β-arrestin into the same endocytic vesicles (Tulipano *et al.*, 2004; Liu *et al.*, 2008; Poll *et al.*, 2010). However, such whole cell phosphorylation assays require high amounts of radioactivity and do not allow the examination of the spatial and temporal dynamics of agonist-driven phosphorylation of individual phosphate acceptor sites.

Recently, two independent groups succeeded in the generation of phosphosite-specific antibodies for S341, S343, S348, T353, T354, T356 and T359 of the sst_2 receptor (Liu *et al.*, 2009; Poll *et al.*, 2010; Nagel *et al.*, 2011) (Figure 1). In the presence of somatostatin-14 (SS-14), phosphorylation of all of these sites occurs very rapidly (<1 min). It appears that S341/S343 phosphorylation precedes the phosphorylation of the ^{353}TTETQRT^{359} motif (Schonbrunn, 2008; Poll *et al.*, 2010; Nagel *et al.*, 2011). Mutation of either serine or threonine residues results in delayed but not reduced phosphorylation of the remaining phosphate acceptor sites. In the presence of phorbol esters, S343 is selectively phosphorylated, indicating that the sst_2 receptor is a substrate for heterologous PKC-mediated phosphorylation (Liu *et al.*, 2009).

In addition to serine and threonine phosphorylation, three tyrosine residues at the sst_2 receptor have been reported to modulate receptor signalling when phosphorylated. While Y71 phosphorylation facilitates interaction with p85 regulatory subunit of phosphoinositide 3-kinase (Bousquet *et al.*, 2006), phosphorylation of Y228 and Y312 leads to recruitment of tyrosine phosphatase SHP-2 and inhibition of cell proliferation (Ferjoux *et al.*, 2003).

Serine/threonine phosphorylation of the sst_2 receptor is remarkably agonist- and species-selective. SS-14 and octreotide promote the phosphorylation of all six carboxyl-terminal serine and threonine residues (S341, S343, T353, T354, T356 and T359) in both rat and human sst_2 receptors (Nagel *et al.*,

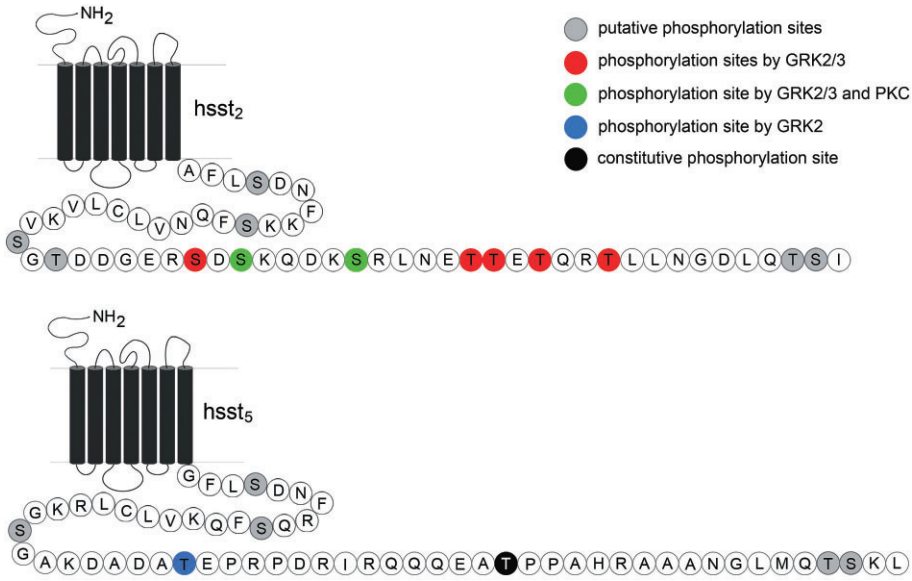

Figure 1

Putative and phospho-specific antibody-proven phosphoacceptor sites in carboxyl-terminal region of human somatostatin receptors sst_2 and sst_5. Schematic representation of the carboxyl-terminal region of human sst_2 and sst_5 receptors. Putative phosphoacceptor sites are marked in grey, constitutive phosphorylated site in sst_5 is depicted in black, and agonist-dependent phosphoacceptor sites are coloured.

2011; Kliewer *et al.*, 2012). In contrast, pasireotide fails to induce any substantial phosphorylation or internalization of the rat sst_2 receptor. Nevertheless, pasireotide is able to stimulate a selective phosphorylation of S341 and S343 of the human sst_2 receptor followed by a clearly detectable receptor sequestration (Nagel *et al.*, 2011; Kliewer *et al.*, 2012). Interestingly, these distinct phosphorylation patterns are paralleled by differential species-selective β-arrestin recruitment. Whereas activation of the human sst_2 receptor by pasireotide facilitates mobilization of β-arrestin (Lesche *et al.*, 2009), such β-arrestin recruitment is not observed at the pasireotide-activated rat sst_2 receptor in HEK293 cells (Poll *et al.*, 2010). In contrast, SS-14 and octreotide promote a robust β-arrestin mobilization at both rat and human sst_2 receptors (Tulipano *et al.*, 2004; Liu *et al.*, 2008; Lesche *et al.*, 2009). Interestingly, overexpression of G-protein coupled receptor kinase (GRK) 2 or 3 but not GRK5 facilitated pasireotide-driven T356/T359 phosphorylation, β-arrestin mobilization and internalization of the rat sst_2 receptor (Poll *et al.*, 2010).

Given the high degree of homology of rat and human sst_2 receptors, the species selectivity of pasireotide is intriguing. Creation of site-directed mutants led to the identification of amino acids 27, 30, 163 and 164, which when exchanged with their human counterparts facilitated pasireotide-driven S341/S343 phosphorylation and internalization of the rat sst_2 receptor (Nagel *et al.*, 2011). Exchange of these amino acids with their rat counterparts completely blocked the pasireotide-mediated internalization of the human sst_2 receptor. Notably, octreotide and somatostatin stimulated a full phosphorylation and internalization of all these mutant sst_2 receptors, strongly suggesting that pasireotide activates the sst_2 receptor via a molecular switch that is structurally and functionally distinct from that turned on during octreotide-driven sst_2 activation.

Agonist-selective sst_2 phosphorylation and internalization is not only observed at receptors heterologously expressed in HEK293 cells but also at endogenous receptors expressed in GH3, INS or AR42J cells (Cescato *et al.*, 2010; Poll *et al.*, 2010; Kao *et al.*, 2011). Interestingly, agonist-selective sst_2 phosphorylation and internalization has also been observed in rat pancreas and pituitary *in vivo* after s.c. application of octreotide or pasireotide (Poll *et al.*, 2010). After full activation of the sst_2 receptor using SS-14 or octreotide, application of increasing concentrations of pasireotide inhibits sst_2 phosphorylation and internalization, indicating that pasireotide acts as partial agonist at the sst_2 receptor (Poll *et al.*, 2010; Kliewer *et al.*, 2012). In a recent study, phosphorylation of S341/S343 was also detected in neuroendocrine tumour samples from octreotide-treated patients (Waser *et al.*, 2012).

These findings have important implications for the clinical utility of octreotide and pasireotide. (i) Tumours that predominantly express sst_2 receptors and exhibit long-lasting responses to octreotide, for example, the majority of GH-secreting adenomas, should remain stable on octreotide. Given the partial agonistic properties of pasireotide, it is conceivable that co-administration of pasireotide and octreotide may potentially limit the clinical benefit of octreotide. (ii) Tumours that show resistance during octreotide treatment and exhibit high levels of sst_5 receptors, for example, octreotide-resistant GH adenomas and carcinoids, are likely to respond to pasireotide. (iii) Given the limited ability of pasireotide to internalize via the sst_2 receptor, pasireotide may be less effective than octreotide for imaging and radiotherapy of sst_2-expressing tumours.

In this regard, pasireotide appears to be unique. Other clinically relevant somatostatin analogues such as somatoprim or dopastatin are more potent sst_2 agonists. However, the functional selectivity of pasireotide at the sst_2

receptor is similar to morphine, which activates the μ-opioid receptor without causing its rapid internalization. Interestingly, different GRKs have been identified that mediate this agonist-selective phosphorylation at the μ-opioid receptor (Doll *et al.*, 2011; 2012; Just *et al.*, 2013). Whereas morphine-driven phosphorylation is preferentially catalysed by GRK5, phosphorylation stimulated by high-efficacy agonists is preferentially catalysed by GRK2 and 3 (Doll *et al.*, 2012). However, such agonist-selective engagement of different GRKs has not been shown at the sst_2 receptor.

Phosphosite-specific antibodies have also been shown to be useful tools to identify the kinases responsible for agonist-induced sst_2 phosphorylation. Combined inhibition of GRK2 and GRK3 expression using specific siRNA sequences was required to produce a significant reduction in SS-14-induced T356/T359 phosphorylation in HEK293 cells (Poll *et al.*, 2010; Nagel *et al.*, 2011). In the same cellular environment, both octreotide- and pasireotide-driven S341/S343 phosphorylation specifically required GRK3. However, in CHO cells, GRK2 also contributes to S341/S343 phosphorylation of the rat sst_2 receptor (Liu *et al.*, 2009). In contrast, inhibition of GRK5 and GRK6 using specific siRNA sequences had no significant effect on sst_2 phosphorylation (Nagel *et al.*, 2011). Thus, the extent and patterns of sst_2 receptor phosphorylation strongly depend on the subcellular complement of GRK2 and GRK3.

The human sst_5 receptor is a major drug target for the novel multireceptor somatostatin analogue pasireotide. However, compared with the closely related sst_2 receptor, little is known about the agonist-driven phosphorylation of its carboxyl-terminal region. Examination of the primary structure of the sst_5 carboxyl-terminal tail revealed the presence of only two potential phosphorylation sites, namely T333 and T347, in the region that corresponds to the phosphorylation-sensitive domain of the sst_2 receptor (Figure 1). Generation of phosphosite-specific antibodies to T333 and T347 revealed that T333 is rapidly phosphorylated in an agonist-dependent manner whereas T347 is constitutively phosphorylated in the absence of agonist (Petrich *et al.*, 2013). In fact, mutation of T333 strongly reduced sst_5 internalization. Interestingly, the previous work of Peverelli and colleagues showed that a truncated sst_5 receptor lacking its carboxyl-terminal 36 amino acids internalized after agonist treatment. This mutant also recruited β-arrestin-2 comparable to wild-type receptor, suggesting an additional role of serine and threonine residues within the third intracellular loop. In fact, mutation of the phosphate acceptor sites within the third intracellular loop including S242 and T247 has also been shown to partially inhibit receptor internalization (Peverelli *et al.*, 2008). Thus, it is possible that these sites play a complementary role in regulating sst_5 internalization. GRK2 was identified as the kinase responsible for T333 phosphorylation in HEK293 cells. There is an excellent correlation between extent and temporal dynamics of carboxyl-terminal T333 phosphorylation of the sst_5 receptor and its trafficking properties. After agonist exposure, sst_5 is phosphorylated at T333 and β-arrestin is recruited to the receptor (Peverelli *et al.*, 2008). Unlike that seen for the sst_2 receptor, the β-arrestin-sst_5 complex is rapidly disrupted and the receptor internalizes without β-arrestin into early endosomes. After 30 min, up to 30% of sst_5 surface receptor is being internalized. In contrast, more than 80% of all sst_2 receptors are subject to SS-14-induced internalization under otherwise identical conditions.

Phosphorylation of the sst_5 receptor is also extremely agonist selective. Natural SS-14 induces a rapid and dose-dependent T333 phosphorylation (Petrich *et al.*, 2013). The multireceptor somatostatin analogue pasireotide and the sst_5-selective ligand L-817,818 are able to promote a clearly detectable T333 phosphorylation. In contrast, no such signal is seen after incubation with octreotide. However, none of these compounds stimulate T333 phosphorylation of sst_5 to the same extent as the natural ligand SS-14. Interestingly, the only compound that was able to stimulate T333 phosphorylation to a similar degree as SS-14 was the sst_5-selective agonist BIM-23268 (Shimon *et al.*, 1997).

To date, little is known about the phosphorylation of somatostatin receptors sst_1 and sst_3. The phosphorylation of sst_1 was shown to be independent of the signalling of the receptor (Liu and Schonbrunn, 2001). For the rat sst_3, three serine and one threonine residue in the cytoplasmatic carboxyl-terminal tail of the receptor were identified as the main phosphorylation sites (Roth *et al.*, 1997).

It is likely that dimerization, palmitoylation and PDZ domain interactions may influence somatostatin receptor phosphorylation. The sst_2 receptor can form heterodimers with sst_5 receptors; indeed, co-expression of sst_5 receptors has been reported to reduce internalization and desensitization of sst_2 receptors in CHO cells (Grant *et al.*, 2004). However, so far phosphorylation and trafficking have been examined in cells expressing either sst_2 or sst_5. It would be interesting to know whether sst_2 or sst_5 receptors would be differently regulated in co-expressing cells. Moreover, palmitoylation of GPCRs has also been shown to influence to phosphorylation of thyrotropin-releasing hormone (TRH; Gehret *et al.*, 2010) and vasopressin receptors (Hawtin *et al.*, 2001). Interestingly, Kokkola *et al.* (2011) identified ZDHHC5 as the palmitoyltransferase that binds to the sst_5 receptor.

Mechanisms of somatostatin receptor dephosphorylation

The regulation of agonist-induced phosphorylation has been studied in great detail for many GPCRs. In contrast, the molecular mechanisms and functional consequences of their dephosphorylation are far from being understood. Earlier studies identified retinal degeneration C (RDGC) as a phosphatase required for rhodopsin dephosphorylation in *Drosophila melanogaster*. The catalytic domain of RDGC exhibits high homology to protein phosphatase 1 (PP1), PP2 and PP3 (Steele *et al.*, 1992; Byk *et al.*, 1993; Vinos *et al.*, 1997). Loss of RDGC causes disturbance of light-signal transduction and leads to light-dependent retinal degeneration (Vinos *et al.*, 1997). At the same time, a PP2-related phosphatase that dephosphorylates the β_2-adrenoceptor was identified and named G-protein coupled receptor phosphatase (GRP) (Pitcher *et al.*, 1995; Krueger *et al.*, 1997). It was proposed that GRP is tethered to vesicular membranes and that receptors have to internalize into an acidic endosomal compartment to become dephosphorylated (Pitcher *et al.*, 1995; Krueger *et al.*,

1997). However, later it was shown that inhibition of β_2-adrenoceptor internalization with dominant-negative dynamin or hypertonic sucrose did not affect the rate of receptor dephosphorylation. Similarly, D_1 dopamine receptor dephosphorylation was not blocked in the presence of concanavalin A, which also inhibits receptor internalization (Gardner *et al.*, 2001). More recent studies have shown that phosphatase inhibitors such as okadaic acid and calyculin A can block the dephosphorylation of a number of GPCRs including the β_2-adrenoceptor, D_1 dopamine receptor, parathyroid hormone receptor 1, thromboxane A receptor and the vasopressin receptor 1 (Innamorati *et al.*, 1998; Gardner *et al.*, 2001; Spurney, 2001; Chauvin *et al.*, 2002; Tran *et al.*, 2007).

For the sst_2 receptor, Ghosh and Schonbrunn (2011) reported different spatial and temporal patterns of receptor dephosphorylation. Specifically, reversal of receptor phosphorylation was determined by the duration of prior agonist exposure. The dephosphorylation of acutely stimulated cells, where most receptors are still located at the surface, occurred only on T353/354 but not on S341/343. In contrast, when cells were stimulated long enough to allow receptor internalization, S341/343 and T353/354 were rapidly dephosphorylated. Surprisingly, T353/354 dephosphorylation was not abolished by treatment with hypertonic sucrose or dynasore, which blocks receptor internalization, whereas S341/343 dephosphorylation was completely prevented under these conditions. In CHO cells, T353/354 but not S341/343 dephosphorylation was sensitive to okadaic acid. These results suggest that receptor dephosphorylation is determined by the duration of agonist stimulation and compartment-specific enzymatic activity. However, these studies did not identify a specific phosphatase responsible for GPCR dephosphorylation.

More recently, Poll *et al.* used a combination of phosphosite-specific antibodies, chemical PP inhibitors and siRNA knock-down screening to identify the GPCR phosphatase that catalyses rapid dephosphorylation of T353, T354, T356 and T359 of the sst_2 receptor (Poll *et al.*, 2011). Complete dephosphorylation of the ^{353}TTETQRT^{359} motif occurs within 30 min after agonist removal. In HEK293 cells, the phosphatase activity required for this rapid dephosphorylation was inhibited in a dose-dependent manner only by calyculin A but not by okadaic acid. Both calyculin A and okadaic acid can effectively block the activity of PP2, PP4 and PP5. In contrast to okadaic acid, calyculin A is also a potent inhibitor of PP1 activity, suggesting that PP1 dephosphorylates the ^{353}TTETQRT^{359} motif of the sst_2 receptor. Three distinct catalytic subunits named α, β and γ are known for PP1. Simultaneous knock-down of all three catalytic subunits confirmed that PP1 activity was required for efficient sst_2 dephosphorylation. Selective inhibition of PP1α or PP1γ expression had no effect on sst_2 dephosphorylation. In contrast, inhibition of PP1β expression resulted in an enhancement of ^{353}TTETQRT^{359} phosphorylation in the presence of agonist and a clearly delayed receptor dephosphorylation after agonist removal. Inhibition of PP2α, PP2β, PP4 or PP5 expression did not alter the time course of sst_2 dephosphorylation. Thus, these findings identify PP1β as bona fide GPCR phosphatase for the β-arrestin acceptor site of the sst_2 receptor.

Inhibition of PP1β expression facilitates detection of phosphorylated sst_2 receptors at the plasma membrane even 5 min after agonist exposure. This enhanced ability to detect phosphorylated sst_2 receptors at the plasma membrane persists 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 of Ghosh and Schonbrunn (2011), showing that T353/T354 dephosphorylation did not require receptor internalization. Interestingly, S341/S343 dephosphorylation occurs with a delayed time course. It is possible that sst_2 dephosphorylation is initiated at the plasma membrane and continues along the endocytic pathway. Alternatively, a second yet unidentified enzyme activity may be responsible for sst_2 dephosphorylation within the cytosol.

GPCR dephosphorylation has long been viewed as an unregulated process of limited functional significance. Initial evidence suggests that PP1β-mediated ^{353}TTETQRT^{359} dephosphorylation may play a role in fine-tuning unconventional β-arrestin-dependent signalling. GRK2/3-driven phosphorylation of the ^{353}TTETQRT^{359} motif is essential for β-arrestin binding (Poll *et al.*, 2010) that facilitates G_i-protein-independent, β-arrestin-dependent ERK activation (Poll *et al.*, 2011). Inhibition of PP1β expression results in a robust increase in β-arrestin-dependent ERK activation in SS-14-treated HEK293 cells that stably express the sst_2 receptor (Poll *et al.*, 2011). This effect was not observed after exposure to EGF 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 (Poll *et al.*, 2011). These findings suggest a model where 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 signalling. This could be a common mechanism for many GPCRs.

A comparative examination of sst_5 and sst_2 receptors reveals strikingly different patterns of dephosphorylation and recycling. Whereas fast sst_5 trafficking correlates with the rapid T333 phosphorylation and dephosphorylation, sst_2 recycling appears to be delayed due to its slow dephosphorylation. Analysis of the sst_5 receptor using chemical inhibitors and siRNA knock-down screening reveals that T333 dephosphorylation is inhibited in a dose-dependent manner only by calyculin A but not by okadaic acid, suggesting that PP1 activity was required. siRNA knock-down experiments revealed that only PP1γ knock-down results in a robust inhibition of sst_5 dephosphorylation (Petrich *et al.*, 2013). In contrast, transfection of PP1α or PP1β siRNA has no effect on sst_5 dephosphorylation. These results indicate that PP1γ is the GPCR phosphatase responsible for rapid T333 dephosphorylation of sst_5. This is an intriguing finding. Thus, after the initial observation of PP1β as GPCR phosphatase for sst_2, PP1γ is the second GPCR phosphatase identified. More recently, Gehret and Hinkle (2013) reported that siRNA knock-down of PP1α inhibits dephosphorylation of the TRH receptor. However, knock-down of all three PP1 catalytic subunits suppresses TRH receptor dephosphorylation much more powerfully than knock-down of PP1α alone, suggesting that different PP1 isoforms could function redundantly. Neverthe-

less, it is unclear which mechanisms regulate PP1 selectivity and specificity. It is possible that either carboxyl-terminal phosphorylation motifs, specific sequences within the intracellular loops of the receptor or the β-arrestin trafficking patterns may contribute to phosphatase selection.

Outstanding issues and questions

1 Does the agonist-selective regulation of sst_2 receptor phosphorylation by octreotide and pasireotide influence responses during long-term treatment of neuroendocrine tumours?
2 What is the biological significance of PKC-mediated S343 phosphorylation of the sst_2 receptor? Which isoforms of PKC are involved? Does it lead to heterologous desensitization?
3 The mechanisms of constitutive T347 phosphorylation of sst_5 are not understood. Does it occur in human tissues *in vivo*?
4 How are phosphorylation and dephosphorylation regulated in target tissues co-expressing sst_2 and sst_5 receptors? Does it affect the response to long-term treatment with somatostatin analogues?
5 What are the determinants for PP1 selectivity? How is the PP1 complex assembled and recruited to phosphorylated GPCRs?

Concluding remarks

GPCRs regulate a myriad of physiological processes. Termination of signalling of activated GPCRs is essential for maintenance of cellular homeostasis. Desensitization of GPCR signalling causes a reduction of receptor response to repeated or long-lasting stimuli. Agonist-induced phosphorylation allows binding of β-arrestin to the receptor that promotes desensitization of G-protein signalling and induces receptor internalization. The classical paradigm of the GPCR life cycle dictates that receptors have to internalize into an acidic endosomal compartment to become dephosphorylated.

Emerging evidence suggests that closely related members of the somatostatin receptor family exhibit strikingly different patterns of phosphorylation and dephosphorylation that result in different spatial and temporal dynamics of their β-arrestin trafficking and recycling. In fact, GRK3-mediated phosphorylation of at least six phosphate acceptor sites promotes a stable association of the sst_2 receptor with β-arrestin. PP1-mediated dephosphorylation requires extended time periods and facilitates slow recycling. Selective GRK2-mediated T333 phosphorylation of sst_5 promotes the formation of instable β-arrestin complexes. Rapid T333 dephosphorylation and recycling of sst_5 specifically require PP1γ.

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Conflict of interest

None.

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Supporting information

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Table S1 Biological and chemical targets and ligands.