

REVIEW

G-protein-coupled receptor phosphorylation: where, when and by whom

AB Tobin

Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, UK

Almost all G-protein coupled receptors (GPCRs) are regulated by phosphorylation and this process is a key event in determining the signalling properties of this receptor super-family. Receptors are multiply phosphorylated at sites that can occur throughout the intracellular regions of the receptor. This diversity of phospho-acceptor sites together with a lack of consensus phosphorylation sequences has led to the suggestion that the precise site of phosphorylation is not important in the phosphorylation-dependent regulation of GPCR function but rather it is the increase in bulk negative charge of the intracellular face of the receptor which is the significant factor. This review investigates the possibility that the multi-site nature of GPCR phosphorylation reflects the importance of specific phosphorylation events which mediate distinct signalling outcomes. In this way receptor phosphorylation may provide for a flexible regulatory mechanism that can be tailored in a tissue specific manner to regulate physiological processes. By understanding the flexible nature of GPCR phosphorylation it may be possible to develop agonists or allosteric modulators that promote a subset of phosphorylation events on the target GPCR and thereby restrict the action of the drug to a particular receptor mediated signalling response.

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Abbreviations: Akt/PKB, protein kinase B; CHO, Chinese hamster ovary; ERK, extracellular-regulated protein kinase; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; PKA, protein kinase A; PKC, protein kinase C

Introduction

It has been known for more than 20 years that G-protein-coupled receptors (GPCRs) are regulated by phosphorylation (Lefkowitz, 2004). From the outset it was clear that for the non-visual GPCRs, the process of stimulus-dependent receptor phosphorylation was mediated by more than one protein kinase family. Studies on the β_2 -adrenoceptor determined that both protein kinase A (PKA) (Benovic *et al.*, 1985) and β -adrenoceptor kinase (Benovic *et al.*, 1986), later to be renamed G-protein-coupled receptor kinase 2 (GRK2), could mediate receptor phosphorylation. At this early stage it was clear that these different protein kinases, activated by distinct mechanisms, were able to phosphorylate different sites on the receptor and that this resulted in different signalling outcomes (Lohse, 1993).

Over the intervening years, however, many of the important concepts illustrated in these early studies have

been lost in an attempt to generate a unifying hypothesis that would account for the regulation of GPCR function across the entire GPCR superfamily. This search for common regulatory mechanisms has resulted in a clear understanding of the role played by the GRK family and arrestins in universal processes such as receptor desensitization and internalization (Ferguson, 2001). GPCRs are, however, a remarkably diverse and widespread protein family involved in the regulation of nearly every biological response (Kristiansen, 2004; Maudsley *et al.*, 2005). The same receptor subtype is often expressed on very different tissues where it regulates defined physiological responses. It seems likely, therefore, that GPCRs will be regulated differentially in the various tissues in which they are expressed and that this will be in line with the tissue-specific function of the receptor.

In this review, we will be considering the possibility that receptor phosphorylation might be mediated by a range of receptor kinases able to phosphorylate distinct sites on GPCRs. This site-specific phosphorylation would result in a specific signalling outcome. In this way, receptor phosphorylation might act as a dynamic and flexible regulatory process that could be tailored in a cell type-specific manner.

Correspondence: Dr AB Tobin, Department of Cell Physiology and Pharmacology, University of Leicester, University Road, PO Box 138, Medical Sciences Building, Leicester LE1 9HN, UK.

E-mail: tba@le.ac.uk

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The role of phosphorylation in GPCR interaction with arrestin

GPCRs essentially function as dynamic scaffolding proteins interacting with an array of signalling molecules in a manner that can be influenced by conformational changes in the receptor induced by agonist occupation (Milligan and White, 2001; Kreienkamp, 2002; Bockaert *et al.*, 2004). The publication of the keenly anticipated crystal structures of the β_2 -adrenoceptor in complex with an inverse agonist (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007) provides hope that in the near future a definitive structural model of an active GPCR will be possible. In the mean time the consensus view is that agonist binding results in a change in the relative positions of transmembrane α -helices III and VI, which results in a re-orientation of the third intracellular loop in a manner that allows for G-protein coupling (Kobilka, 2002). This agonist-induced conformational change is also thought to unmask sites on the intracellular domains that can be modified by phosphorylation (Pitcher *et al.*, 1998). This combined process of conformational change and post-translational modification allows for the receptor to interact with proteins that previously were unable to interact with the receptor. Alternatively, the conformational change in the receptor following agonist binding can induce a conformational change in interacting partners that are present in a pre-assembled complex with the receptor and in so doing 'activate' the signalling properties of the partners. Among a growing list of GPCR interacting proteins (Milligan and White, 2001; Kristiansen, 2004), the arrestin family is the most extensively studied and represents the archetypical phosphorylation-dependent interacting partner.

There are four members of the arrestin family. Arrestin1 and arrestin4 are the so-called visual arrestins expressed only in rod and cone photoreceptors where the interaction with activated and phosphorylated rhodopsin and cone opsins results in termination of phototransduction (Luttrell and Lefkowitz, 2002). The remaining two arrestins, arrestin2 and arrestin3, are able to regulate the activity of the many hundreds of non-visual GPCRs (Gurevich and Gurevich, 2006). Early studies determined that arrestin binding is a two-component process where both the phosphorylated receptor and the ligand-activated conformation of the receptor were recognized by distinct regions on the arrestin molecule (Gurevich and Benovic, 1993). This cooperative binding served to change the conformation of the arrestin molecule to an 'active' state with a high affinity for the receptor (Hirsch *et al.*, 1999). Modelling studies carried out primarily by the Gurevich group demonstrated how the association of arrestin with multiple sites on the intracellular regions of GPCRs results in a complex that effectively 'caps' the GPCR and hence precludes G-protein coupling (Gurevich and Gurevich, 2006). In this way, arrestin is able to uncouple or desensitize the receptor from G-protein-dependent signalling pathways (Krupnick and Benovic, 1998). It has also become clear that in addition to sterically hindering G-protein interaction, arrestins also act as scaffolding proteins for molecules, such as src and components of the mitogen-activated protein kinase cascade and through this route are able to activate G-protein-independent

signalling (Lefkowitz and Whalen, 2004; Lefkowitz and Shenoy, 2005).

The relationship between receptor phosphorylation and arrestin binding for the visual arrestins is relatively clear. In this case, phosphorylation of serines and threonines contained in a cluster in the C-terminal tail of rhodopsin (Ohguro *et al.*, 1993, 1996) results in a 10-fold increase in the affinity of arrestin1 for the light-activated receptor (Gurevich *et al.*, 1995). The phosphate sensor on arrestin lies buried in a polar core and consists primarily of a salt bridge between a conserved arginine (R175 in arrestin1) and an aspartate (D296) (Vishnivetskiy *et al.*, 1999, 2002). Phosphates contained on the receptor phospho-acceptor sites are proposed to be 'guided' to the polar core on arrestin by interaction with lysine residues on the concave surface of the arrestin molecule (Gurevich and Gurevich, 2006). The disruption of the salt bridge at the polar core together with the driving force derived from multiple interactions of the concave surface of arrestin with the activated receptor conformation (the so-called activation sensor) (Vishnivetskiy *et al.*, 2004) results in the conformational change in arrestin that allows for high-affinity receptor binding (Hirsch *et al.*, 1999; Gurevich and Gurevich, 2006) (Figure 1).

Whereas the role of rhodopsin phosphorylation in recruiting arrestin1 is clear, this is not the case for the non-visual

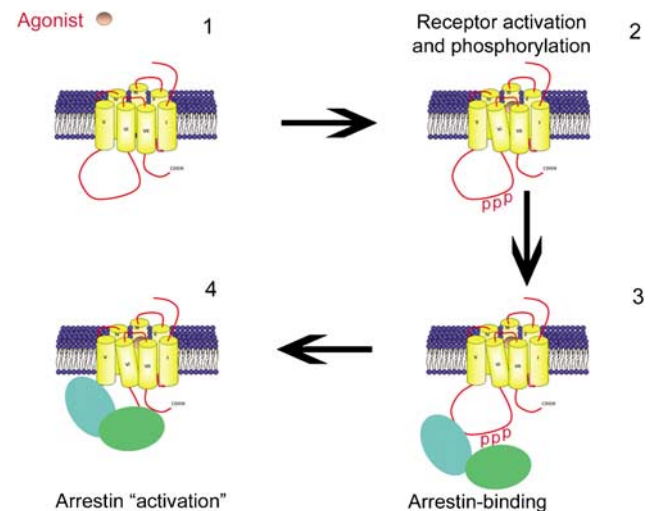


Figure 1 Interaction of arrestin with an activated and phosphorylated G-protein-coupled receptor. (1) Agonist binding to the receptor results in a conformational change that particularly affects the orientation of transmembrane helices III and VI. (2) This conformational change is thought to result in as yet undefined changes in the intracellular loop regions and C-terminal tail that unmask phosphorylation sites and results in multi-site phosphorylation (P). (3) The active conformation of the receptor is detected by the activation sensor on arrestin. The activation sensor consists of multiple interaction sites situated on the concave surface of the arrestin molecule that are able to engage with the active conformation of the receptor. Also, phosphorylated residues are shown initially interacting with residues (lysines) situated on the outer surface of the arrestin molecule. (4) Arrestin binds with high affinity to the receptor and adopts an active conformation through further interactions with the activated receptor via the activation sensor and by phosphates interacting with the phosphate sensor situated in the polar core. The active conformation of arrestin is proposed to involve re-orientation of the two domains of arrestin.

GPCRs. Unlike the visual system where rhodopsin phosphorylation dramatically increases the affinity of the activated receptor for arrestin, the phosphorylation of non-visual GPCRs has only a small effect on arrestin affinity, often amounting to no more than a 2- to 3-fold increase (Gurevich and Gurevich, 2006). This modest increase in affinity occurs despite the fact that the essential features of the molecular mechanism of receptor/arrestin binding appear to be conserved between the visual and non-visual systems. Thus, arrestin2 and 3 are thought to make multiple contacts with the ligand-occupied receptor through residues on the concave surface of arrestin that act as the activation sensor. This operates cooperatively with the interaction of receptor phosphates at the phosphate sensor situated at the polar core (Gurevich and Gurevich, 2006). The issue that faces arrestin2 and 3 is that these two molecules are regulators of 600–800 GPCR subtypes, which show very little sequence homology in their intracellular domains and that are phosphorylated at multiple sites within different receptor regions. Thus, GPCRs have been shown to be phosphorylated on the C-terminal tail (Bouvier *et al.*, 1988; Fredericks *et al.*, 1996; Seibold *et al.*, 2000), third intracellular loop (Budd *et al.*, 2001; Kim *et al.*, 2001; Trester-Zedlitz *et al.*, 2005; Torrecilla *et al.*, 2007), first intracellular loop (Nakamura *et al.*, 1998) and possibly on the second intracellular loop (Kim *et al.*, 2001). Receptor phosphorylation can also be mediated by very different protein kinases (Hausdorff *et al.*, 1989; Torrecilla *et al.*, 2007), which in some cases have been demonstrated to promote arrestin binding (Benovic *et al.*, 1987; Lohse *et al.*, 1992; Gurevich *et al.*, 1995; Krasel *et al.*, 2005) and in others to have no effect on arrestin interaction (Lohse *et al.*, 1992). The result of this heterogeneity is a range of receptor–arrestin affinities where certain receptors such as, V2 vasopressin receptor, have a sufficiently high affinity for the arrestin–receptor complex to remain intact during the trafficking of the receptor to intracellular compartments following agonist stimulation (Oakley *et al.*, 1999). Such receptors have been classified as Class B receptors (Oakley *et al.*, 2000). This compares with Class A receptors, such as β_2 -adrenoceptor, that show a relatively low affinity for arrestin and where the receptor–arrestin complexes are much harder to visualize (Oakley *et al.*, 1999, 2000).

It is also clear that for some receptors, phosphorylation is not an absolute requirement for arrestin binding. This is seen for the substance P (Richardson *et al.*, 2003), lutropin (Mukherjee *et al.*, 2002) and leukotriene B4 (Jala *et al.*, 2005) receptors where phosphorylation-deficient mutants still maintain the ability to interact with arrestin. Furthermore, arrestin binding is maintained in mutants of the oxrexin-1 (Milasta *et al.*, 2005) and protease-activated receptor-2 receptors (Stalheim *et al.*, 2005) where the putative phosphorylation sites had been removed. It is possible that the multiple interactions of the receptor with the activator sensors on arrestin are sufficient to allow for a stable complex in the absence of phosphorylation. This notion has been extended to suggest that each elementary interaction between the receptor and arrestin has low affinity in the micromolar range (Wu *et al.*, 1997; Cen *et al.*, 2001; Liu *et al.*, 2004). It is the cooperative binding of these elementary interactions that results in the overall

high-affinity interaction of the receptor with arrestin. In this model, elements of arrestin interaction could be lost (that is, phosphorylation-dependent interaction) with a consequent loss in the affinity of the receptor for arrestin. However, the reduction in affinity may not be sufficient to completely prevent arrestin binding (Gurevich and Gurevich, 2006).

Phosphorylation-independent binding of some receptors may, however, be explained by the presence of negatively charged residues that act as phospho-mimetics. An acidic cluster in the C-terminal tail of the chemokine receptor D6, which appears constitutively bound to arrestin, plays a key role in arrestin interaction (Galliera *et al.*, 2004). Similarly, an aspartate in the third intracellular loop of the lutropin receptor is thought to interact with the phosphate sensor in arrestin (Mukherjee *et al.*, 2002) and that this interaction drives phosphorylation-independent receptor internalization. Given the diversity of GPCR supergene family, it is likely that the D6 and lutropin receptors are not the only examples of receptors that mediate arrestin binding via charged amino-acid residues.

Does site-specific phosphorylation contribute to specific properties of receptor–arrestin interaction?

It is remarkable that arrestin binding to the phosphorylated form of non-visual GPCRs can tolerate the diverse nature of GPCR phosphorylation. This flexibility in arrestin binding has prompted the hypothesis that arrestin can interact with negative charge placed at a number of different positions on the receptor. It has been suggested that it is the amount of negative charge introduced through phosphorylation rather than the precise position or site of phosphorylation, which is the important factor in recruiting arrestin to the receptor (Gurevich and Gurevich, 2006). Thus, arrestin binding appears to require at least two phosphorylated residues in close proximity (Gurevich and Benovic, 1993). The phospho-acceptor sites on GPCRs very often occur at serine/threonine clusters where more than one of the residues are phosphorylated (Wilden and Kuhn, 1982; Smith *et al.*, 1998; Guo *et al.*, 2000; Lee *et al.*, 2000b; Braun *et al.*, 2003). Because these phosphorylation sites appear in different intracellular domains (C-terminal tail, third, second and first intracellular loops), it has been speculated that it is the 'bulk' negative charge that is important rather than the specific amino-acid sequence of the phospho-acceptor sites (Gurevich and Gurevich, 2006).

It appears, however, that not all phosphorylation events are equal. The first indication of this was that phosphorylation of the β_2 -adrenoceptor at the PKA/protein kinase C (PKC) site in the distal portion of the third intracellular loop and the PKA site at the proximal region of the C-terminal tail (Yuan *et al.*, 1994; Moffett *et al.*, 1996; Tran *et al.*, 2004) did not result in the recruitment of arrestin (Lohse *et al.*, 1992). In contrast, GRK-mediated phosphorylation at the C-terminal tail mediated arrestin binding. Although, both phosphorylation by the GRKs and PKA/PKC results in β_2 -adrenoceptor desensitization the mechanisms employed by these receptor kinases are distinct (Roth *et al.*, 1991; Lefkowitz, 1998; Pitcher *et al.*, 1998).

More recently, studies on the angiotensin AT_{1A} and vasopressin V2 receptors have demonstrated that GRK2 is the protein kinase primarily responsible for receptor phosphorylation and arrestin recruitment in transfected HEK293 cells (Oppermann *et al.*, 1996; Kim *et al.*, 2005; Ren *et al.*, 2005). These receptors stimulate a biphasic extracellular-regulated protein kinase (ERK) response where the initial peak response is dependent on G-protein coupling and the prolonged response is mediated by the recruitment of arrestin (Wei *et al.*, 2003; Kim *et al.*, 2005; Ren *et al.*, 2005). In this case, it might be predicted that disrupting GRK2-mediated receptor phosphorylation and arrestin recruitment would disrupt the prolonged phase of ERK activity. Surprisingly, inhibition of GRK2 activity by siRNA had little effect on AT_{1A} and V2 receptor-mediated ERK activity (Wei *et al.*, 2003; Ren *et al.*, 2005). In contrast, inhibition of GRK5 and 6, despite having no detectable effect on receptor phosphorylation, reduced arrestin recruitment by 30–50% and inhibited the prolonged (arrestin-dependent) phase of the ERK response (Kim *et al.*, 2005; Ren *et al.*, 2005). Other arrestin-mediated processes such as receptor internalization and desensitization were, however, attributed to GRK2-mediated phosphorylation and not GRK5/6 activity (Kim *et al.*, 2005; Ren *et al.*, 2005). One possible interpretation of these data is that GRK2 phosphorylates the AT_{1A} and V2 receptors at different sites from those phosphorylated by GRK5/6. As a result, it has been suggested that the arrestin bound to the receptor phosphorylated by GRK2 could adopt a conformation that is different from that adopted when arrestin is bound to the receptor phosphorylated by GRK5/6 (Wei *et al.*, 2003; Kim *et al.*, 2005). In the case of the AT_{1A} and V2 receptors, the arrestin conformation adopted in response to GRK2 phosphorylation would be important for G-protein uncoupling and that adopted following GRK5/6 phosphorylation would be important in coupling the receptor to the ERK pathway (Wei *et al.*, 2003; Kim *et al.*, 2005; Ren *et al.*, 2005).

Further experiments on the β_2 -adrenoceptor have demonstrated that the recruitment of arrestin itself may be dependent on the isotype of GRK involved in receptor phosphorylation. Individual siRNA knockdown of GRK2, 3, 5 and 6 had differential effects on the kinetics of arrestin recruitment to the receptor (Violin *et al.*, 2006). This could be interpreted as the GRKs mediating differential phosphorylation of the receptor and this in turn affecting different rates of arrestin recruitment. A cautionary note sounded by the authors of these experiments is that there is currently no evidence from intact cells that the various members of the GRK family can phosphorylate receptors differentially (Kim *et al.*, 2005; Ren *et al.*, 2005; Violin *et al.*, 2006).

Further evidence that the specific site on which a receptor is phosphorylated is able to mediate specific signalling outcomes has emerged from studies on the M₂-muscarinic receptor. In this case, mutation of serines/threonines to alanines in two regions of the third intracellular loop known as the N- and C-clusters prevented agonist-dependent receptor phosphorylation, internalization and desensitization (Pals-Rylaarsdam and Hosey, 1997). The preferred phosphorylation sites are at the C-cluster where phosphorylation mediates the interaction of the receptor with

arrestin (Lee *et al.*, 2000a). Removal of the C-cluster prevents the association of wild-type arrestin with the M₂-muscarinic receptor, but surprisingly it also prevented interaction with the phosphorylation-independent arrestin mutant arrestin-R169E (Kovoor *et al.*, 1999). This arrestin construct, which is mutated in the phosphate sensor, is constitutively active adopting a conformation that mimicks that of the phosphate-docked arrestin (Kovoor *et al.*, 1999). Deletion of a 16 amino-acid region in the M₂-muscarinic receptor, which included at its heart the C-cluster, restored the ability of the M₂-muscarinic receptor to interact with both wild-type arrestin and arrestin-R169E (Lee *et al.*, 2000b). These results indicated that far from the C-cluster containing phosphorylation sites that allowed for the receptor to interact with the phosphate sensor in arrestin in a conventional way, the C-cluster rather was at the centre of a region that acted as a brake on arrestin binding. Phosphorylation of the C-cluster released the brake and allowed the receptor to interact with arrestin. This was the first demonstration of such a brake mechanism that appears to be in operation in a number of other receptor types, including the δ -opioid (Whistler *et al.*, 2001) and follitropin receptors (Kishi *et al.*, 2002).

It appears, therefore, that the precise sites of phosphorylation on any one particular receptor subtype can be important in the interaction of the receptor with arrestin. Hence, although bulk negative charge can be seen to contribute to arrestin binding it is very likely that, at least for some receptors, the amino-acid context in which the phosphorylation takes place will also be important in dictating the role of phosphorylation in regulating the signalling properties of the receptor.

Regulation of signalling associated with site-specific phosphorylation of the β_2 -adrenoceptor

It appears that desensitization of the adenylate cyclase response mediated by the β_2 -adrenoceptor involves both uncoupling from G_s protein and also increased degradation of cyclic AMP. One of the multitude of arrestin-binding partners are isotypes of phosphodiesterases responsible for the hydrolysis of cyclic-AMP (Perry *et al.*, 2002). Recruitment of phosphodiesterases to the activated receptor provides a negative feedback loop, the components of which are assembled at the site of agonist-stimulated adenylate cyclase activity. This regulatory process has recently been linked with the controversial (see Friedman *et al.*, 2002; Lefkowitz *et al.*, 2002) proposal that the β_2 -adrenoceptor is able to switch between G_s- and G_i-protein signalling via site-specific phosphorylation mediated by PKA (Daaka *et al.*, 1997). Thus, it appears that phosphorylation may have a complex regulatory role in β_2 -adrenoceptor adenylate cyclase signalling. GRK-mediated phosphorylation of the β_2 -adrenoceptor allows for the recruitment of arrestin that uncouples the receptor from G_s protein and recruits phosphodiesterases that enhances the degradation of cyclic AMP. This negative feedback loop operates in an apparently simultaneous manner with PKA-mediated phosphorylation of the β_2 -adrenoceptor which uncouples the receptor from G_s protein (in a manner distinct from GRK-mediated

desensitization) and switches signalling to Gi proteins (Baillie *et al.*, 2003).

An example of a specific GRK-mediated phosphorylation event resulting in signal regulation has been reported for GRK5 in the interaction of Na⁺/H⁺-exchanger regulatory factor EBP50 with the β_2 -adrenoceptor. The PDZ domain of EBP50 interacts with the C-terminal tail of the β_2 -adrenoceptor and this interaction is important in the intracellular sorting of the receptor following agonist-stimulated internalization (Hall *et al.*, 1998; Cao *et al.*, 1999). An essential element in the interaction is serine 411 in the C-terminal tail of the receptor where mutation to an alanine or the phospho-mimetic residue aspartate is seen to disrupt the interaction with EBP50 (Cao *et al.*, 1999). This residue (serine 411) is known to be phosphorylated specifically by GRK5 *in vitro* (Fredericks *et al.*, 1996) and therefore the suggestion has been made that GRK5-mediated phosphorylation of serine 411 regulates the interaction of EBP50 with the receptor and in so doing regulates receptor sorting and recycling. A caveat to this conclusion is that subsequent *in vivo* analysis of the agonist-mediated phosphorylation sites on the β_2 -adrenoceptor has not established serine 411 as a GRK site in intact cells (Seibold *et al.*, 2000; Trester-Zedlitz *et al.*, 2005).

Multi-site nature of GPCR phosphorylation

Mutagenesis (Bouvier *et al.*, 1988; Eason *et al.*, 1995; Prossnitz *et al.*, 1995; Malecz *et al.*, 1998; Smith *et al.*, 1998; Guo *et al.*, 2000; Mendez *et al.*, 2000; Seibold *et al.*, 2000), mass spectrometry (Papac *et al.*, 1993; Karoor and Malbon, 1996; Trester-Zedlitz *et al.*, 2005), phosphopeptide maps (Giannini *et al.*, 1995; Ozcelebi and Miller, 1995; Blaukat *et al.*, 2001; Torrecilla *et al.*, 2007) and antibody studies (Tran *et al.*, 2004) on a wide range of receptor types have demonstrated that GPCRs are multiply phosphorylated. Early studies on rhodopsin demonstrated that this phosphorylation was sequential or hierarchical (Ohguro *et al.*, 1993). There is now a growing body of evidence indicating that this is also the case for the non-visual GPCRs. The chemokine N-formyl peptide C5a receptor is phosphorylated in the basal state by an unknown protein kinase (Giannini *et al.*, 1995), which appears to prime the receptor for ligand-induced phosphorylation that may (Prossnitz *et al.*, 1995) or may not (Milcent *et al.*, 1999) proceed via the GRKs. The primary site for agonist-dependent phosphorylation of the δ -opioid receptor is Ser363 on the C-terminal tail. Mutation of this serine to alanine prevented ligand-dependent phosphorylation at other sites on the C-terminal tail (Kouhen *et al.*, 2000). *In vitro* phosphorylation of the third intracellular loop of the M₃-muscarinic receptor by protein kinase CK2 revealed that mutation of one CK2 consensus site resulted in the loss of a number of phosphorylation events, suggesting that this kinase was mediating multiple hierarchical phosphorylation (Torrecilla *et al.*, 2007). An elegant study of the bradykinin B₂ receptor revealed that basal phosphorylation at Ser346 on the C-terminal tail is followed by multiple phosphorylations that were both time and ligand concentration dependent. This complex

phosphorylation pattern is due to the multiple kinases that are employed in the phosphorylation of this receptor, which include GRKs, PKC and at least one unidentified protein kinase (Blaukat *et al.*, 2001).

Involvement of multiple protein kinases in GPCR phosphorylation

The multi-site phosphorylation of GPCRs likely reflects the action of a number of protein kinase families (Figure 2). Although the role of PKA/PKC and the GRKs in GPCR phosphorylation has been known for two decades, evidence that other protein kinase families are involved has been slow to emerge. Probably the clearest example that receptor kinases other than PKA/PKC and the GRKs have a role in GPCR phosphorylation was the discovery that some GPCRs are tyrosine phosphorylated (Paxton *et al.*, 1994; Fan *et al.*, 2001). In particular, the discovery that the β_2 -adrenoceptor was phosphorylated on tyrosine demonstrated the importance of investigating GPCR regulation in the correct cellular context.

Glucose metabolism is controlled primarily by insulin that stimulates glycogen synthesis, glucose uptake and lipid storage, whereas adrenaline counteracts these actions by promoting glycogen breakdown, gluconeogenesis and lipolysis (White and Kahn, 1994; Saltiel and Kahn, 2001). The actions of insulin are augmented by the ability of the insulin receptor to stimulate the tyrosine phosphorylation and internalization of the β_2 -adrenoceptor (Karoor and Malbon, 1996; Karoor *et al.*, 1998). Further studies established an intricate mechanism whereby insulin receptor activation of PI-3 kinase drives the activity of Akt/PKB (protein kinase B), which was able to directly phosphorylate the β_2 -adrenoceptor at serines 345/346 on the C-terminal tail (Doronin *et al.*, 2002). The ability of Akt/PKB to phosphorylate the receptor was obligate on insulin-mediated tyrosine 350 phosphorylation (Doronin *et al.*, 2002). This combined hierarchical phosphorylation process was essential in the internalization of the β_2 -adrenoceptor. The fact that the Akt/PKB sites of phosphorylation on the β_2 -adrenoceptor are different from those of the GRK sites (Seibold *et al.*, 2000; Doronin *et al.*, 2002) suggested that the mechanism of receptor

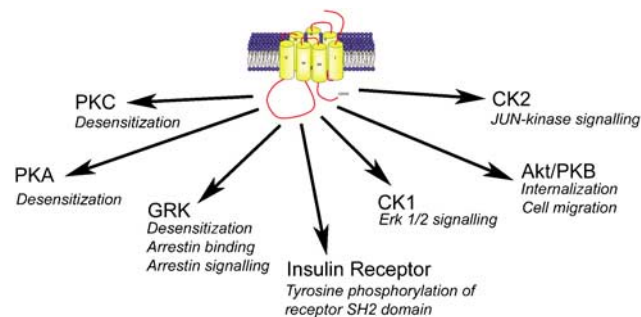


Figure 2 Summary of protein kinases known to phosphorylate G-protein-coupled receptors (GPCRs). Shown are protein kinases that have been demonstrated to phosphorylate GPCRs and the main functional consequences of the phosphorylation event.

internalization was distinct from that mediated by the GRKs. This view was supported by the fact that receptor internalization mediated by insulin and that mediated via the GRKs showed different sensitivities to agents that disrupt cellular cytoskeletal networks (Shumay *et al.*, 2004). One proposed mechanism was that phosphorylation of tyrosine 350 creates an SH2 (src homology domain 2)-binding motif (Karoor *et al.*, 1998) that is able to promote the association of c-Src, Grb2 and dynamin (Karoor *et al.*, 1998; Fan *et al.*, 2001; Gurevich and Gurevich, 2006). The creation of an SH2 domain appears to act in concert with Akt/PKB phosphorylation to mediate receptor internalization in response to insulin (Gavi *et al.*, 2006). One interesting proposition that comes from the study of insulin regulation of the β_2 -adrenoceptor is that via site-specific phosphorylation of the receptor by a number of protein kinases the receptor can firstly access unique internalization and recycling mechanisms, but that also by the creation of a protein interaction motif, which in this case is a tyrosine-phosphorylated SH2 domain, the receptor acts as a scaffold protein able to signal to pathways such as the mitogen-activated protein kinase pathway in the absence of its own ligand (Wang *et al.*, 2000). Thus in a physiological context, insulin can effectively counter-regulate β_2 -adrenoceptor signalling through receptor internalization and also is able to enhance its own signalling potential by recruiting the β_2 -adrenoceptor as a scaffolding protein that augments insulin-mediated ERK activity.

The involvement of Akt/PKB in directly phosphorylating GPCRs is not restricted to the β_2 -adrenoceptor. Insulin like growth factor-I has been proposed to stimulate Akt/PKB-mediated phosphorylation of the β_1 -adrenoceptor at serine 412 and this mediates functional antagonism of the β_1 -adrenoceptor (Gavi *et al.*, 2006). Furthermore, the finding that the α_{1A} -adrenoceptor is desensitized following phosphorylation by Akt/PKB (Garcia-Sainz, 2005) and that sphingosine-1-phosphate-stimulated cell migration is regulated by Akt/PKB-mediated S1P1 (EDG-1) receptor phosphorylation (Lee *et al.*, 2001) has highlighted the importance of this serine/threonine kinase in the regulation of a number of GPCRs.

Recent evidence has pointed to a role of the casein kinase family in GPCR phosphorylation. Studies on the mating factor receptors of the budding yeast *Saccharomyces cerevisiae* have demonstrated an essential role for CK1 in the phosphorylation of serines in the C-terminal tail. These phosphorylation events drive mono-ubiquitination of lysine residues downstream of the phosphorylation sites and this in turn mediates receptor internalization (Panek *et al.*, 1997; Hicke *et al.*, 1998; Roth and Davis, 2000; Feng and Davis, 2000). Our own studies on the M_3 -muscarinic receptor have demonstrated by the use of cell-free assays and a CK1 α -dominant-negative mutant that CK1 α was responsible, at least in part, for the agonist-stimulated phosphorylation of the M_1 - (Waugh *et al.*, 1999; Mou *et al.*, 2006) and M_3 -muscarinic receptors (Tobin *et al.*, 1997; Budd *et al.*, 2001). In the case of the M_3 -muscarinic receptor, this phosphorylation event appeared to regulate the ability of the receptor to couple to the ERK-1/2 pathway (Budd *et al.*, 2001).

The first indication that protein kinase CK2 might be involved in GPCR phosphorylation came from studies on the thyrotropin-releasing hormone receptor where mutation of putative protein kinase CK2 sites in the C-terminal tail resulted in a decrease in receptor phosphorylation and a loss of arrestin-mediated internalization (Hanyaloglu *et al.*, 2001). In this case, it appeared that at least three sites in a tight cluster (T365, T371 and S383) were required to be phosphorylated to allow for arrestin-dependent receptor internalization. Notably, internalization mediated by the phosphorylation-independent mutant, arrestin-R169E, was not affected by removal of these phosphorylation sites. Thus, in the case of the thyrotropin-releasing hormone receptor CK2 appeared to substitute for the GRKs in driving receptor phosphorylation of a region that was involved in arrestin activation and receptor internalization (Hanyaloglu *et al.*, 2001).

Our studies on the M_3 -muscarinic receptor have demonstrated that protein kinase CK2 inhibition through siRNA knockdown or pharmacological agents could partly reduce agonist-mediated receptor phosphorylation (Torrecilla *et al.*, 2007). Phospho-peptide maps revealed that CK2 was likely to phosphorylate just a subset of phospho-acceptor sites in the third intracellular loop of the receptor (Torrecilla *et al.*, 2007). Thus, protein kinase CK2 can be added to a growing list of protein kinases, which includes GRK2/6 (Willets *et al.*, 2002, 2003) and CK1 α (Tobin *et al.*, 1997; Budd *et al.*, 2001), that are known to phosphorylate the M_3 -muscarinic receptor in an agonist-dependent manner. Interestingly, protein kinase CK2-mediated phosphorylation did not appear to result in receptor internalization nor regulation of the ERK-1/2 pathways (Torrecilla *et al.*, 2007). Rather, CK2 inhibition resulted in enhanced coupling of the receptor to the JUN kinase pathway (Torrecilla *et al.*, 2007). Combined these data suggested that protein kinase CK2 phosphorylated a subset of phospho-acceptor sites and that these were involved in the specific regulation of receptor coupling to the JUN kinase pathway and not involved in other phosphorylation-dependent processes such as receptor internalization and ERK activation.

Conclusions and perspectives

There is now a gathering body of evidence that indicates that GPCR phosphorylation is a complex process involving a range of different protein kinases able to phosphorylate the same receptor at different sites and that this results in differential signalling outcomes (see Figure 2). In this regard, GPCR phosphorylation presents a flexible regulatory process where the recruitment of different protein kinases in cell types could tailor the signalling response of the receptor to suit a particular physiological role. If this were to be true, then one would expect to see different patterns of receptor phosphorylation in different cell types. That this may be the case has emerged from phospho-peptide mapping studies that have produced a 'phosphorylation signature' of the M_3 -muscarinic receptor expressed in Chinese hamster ovary (CHO) cells and cerebella granule neurons (Torrecilla *et al.*, 2007) (Figure 3). These maps have determined that some phosphorylation events, marked as numbers in Figure 3, are

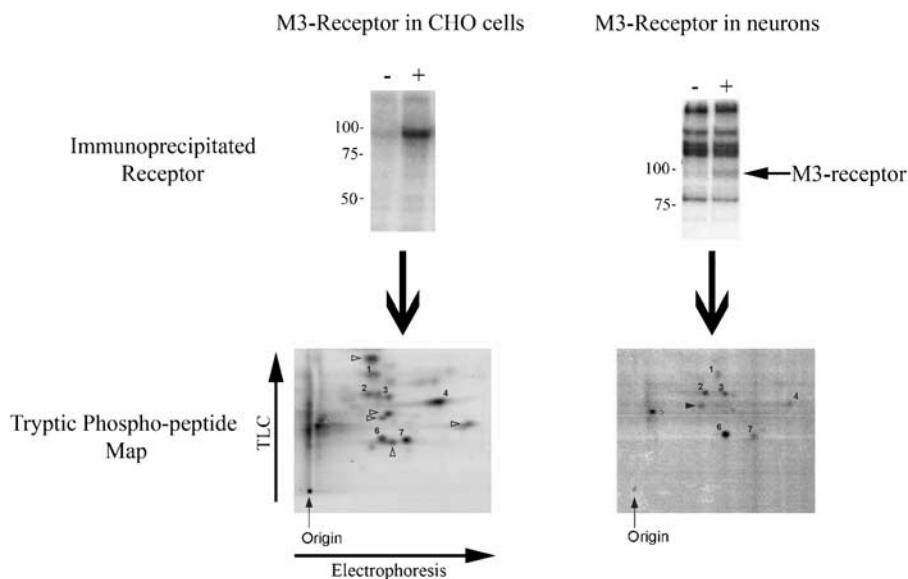


Figure 3 M₃-muscarinic receptors expressed in Chinese hamster ovary (CHO) cells and cerebellar granule neurons are differentially phosphorylated. Cerebellar granule neurons were cultured from 6-day-old neonatal mice and maintained for 6 days before being labelled for phosphorylation studies. CHO cells expressing the recombinant mouse M₃-muscarinic receptor or cerebellar granule neurons were labelled with [³²P]orthophosphate for 1–2 h (100–200 μCi ml⁻¹). The cells were then stimulated for 5 min with the muscarinic agonist methacholine (100 μM). Cells were then solubilized and the M₃-muscarinic receptor immunoprecipitated using a receptor-specific antibody. The immunoprecipitate was resolved by 8% SDS-PAGE and the radioactive band associated with the receptor was excised. The receptor band was then digested with trypsin and the resulting peptides resolved in the first dimension by electrophoresis and in the second dimension by thin layer chromatography. The position of the radioactive phospho-peptides was then determined in a phospho-imager. The numbered peptides are those that run in the same position and represent phosphorylation sites that are the same in the two cell types. The arrows represent phosphorylation events that are specific to the cell in which the receptor is expressed. The experiment shown was adapted from Torrecilla *et al.* (2007) where details of the methods can be obtained.

conserved between the two cell types whereas others, marked with arrows, are exclusive to either the CHO cells or the cerebellar granule neurons (Torrecilla *et al.*, 2007). The question from these studies is whether the common phosphorylation events regulate common receptor functions, such as receptor internalization, and the specific phosphorylations are associated with cell-type-specific signalling. This question is likely to be answered only in the study of the receptors in the experimentally challenging environment of native cell types.

This may prove to be a very important point in terms of drug discovery and design. GPCRs are clearly acting in a tissue-specific manner. Currently, little attention is paid to the fact that by identification of tissue-specific processes and targeting these in therapeutic strategies we may limit the side effects common to many GPCR drugs. Phosphorylation may well be one of the tissue-specific phenomena that will allow for more specific drug design. By generating agonists or allosteric modulators that will promote a given subset of phosphorylation events on the target, GPCR pharmaceutical compounds may be made specific to certain signalling pathways (a form of agonist trafficking) or specific to the target GPCR subtype when expressed only in one particular tissue.

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Conflict of interest

The authors state no conflict of interest.

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