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COMMENTARY

The subtleties of μ-opioid receptor phosphorylation

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The link between μ-opioid receptor phosphorylation and function is of critical importance to our understanding of the mechanisms underlying tolerance to opioid drugs. Increasingly sophisticated techniques are needed to assess the phosphorylation status of GPCRs, such as the use of phosphosite-specific antibodies that can monitor the kinetics of phosphorylation and dephosphorylation of individual residues in a receptor. Here the use of phosphosite-specific antibodies, raised against phosphorylated residues in the COOH-terminus of the μ-opioid receptor is discussed, along with some of the important findings that this approach has so far revealed. These include the finding that the μ-opioid receptor is constitutively phosphorylated, and that upon agonist removal it undergoes dephosphorylation equally well whether it is at the cell surface or internalized in endosomes. Thus already these phosphosite-specific antibodies are providing important new information about μ-opioid receptor function and the actions of opioid drugs.

LINKED ARTICLE

This article is a commentary on Doll *et al.*, pp. 298–307 of this issue. To view this paper visit http://dx.doi.org/10.1111/j.1476-5381.2011.01382.x

Abbreviations

DAMGO, [D-Ala^2-MePhe^4-Gly-ol]enkephalin; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase

The phosphorylation of GPCRs appears to be more complex than originally thought, but is also much more interesting. Apart from representing an important component of the mechanism of receptor desensitization, GPCR phosphorylation can also initiate alternative signalling pathways, such as the arrestin-dependent activation of MAPKs (DeWire *et al.*, 2007). In addition, it is now known that at least some GPCRs can be phosphorylated on multiple residues and by distinct kinases (Iyer *et al.*, 2006). Furthermore, the pattern of phosphorylation can be cell context-dependent (Butcher *et al.*, 2011), while recent developments in the area of biased agonism (Urban *et al.*, 2007) indicate that different ligands acting at the same subtype of GPCR can induce distinct patterns of phosphorylation (Butcher *et al.*, 2011). Together, these recent advances in our understanding of the complexities of GPCR function underline the need to develop powerful new tools to analyse the phosphorylation of these proteins.

The μ-opioid receptor is a particularly important GPCR, being the target for morphine and related opioid drugs in the management of pain, and also in the production of euphoria experienced by opioid drug users. Phosphorylation of μ-opioid receptors is likely to contribute to the phenomenon of opioid tolerance, whereby escalating doses of the drug must be administered to achieve effective analgesia, or euphoria. Interestingly, an expanding number of kinases have been implicated in the development and maintenance of tolerance, including G protein-coupledreceptor kinases (GRKs), PKC, extracellular signal-regulated kinase and c-Jun-N-terminal kinase (Bailey *et al.*, 2009; Dang *et al.*, 2009; Melief *et al.*, 2010) with the strong possibility that the μ-opioid receptor itself is the target of these kinases. In order to understand the molecular mechanisms underlying tolerance it is therefore important to identify sites in the μ-opioid receptor that are phosphorylated, and to determine the functional consequences of these phosphorylation events. Although it has long been known that the μ-opioid receptor is phosphorylated in response to an agonist (El Kouhen *et al.*, 2001), the precise identity of these sites and their role in μ-opioid receptor function as well as opioid tolerance remain the subject of intense debate. In the current issue of the *British Journal of Pharmacology*, Doll *et al.* (2011) have gone some way to uncovering the complexities of μ-opioid receptor phosphorylation, as well as the link between

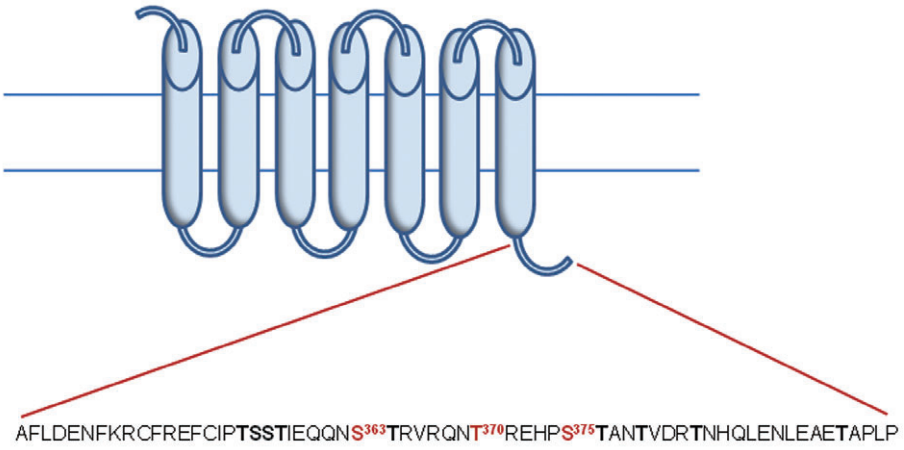

Figure 1

Diagrammatic representation of the rat μ-opioid receptor with the amino acid sequence of the intracellular COOH-terminus shown. The three amino acids against which phosphosite-specific antibodies were raised are shown in red and numbered. Other serine and threonine residues in the COOH-terminus are shown as bold and in black.

phosphorylation and function. The authors have developed phosphosite-specific antibodies to investigate the phosphorylation status of three previously identified (El Kouhen *et al.*, 2001) phosphoacceptor sites in the COOH-terminus of the μ-opioid receptor. Antibodies were developed against phospho-Ser363, phospho-Thr370 and phospho-Ser375 (Figure 1). A phosphosite-specific antibody had previously been raised against phospho-Ser375 by the same group (Schulz *et al.*, 2004).

What did this study show? Firstly, that Ser363 is phosphorylated in the absence of agonist. This constitutive phosphorylation of Ser363 is significant because a recent study implicates this residue in PKC-mediated phosphorylation and desensitization (Feng *et al.*, 2011), while the role of ongoing PKC activation in morphine tolerance is well established (Bailey *et al.*, 2006; Bailey *et al.*, 2009; Zheng *et al.*, 2011). While it is tempting to speculate that PKC-mediated phosphorylation of Ser363 is a key event in triggering morphine tolerance, this will require further rigorous investigation. With regard to agonist-induced phosphorylation, Doll *et al.* (2011) showed that Ser375 is phosphorylated following morphine treatment of cells, while both Ser375 and Thr370 are phosphorylated in response to the higher efficacy agonists [D-Ala$_2$-MePhe$_4$-Gly-ol]enkephalin (DAMGO) and etonitazene. Do these data provide evidence for biased agonism at μ-opioid receptors? On the face of it they do, given the differential effect of morphine versus the other agonists on Thr370 phosphorylation. However, it could be argued that the inability of morphine to induce phosphorylation of Thr370 is simply a reflection of its lower efficacy at μ-opioid receptors rather than to biased agonism (McPherson *et al.*, 2010). Nevertheless, the phosphosite-specific antibodies developed by the authors will enable the issue of biased agonism at μ-opioid receptors to be explored in detail at the level of receptor phosphorylation, using the large number of μ-opioid receptor ligands that are currently available.

Another advantage of the approach used in the present study is that it allows the kinetics of phosphorylation to be determined. Although morphine did induce phosphorylation of Ser375, Doll *et al.* (2011) show that the phosphorylation was weaker and developed more slowly than that induced by the other agonists. Furthermore, in a clever experiment they reduced the temperature to slow the overall rate of phosphorylation and were able to show that Ser375 is phosphorylated more rapidly than Thr370 in response to DAMGO. Because the phosphorylation of Thr370 was also reduced in the Ser375Ala mutant, it was suggested that Ser375 is the primary site for agonist-induced phosphorylation of μ-opioid receptors, and that phosphorylation of Thr370 is in part dependent upon the prior phosphorylation of Ser375. This type of hierarchical phosphorylation has not been widely studied in GPCRs, but the advent of phosphosite-specific antibodies will facilitate study of this phenomenon and its functional significance.

What is known about the function of the three residues in the COOH-terminus of μ-opioid receptors identified here as phosphoacceptors? Ser375 is strongly implicated in trafficking as mutations of this residue reduce agonist-induced μ-opioid receptor internalization (El Kouhen *et al.*, 2001; Schulz *et al.*, 2004). However, mutation of Thr370 did not affect agonist-induced μ-opioid receptor internalization whereas mutation of Ser363 actually increased agonist-induced internalization (El Kouhen *et al.*, 2001). With regard to desensitization, mutation of these three residues together was reported to block μ-opioid receptor desensitization in response to DAMGO but not morphine (Zheng *et al.*, 2011), whereas in another study mutation of Ser375 blocked desensitization to morphine but not DAMGO (Schulz *et al.*, 2004). Therefore, the relationship between phosphorylation of these residues and μ-opioid receptor coupling and desensitization requires resolution.

A further advantage of the phosphosite-specific antibody approach used by Doll *et al.* (2011) is that it allows the

phosphorylation status of the μ-opioid receptor to be correlated with the subcellular location of the receptor. Importantly, they demonstrated that dephosphorylation of this receptor can occur at or close to the cell surface, that is, in the absence of significant receptor internalization. This is shown in two ways; firstly because after removal of morphine, Ser375 dephosphorylated rapidly, under conditions where there has presumably been little μ-opioid receptor internalization, and secondly because in the presence of concanavalin A to inhibit internalization, DAMGO removal precipitated the dephosphorylation of Ser375 at the same rate as that observed for the μ-opioid receptor in the absence of concanavalin A. Thus, the phosphatases necessary for μ-opioid receptor dephosphorylation are present at the cell membrane, and internalization is not necessary for this dephosphorylation to occur. It had previously been reported that concanavalin A treatment does not interfere with the desensitization or resensitization of μ-opioid receptors in neurones (Arttamangkul *et al*., 2006). Does the classical model of GPCR activation, desensitization and trafficking therefore need to be modified to incorporate a model whereby receptors do not require to be internalized for access to phosphatases? Similar conclusions with regard to dephosphorylation and trafficking have been reached with other GPCRs (Iyer *et al*., 2006). Furthermore, these results do not support the hypothesis that morphine's ability to induce marked tolerance is because this agonist is unable to trigger internalization and hence dephosphorylation and resensitization. Instead, recent studies suggest that morphine is able to induce tolerance by a PKC-dependent mechanism different from that induced by high-efficacy agonists (Bailey *et al*., 2006; Bailey *et al*., 2009), which could instead explain differences in the degree of tolerance observed with different agonists.

A limitation of the study by Doll *et al*. (2011) is that there may well be other phosphoacceptor sites in the intracellular regions of the μ-opioid receptor, which could be important for the signalling or regulation of this receptor. Therefore, for a comprehensive analysis of this receptor, all phosphoacceptor sites need to be identified, to enable the development of a complete array of phosphosite-specific antibodies. Certainly, there are potential phosphoacceptor sites in the intracellular regions of the μ-opioid receptor in addition to the phosphoacceptor sites identified in this study. A further limitation is the absence of identification of the kinases involved in phosphorylating these residues. Treatment of cells with the PKC activator phorbol 12-myristate 13-acetate was able to induced phosphorylation of Thr370 but not Ser375, so it is suggested that the former is a target for endogenous PKC. However, it would be better to test the ability of selective kinase inhibitors or kinase siRNA constructs to block agonist-induced phosphorylation of the phosphoacceptor sites, or use tissue from kinase knockout animals.

Finally, although in the present study Doll and colleagues restricted their experiments to μ-opioid receptors expressed in HEK293 cells, these phosphosite-specific antibodies open the way for the assessment of μ-opioid receptor phosphorylation in neurones and in areas of the brain relevant to μ-opioid receptor function. In particular, it will be important to determine the phosphorylation status of individual amino acid residues in μ-opioid receptors in the brain under different conditions of opioid-induced tolerance and dependence, and following withdrawal from opioid treatment.

Conflict of interest

There is no conflict of interest with regard to this work.

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