British Journal of Pharmacology (2004) 143, 685–696
www.nature.com/bjp

REVIEW

μ-Opioid receptor desensitization: Is morphine different?

*,[1]Mark Connor, [1]Peregrine B. Osborne & [1]MacDonald J. Christie

[1]Pain Management Research Institute, E25, Kolling Institute, University of Sydney at Royal North Shore Hospital, Pacific Highway, St Leonards, NSW, 2065, Australia

Opioid tolerance and dependence are important phenomena. The contribution of acute μ-opioid receptor regulatory mechanisms to the development of analgesic tolerance or physical dependence are unknown, and even the mechanisms underlying relatively rapid receptor desensitization in single cells are unresolved. To a large degree, the uncertainty surrounding the mechanisms and consequences of short-term regulation of μ-opioid receptors in single cells arises from the limitations in the experimental design in many of the studies that have investigated these events. Receptor overexpression and use of assays in which regulatory mechanisms are likely to blunt control determinations have led to measurements of opioid receptor activity that are likely to be insensitive to receptor uncoupling. Together with uncertainties concerning molecular details of μ-opioid receptor interactions with potential regulatory molecules such as G protein-coupled receptor kinases and arrestins, we are left with an incomplete picture crudely copied from the well-worked-out regulatory schema for β_2-adrenoceptors. As a consequence, suggestions that clinically relevant μ-opioid receptor agonists may have different propensities to produce tolerance and dependence that arise from their differential recruitment of regulatory mechanisms are premature, and have not yet been appropriately assessed, nor explained in the context of a thoroughly established regulatory scheme. In this commentary, we outline the experimental limitations that have given rise to conflicting ideas about how μ-opioid receptors are regulated, and identify the issues we feel still need to be addressed before we can understand why morphine promotes receptor trafficking differently to other opioids.
British Journal of Pharmacology (2004) **143,** 685–696. doi:10.1038/sj.bjp.0705938

Keywords: Internalization; efficacy; opioid tolerance; opioid dependence; DAMGO; etorphine; phosphorylation

Abbreviations: CHO, Chinese hamster ovary; DAMGO, Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin; GIRK, G protein-gated inwardly rectifying potassium channel; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK 293, human embryonic kidney 293 cell line; I_{Ca}, voltage-gated calcium channels; LC, locus coeruleus; MAPK, mitogen-activated protein kinase

Introduction

Opioid tolerance is an important phenomenon (McQuay, 1999). Clinically, many patients taking opioids for pain control require increasing doses of drug to maintain adequate analgesia, and this dose escalation can eventually lead to inadequate pain relief and unacceptable toxic effects. In addition, opioid abusers often require increasing doses of drugs to maintain their addiction, again with potentially toxic (or fatal) consequences, in particular overdoses following a period of drug abstinence. Many processes underlie opioid tolerance *in vivo*, including adaptations in neural circuitry and neurotransmitter systems related to opioid systems. However, recent work has suggested a direct relationship between the effects of μ-opioid agonists on μ-opioid (MOP) receptor regulation and their clinical efficacy, development of tolerance and propensity to produce dependence (Keith *et al*., 1998; Zhang *et al*., 1998; Whistler *et al*., 1999). In particular, it has been proposed that the prototypic μ-opioid analgesic morphine produces more tolerance and has a greater dependence liability than other opioids of similar efficacy. These proposals have been made in the light of the observation that morphine does not efficiently promote the removal of μ-opioid receptors from the plasma membrane of cells in which the receptor has been heterologously expressed.

The agonist-induced regulation of opioid receptors has been reviewed extensively (von Zastrow *et al*., 2003; Waldhoer *et al*., 2004). The generally accepted regulatory pathway for μ-opioid receptors is outlined in Figure 1. Briefly, following the binding of an efficacious agonist such as DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin) to μ-opioid receptors, the receptor signals *via* activation of heterotrimeric G proteins of the Gi/Go family. With continued exposure to agonist, μ-opioid receptors are rapidly phosphorylated by a G protein-coupled receptor kinase (GRK), and this phosphorylation stimulates the binding of arrestins to the receptor. The μ-opioid receptor/arrestin complex is then recruited to a constitutive pathway that utilizes clathrin-coated pits to endocytose a wide variety of cell surface proteins. In the early endosome, DAMGO unbinds, most receptors are dephosphorylated and then quickly returned to the cell surface, ready to signal. Once μ-opioid receptors are bound to either GRK or arrestin, they probably cannot bind to G proteins and are presumed to be uncoupled from their effectors. Further, there is little evidence for μ-opioid receptor signalling once the receptor is internalized. In the context of this scheme, the principal difference between morphine and other μ-opioid agonists is that

*Author for correspondence; E-mail: markc@med.usyd.edu.au
Advance online publication: 25 October 2004

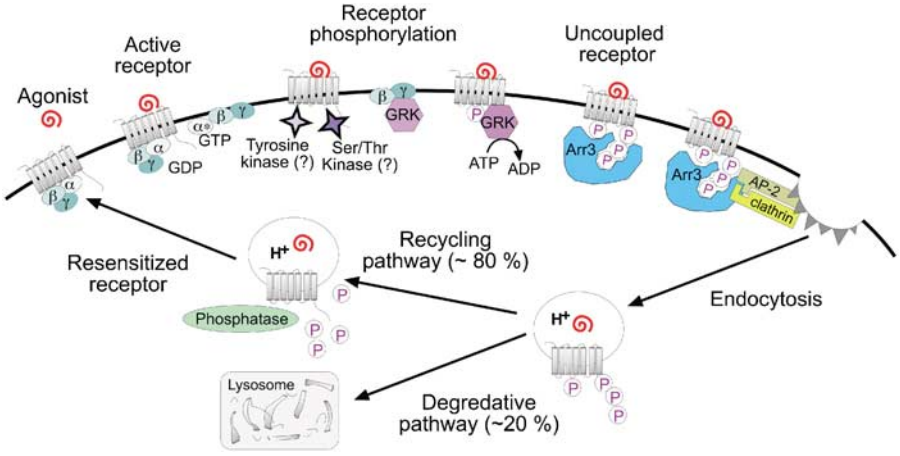

Figure 1 The generally accepted pathway for acute μ-opioid receptor regulation. Evidence for the involvement of the proteins illustrated is discussed in text. While the phenomena of opioid receptor activation, uncoupling and internalization are well described, the precise mechanisms underlying the experimental observations are largely undefined. Serine/threonine protein kinases that might phosphorylate the μ-opioid receptor include PKA, calmodulin-dependent protein kinase, and protein kinase C; specific tyrosine kinases that might phosphorylate the μ-opioid receptor have not been identified. Abbreviations are AP-2, adaptor protein 2; Arr3, arrestin 3; GRK, G protein-coupled receptor kinase; P, phosphate. Adapted from figures in von Zastrow *et al.* (2003).

morphine is disproportionally poor at promoting rapid receptor internalization (Arden *et al.*, 1995; Keith *et al.*, 1996), although it is clearly not completely incapable (Bushell *et al.*, 2002; Borgland *et al.*, 2003; Celver *et al.*, 2004).

Paradoxically, despite the general understanding that μ-opioid receptors are uncoupled by arrestin binding before internalization, it is the inability of morphine to promote efficient receptor internalization that has led to suggestions that morphine is particularly prone to promoting μ-opioid receptor desensitization and tolerance. One theory suggests that because morphine-activated μ-opioid receptors do not internalize, they continue to signal in the continued presence of agonist and promote adaptations in cells that oppose the effects of μ-opioid receptor activation, thus leading to cellular tolerance to the effects of μ-agonists (Whistler *et al.*, 1999; Finn & Whistler, 2001). A second theory proposes that because receptor uncoupling is mediated by phosphorylation, and internalization is the only way in which μ-opioid receptors can be dephosphorylated, agonists that promote the phosphorylation but not internalization of μ-opioid receptors will cause more desensitization (Koch *et al.*, 1998; 2001; Zhang *et al.*, 1998). These ideas are not obviously complementary, one requires continued signalling of uninternalized receptors to produce adaptation, while the other postulates that the lack of receptor internalization leads to loss of receptor activity. Conversely, if internalization of μ-opioid receptors enables them to signal again, then cellular adaptations should be readily produced by internalizing agonists because of the overall high level of receptor signalling that should be preserved.

We feel that substantial uncertainty exists regarding the mechanisms underlying agonist-induced regulation of μ-opioid receptors, as well as the consequences of this regulation for long-term opioid exposure (see Figure 1). This has arisen partly as a result of the experimental approaches used to address the question of opioid receptor regulation. In addition, many of the experiments that have provided detailed evidence about the mechanisms of regulation of other G protein-coupled receptors (GPCRs), such as the β_2-adrenoceptor, have simply not been reported for MOP receptors. As a consequence, much of what is 'known' about μ-opioid receptors is extrapolation from more completely described systems. Without a clear understanding of the events that follow agonist binding to the μ-opioid receptor, it will be impossible to determine whether morphine does recruit different regulatory processes to other opioid agonists. In this commentary, we will outline the experimental uncertainties that have given rise to conflicting ideas about how μ-opioid receptors are regulated, and will identify what we consider to be the issues that still need to be addressed before we can understand what may be different about morphine.

Biochemical analyses of μ-opioid receptor function

μ-*Opioid receptor signalling*

To date, μ-opioid receptors have been demonstrated to couple to effectors exclusively through heterotrimeric G proteins, usually of the Gi/Go family (Connor & Christie, 1999). There are differences in the capacity of some μ-agonists to activate different G protein subtypes, but available evidence indicates that both morphine and the prototypic high-efficacy peptide agonist DAMGO can promote coupling of μ-opioid receptors to all Gi/Go subunits (Chakrabarti *et al.*, 1995; Massotte *et al.*, 2002). Consequently, morphine modulates a similar range of downstream effectors as DAMGO, including adenylyl cyclase (AC), potassium channels, calcium channels (I_{Ca}) and mitogen-activated protein kinase (MAPK). However, DAMGO but not morphine has been reported to activate recombinant PLD_2 *via* μ-opioid receptors heterologously expressed in human embryonic kidney (HEK) 293 cells (Koch *et al.*, 2003). μ-Opioid receptors appeared to bind to PLD_2 directly, but the activation of PLD_2 by μ-opioid receptors *via* a G protein-independent mechanism has not been established. This finding may have

important implications for differential effects of morphine and DAMGO on receptor regulation, as discussed below.

μ-*Opioid receptor phosphorylation*

Phosphorylation of GPCRs is a major first step in mechanisms that regulate receptor activity. Phosphate groups on receptors provide binding sites for molecules such as arrestin that can uncouple receptors, or direct receptor signalling down specific G protein-dependent pathways. μ-Opioid receptors have about 20 serine, threonine and tyrosine residues located in regions conceivably accessible to protein kinases. Basal phosphorylation of native (Deng *et al*., 2001) and recombinant μ-opioid receptors increases substantially after agonist binding (Arden *et al*., 1995; Zhang *et al*., 1996; 1998; Yu *et al*., 1997; Whistler *et al*., 1999; Koch *et al*., 2001), is detectable within a few minutes and maximal after 10–15 min. Morphine has repeatedly been reported to induce significant phosphorylation of μ-opioid receptors (Arden *et al*., 1995; Zhang *et al*., 1996; Deng *et al*., 2001, but see Zhang *et al*., 1998; Whistler *et al*., 1999), although morphine may induce less phosphorylation than DAMGO (Yu *et al*., 1997; Koch *et al*., 2001). The identity of protein kinase(s) that phosphorylate μ-opioid receptors in cells has not been established, but the receptor carboxyl-terminal domains have been suggested to contain putative GRK phosphorylation sites, and mutation of these sites reduces agonist-induced phosphorylation in some cell lines (Deng *et al*., 2000; El Kouhen *et al*., 2001; Wang *et al*., 2002). Further, GRK2 or GRK3 overexpression potentiates DAMGO- and morphine-induced μ-opioid receptor phosphorylation (Zhang *et al*., 1998; El Kouhen *et al*., 1999). While available data suggest that GRKs can phosphorylate the μ-opioid receptor, evidence for a relatively weak interaction between μ-opioid receptors and GRKs was provided by the observation that, in contrast to the δ-opioid receptor (DOP), agonist stimulation of μ-opioid receptors did not recruit fluorescently tagged GRK2 or 3 to the plasma membrane of HEK 293 cells (Schulz *et al*., 2002). Thus, in contrast to κ-opioid receptors (KOP; McLaughlin *et al*., 2004), δ-opioid receptors (Li *et al*., 2003) and β_2-adrenoceptors (Fredericks *et al*., 1996), there are no studies demonstrating that purified GRK can directly phosphorylate μ-opioid receptors, or that μ-opioid receptors and GRKs bind to each other.

Arrestin binding to μ-*opioid receptors*

Arrestin binding to GPCRs uncouples them from G proteins and is an important step in directing receptors to endocytic pathways. Arrestins bind with much higher affinity (<1 nM) to GRK-phosphorylated GPCRs than to unphosphorylated receptors, and the presence of multiple phosphate groups on the receptor (i.e. >3 per receptor) is thought to be the primary trigger for high-affinity arrestin binding (reviewed in Gurevich & Gurevich, 2004). Arrestin affinity for phosphorylated receptors is also increased further by agonist binding. μ-Opioid receptor activation promotes translocation of arrestin 3 (but not arrestin 2) from the cytosol to the plasma membrane of HEK 293 cells (Zhang *et al*., 1998; Whistler *et al*., 1999; Oakley *et al*., 2000; Bohn *et al*., 2004), but this finding has not been reproduced in neurons (Bushell *et al*., 2002). In HEK 293 cells, morphine only promotes the translocation of arrestin 3 in the presence of overexpressed GRK2 (Zhang *et al*., 1998; Bohn *et al*., 2004). Interestingly, morphine was shown to promote modest translocation of GFP-tagged arrestin 3 when the μ-opioid receptor was transfected into fibroblasts derived from embryonic mice lacking both arrestin 2 and arrestin 3 (Bohn *et al*., 2004). Further, etorphine (but not morphine) also induced a modest translocation of GFP-tagged arrestin 2 in the arrestin-negative fibroblasts, revealing a hitherto unreported interaction revealed by the lack of native arrestin 3. These data suggest that morphine binding to μ-opioid receptors can promote the translocation of arrestin 3 to plasma membrane, which is consistent with a direct arrestin 3 interaction with the receptor; however, the binding of arrestin to intact μ-opioid receptors remains to be directly demonstrated, and in a study that reported isolated intracellular domains of δ- and κ- receptors could bind to arrestin *in vitro*, similar μ-opioid receptor domains did not (Cen *et al*., 2001).

μ-*Opioid receptor internalization, recycling and downregulation*

μ-Opioid receptors leave the plasma membrane in response to agonist activation in many cell lines (Arden *et al*., 1995; Keith *et al*., 1996) and native neurons (e.g. Sternini *et al*., 1996; Haberstock-Debic *et al*., 2003). μ-Opioid receptor internalization in cell lines is inhibited by dominant-negative mutants of GRK, arrestin or dynamin, and further promoted by overexpression of either GRK2 or arrestin (Whistler & von Zastrow, 1998; Zhang *et al*., 1998; Celver *et al*., 2004). Internalization is usually rapid, with up to 50% of receptors that will be internalized leaving the cell surface 5 min after exposure to DAMGO or etorphine. Internalization and recycling can usually achieve a dynamic steady state within 30 min (Keith *et al*., 1998; Trapaidze *et al*., 2000; Borgland *et al*., 2003). DAMGO, etorphine, β-endorphin, fentanyl and methadone generally promote robust internalization of μ-opioid receptors, while morphine, pentazocine and buprenorphine promote slower and much less complete internalization (Arden *et al*., 1995; Keith *et al*., 1996; 1998; Bushell *et al*., 2002; Borgland *et al*., 2003; Celver *et al*., 2004), although a substantial morphine-induced internalization of receptor can be produced by overexpression of GRK2 and/or arrestin (Whistler & von Zastrow, 1998; Zhang *et al*., 1998). These data suggest that agonist-activated μ-opioid receptors can engage an endogenous clathrin-dependent endocytosis pathway shared by many GPCRs, provided there is sufficient and/or appropriate receptor phosphorylation and enough cellular arrestin. However, the data indicate that μ-opioid receptor phosphorylation and internalization proceed with submaximal efficiency in heterologous systems, because responses to even the most efficacious agonists such as etorphine and DAMGO are significantly enhanced by overexpression of GRK or arrestin. Thus, in general, the μ-opioid receptor may be a relatively poor substrate for potential regulation by internalization.

Following internalization, μ-opioid receptors are either recycled from endosomes to the plasma membrane or trafficked to lysosomes/proteosomes for degradation. Up to 80% of internalized receptors are recycled after 30 min of DAMGO treatment, but receptors can be substantially downregulated (i.e. $>60\%$) after only 2 h of DAMGO treatment (Polakiewicz *et al*., 1998; Pak *et al*., 1999), although this is not

a universal finding (Tanowitz & von Zastrow, 2003). β_2-adrenoceptors are internalized following multiple receptor phosphorylation events, then dephosphorylated in endosomes and recycled to the plasma membrane *via* a specific pathway (Krueger *et al*., 1997; Tanowitz & von Zastrow, 2003). μ-Opioid receptors are recycled by a specific pathway distinct from that for β_2-adrenoceptors (Tanowitz & von Zastrow, 2003), and while it is assumed that they are also dephosphorylated in the early endosome before recycling, there is no direct evidence for this. μ-Opioid receptors phosphorylated in response to morphine have been reported to be dephosphorylated in about 30 min, presumably *via* a process not involving internalization and recycling (Yu *et al*., 1997).

Where does desensitization occur?

As μ-opioid receptors couple to effectors *via* G proteins, the most direct mechanism for abrogating agonist-dependent receptor signalling is by disruption of the receptor–G protein interaction. Binding of a GRK (or other kinase) to the μ-opioid receptor, phosphorylation of the receptor or arrestin binding to the receptor could physically prevent receptors and G proteins from interacting. Although there are clearly other ways in which opioid receptor signalling is regulated (see below), based on the information outlined above, it seems likely that any differences in the relative ability of morphine and other agonists to recruit processes involved in the acute inhibition of receptor function will be reflected in the efficiency with which different agonists promote receptor phosphorylation or arrestin binding.

The studies described above have provided significant information about μ-opioid receptor signalling and regulation, and they provide the basis for experiments designed to determine the mechanisms through which opioid receptor signalling is modulated. Loss of agonist responses can occur very quickly for the μ-opioid receptor, which is not unexpected for a receptor that can be rapidly phosphorylated and removed from the cell surface. However, in contrast to the information about the biochemical basis of μ-opioid receptor signalling, the most direct measurements of opioid receptor desensitization have been obtained in neurons, neuron-like cell lines and systems where elements of opioid receptor signalling cascades can be re-constituted, such as *Xenopus* oocytes. These experiments have revealed that acute regulation of the μ-opioid receptor signalling is complex, and may proceed through different mechanisms depending on the cellular environment.

Acute modulation of μ-opioid receptor signalling

Many factors affect the interpretation of experiments purporting to measure modulation of acute μ-opioid receptor signalling, including the choice of assay system, as well as the nature and stoichiometry of regulatory, transduction and effector proteins in different cell types. An important step in being able to compare these different processes would be the adoption of a precise vocabulary to describe these events. The term 'desensitization' provides an operationally correct description for any loss of receptor sensitivity and encompasses a number of receptor regulatory events. These include rapid loss of receptor coupling, for which better terms would be 'rapid desensitization', or 'uncoupling'; removal of receptor from the cell surface, better described as 'internalization'; and loss of total receptor protein, best described as 'downregulation'. 'Sequestration' is often used interchangeably with internalization, but this term denotes removal of the receptor from an environment where it can signal productively, rather than movement of the receptor inside the cell. 'Desensitization' can also refer to processes that disrupt the coupling of multiple receptor types or change the signalling capacity of stimulus transduction proteins downstream of the receptor itself; it is important that such processes be identified as 'heterologous desensitization' where appropriate (Lohse *et al*., 1990). Similarly, the term tolerance has been used to loosely to describe the result of both acute (seconds to minutes) regulatory events and prolonged adaptive changes (days to weeks). Given the complex nature of processes likely to underlie tolerance to opioids in the whole animal, 'tolerance' is probably not a useful term to use in reference to a single molecular event contributing to an overall decrease in the response to opioid agonists, but should be reserved for adaptations associated with long-term (days to weeks) treatment with opioids.

The interpretation of many functional studies of μ-opioid receptor regulation are also hampered by the failure to consider two issues critical in measurement of receptor regulation: (1) temporal resolution of regulatory events, and (2) appropriate measurement of specificity and magnitude of loss of receptor activation. We feel that the temporal correlation of agonist-induced phosphorylation with internalization and desensitization of the μ-opioid receptor has only been weakly established, and, furthermore, there is little evidence in most systems that loss of agonist responsiveness is related to changes in the receptor itself. Ultimately, identification of fundamental differences between the regulatory processes engaged by morphine and other μ-opioid agonists requires studies that are not confounded by inappropriate measures of receptor activity.

Considerations in the functional measurement of rapid μ-opioid receptor desensitization

Rapid desensitization of GPCRs that couple to ion channels can be measured on the time scale of several seconds to minutes, and has been described in some detail in neurons, AtT20 cells and *Xenopus* oocytes. Few other methods of assessing GPCR activity achieve this temporal resolution (Figure 2). The major requirement for temporal resolution of GPCR signalling is an assay method that measures receptor activity at discrete time points over a period of minutes, rather than the integrated activity at the end of this period. Opioid receptor modulation of G protein gated inwardly rectifying potassium (GIRK) channels or I_{Ca} satisfies these requirements. In each case, channel modulation is mediated directly by rapid (millisecond scale) lateral diffusion of the G protein $\beta\gamma$ subunits activated by μ-opioid receptors, enabling reliable, discrete measurements to be made with a resolution of seconds or less (Wilding *et al*., 1995; Ingram *et al*., 1997). Furthermore, with recordings of GIRK currents, an essentially continuous readout of μ-opioid receptor activity can be obtained. By contrast, the nonexcitable cells used for most studies of receptor regulation do not express μ-opioid receptor-sensitive ion channels, so μ-opioid receptor signal transduction is

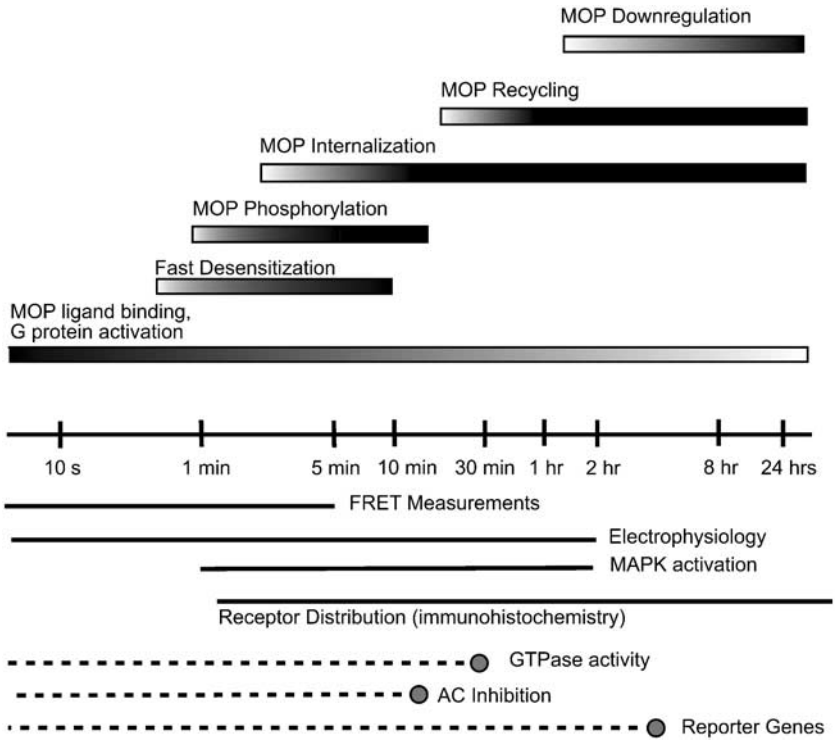

Figure 2 A timeline of opioid receptor activation, signalling and regulation, and the experimental approaches that can be potentially used to measure these processes. Time is represented on a log scale. The bars above the timeline indicate the general periods over which the processes are thought to occur and those below the timeline represent the capacity of different methodological approaches to resolve regulatory events. Unbroken lines indicate that measurements are made continuously or at any time during the indicated period, broken lines indicate that the assay is proceeding but measurements are usually only made at the time indicted by the circle. FRET can detect conformational changes within receptors or potential associations between receptors and other proteins at subsecond resolution, measurements of GTPase activity report the interaction between receptors and G proteins, while electrophysiological techniques and biochemical measurements of adenylyl cyclase activity and MAPK activation measure the consequences of receptor activation of G proteins. Reporter gene assays provide a measure of the overall effect of modulation of many pathways on one or more transcription factors. Immunohistochemistry is used to localize receptors within cells. Abbreviations are FRET, fluorescence resonance energy transfer; MAPK, mitogen-activated protein kinase; MOP, μ-opioid receptor. Selected references for each technique: FRET, Vilardaga *et al*., 2003; electrophysiology, Wilding *et al*., 1995; Ingram *et al*., 1997; MAPK activation, Trapaidze *et al*., 2000; receptor immunohistochemistry, Keith *et al*., 1996; Borgland *et al*., 2003; GTPase activity/GTPγS binding, Elliot *et al*., 1997; Clark *et al*., 2003; adenylyl cyclase activity, Koch *et al*., 1998; Zhang *et al*., 1998; reporter gene activation, Finn & Whistler, 2001; He *et al*., 2002.

examined by measuring activation of G proteins, either directly using GTPγS-binding assays or determination of GTPase activity, or by less direct methods such as measuring the inhibition of AC activity or stimulation of MAPK activation. Some studies even purport to measure 'acute' opioid receptor signalling by activation of the transcription of a reporter gene, whose activity is measured hours after the agonist stimulus (Finn & Whistler, 2001; He *et al*., 2002).

In contrast to measurements of GIRK channel activation or I_{Ca} inhibition, assays of GTPγS binding, GTPase activity or AC inhibition measure the cumulative effect of μ-opioid receptor stimulation over the entire period of the assay. Although it is possible to measure very rapid opioid stimulation of G proteins (i.e. <30 s, Clark *et al*., 2003) or inhibition of AC activity (Zhang *et al*., 1998), this is not usually carried out, and μ-opioid receptor activity is routinely determined over periods ranging from 10 min to 1 h. A major assumption that is made with the biochemical determinations of receptor activity is that regulatory events do not occur during the assay, and that the assays provide a snapshot of the receptor signalling status of the cell as it was at the beginning of the experiment. This assumption is not justified for assays performed on whole cells, as the time course of most of the biochemical assays currently used cross the temporal boundaries of several underlying receptor regulatory processes, including the rapid agonist-induced phosphorylation, uncoupling and internalization of μ-opioid receptors. This is summarized in the lower panel of Figure 2. Thus, in many studies, the 'control' condition actually represents a situation where a significant proportion of the receptors are likely to have been uncoupled.

Many studies of opioid receptor regulation also fail to appreciate the influence of receptor and drug concentrations on the sensitivity of measurements of receptor activity. The need to perform functional assays of receptor sensitivity under conditions that can actually detect changes in the magnitude of receptor responses has been widely recognized (Christopoulos & El-Fakahany, 1999; Clark *et al*., 1999; Trzeciakowski, 1999; Kenakin, 2002), but few studies of μ-opioid receptors have considered this issue experimentally. Most studies of μ-opioid receptor desensitization in heterologous expression systems have used cells expressing high densities of receptor, with only supramaximal concentrations of agonist being examined. Figure 3, which summarizes simulations extrapolated from

data obtained in locus coeruleus (LC) neurons, illustrates this common problem associated with studies of opioid receptor regulation in artificial (and potentially native) expression systems. In cells where there is significant receptor reserve for the measured effector, probing desensitization with a supramaximally effective concentration of agonist will only produce a decrease in the measured response when more than 90% of receptors are uncoupled from the effector. Conversely, where there is no receptor reserve, low concentrations of agonist may provide only a restricted response range to measure. Concentration–response curves for agonist inhibition of AC have rarely been used to determine appropriate agonist concentrations to desensitize AC or to probe the desensitization (e.g. Koch *et al*., 1997; 1998; 2001; 2003; Whistler & von Zastrow, 1998; El Kouhen *et al*., 1999, but see Wang *et al*., 2002). However, these issues were addressed directly by Law *et al*. (2000), who used β-chlornaltrexamine to irreversibly reduce cell surface expression of μ-opioid receptor. Even under these conditions, μ-opioid receptor desensitization was not very rapid, possibly because a high concentration of etorphine (1 μM) was used to probe desensitization, and thus even a substantial loss of receptor would have produced a small decrease in the probe response. As shown in Figure 3, the most efficient approach to circumvent the problem of the magnitude of receptor response in conditions where it is not feasible to investigate changes in receptor density or to change receptor expression level is to use appropriate, submaximal concentrations of agonist to probe receptor sensitivity.

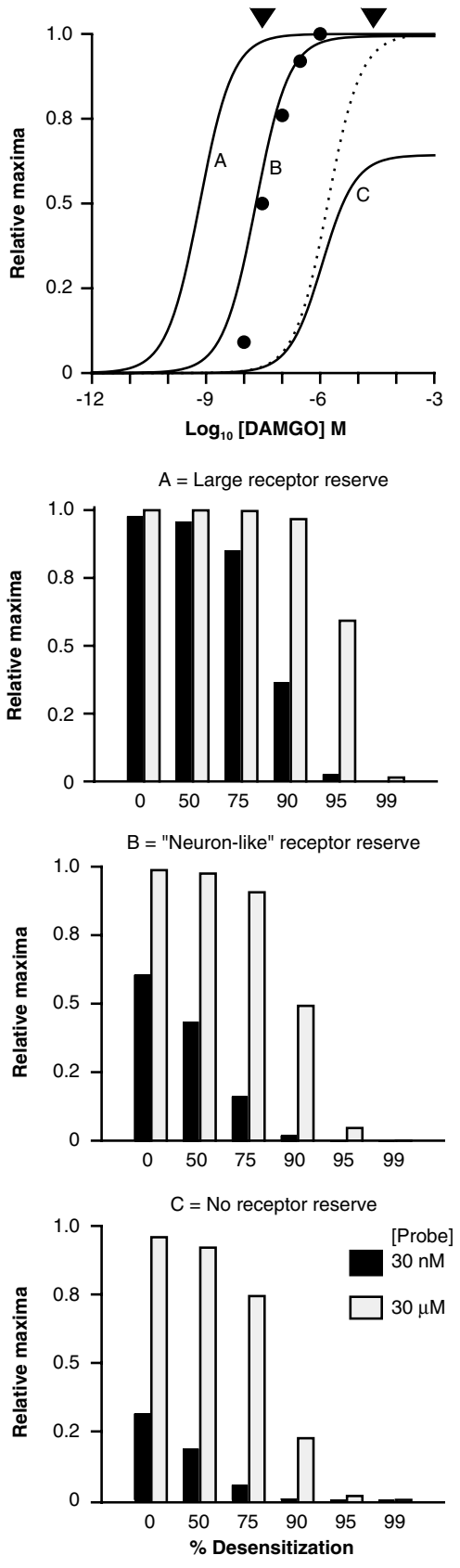

Figure 3 Influence of the level of receptor expression (or coupling efficiency) and agonist test concentrations on measurement of desensitization. In the top panel, DAMGO currents measured in LC neurons (circles) (Osborne & Williams, 1995) are shown, together with concentration–response curves (solid lines) simulated to represent the responses produced if there was a 30-fold increase (curve A) or a 70% decrease in μ-opioid receptor number relative to the native neurons (curve B). Analysis of receptor inactivation by desensitization in LC neurons (Osborne & Williams, 1995) or alkylation in AtT20 cells (Borgland *et al*., 2003) has shown that DAMGO is a full agonist in these cells with a receptor reserve, which is represented by the displacement between curve B and the receptor occupancy curve (broken line). In the lower panels, the apparent effects of desensitization for each of the three amounts of μ-opioid receptor were simulated by progressively uncoupling 0–99% of the receptors. Desensitization was 'measured' by the decrease in responses obtained with supermaximal (30 μM) or submaximal (30 nM) probe concentrations of DAMGO. In systems with massive amounts of receptor (panel A), the relative response to 30 μM DAMGO is almost completely insensitive to desensitization, even after 90% of functional receptors are lost, whereas the response to 30 nM is quite sensitive to loss of function. With realistic levels of receptor reserve (panel B), 30 nM, but not 30 μM, DAMGO still sensitively reflects desensitization. In systems with no receptor reserve, DAMGO is a partial agonist and the maximally effective 30 μM concentration is a more useful probe than 30 nM (panel C). Note: curves were generated with ProFit software using a modified form of the operational model of agonism (Trzeciakowski, 1999), where:

$$E = \frac{E_m \tau [A]}{K_{app} + (1+\tau)[A]}$$

in which E_m is the operational maximum response of the tissue, τ is the operational efficacy and K_{app} is the apparent dissociation constant of the agonist. Curve B was calculated with $K_{app} = 2.9\,\mu M$ and $\tau = 155$ (Osborne & Williams, 1995) and E_m normalized to 1. As $\tau = [R]_T/K_E$, where $[R]_T$ is the total number of receptors and K_E is the concentration of occupied receptors required to produce half the maximal response, curves A and C were generated using a value of τ multiplied by 30 and 0.3, respectively. Similarly, changes induced by desensitization were computed by substituting $\tau_{desensitized} = \tau_{control}$ (1–fraction receptors desensitised) (Lohse *et al*., 1990; Osborne & Williams, 1995).

Studies of receptor desensitization in any cells can also be confounded by not determining whether loss of function is due to loss of sensitivity associated specifically with the μ-opioid receptor or changes in elements of signalling cascades that affect the end point of the assay, which may also affect the signalling of other GPCRs in the cell (e.g. Nomura *et al*., 1994; Kovoor *et al*., 1995; Samoriski & Gross, 2000; Blanchet & Luscher, 2002). Homologous desensitization is defined as processes that affect the activated receptor and only the activated receptor, such as GRK-dependent phosphorylation followed by arrestin binding. Heterologous desensitization originally referred to receptor modifications that were not specific for the agonist-activated receptor, such as receptor phosphorylation by protein kinase C, which can act on receptors regardless of whether agonists are bound. However, heterologous desensitization is also an appropriate term for changes in effector cascades that reduce the responses of multiple receptors. While heterologous desensitization may be a physiologically important response, its presence compromises information that can be obtained about what happens to the μ-opioid receptor when agonist binds. For example, it is well established that treatment of cells for only a few hours with μ-agonists produces increases or decreases in the intrinsic activity of AC isoforms acutely regulated by opioids (Avidor-Reiss *et al*., 1997), but the homologous or heterologous nature of reduced opioid inhibition of AC during chronic treatments has rarely been assessed (Chakrabarti *et al*., 1995), compromising conclusions about whether changes in measured AC activity actually represent changes to the μ-opioid receptor.

Finally, studies of the modulation of acute μ-opioid receptor signalling can potentially be confounded by receptor downregulation, or absolute loss of receptor from the cell. μ-Opioid receptor downregulation can be rapid, and loss of μ-opioid receptor produced by continuous agonist exposure could explain a significant component of desensitization observed at times greater than a couple of hours, a possibility which is almost never ruled out experimentally. Studies using 4 h 'acute' opioid challenges (Finn & Whistler, 2001; He *et al*., 2002) or those that use similar agonist pre-incubations to desensitize the receptor (e.g. Koch *et al*., 1998; 2001) cannot be assumed to be only measuring receptor properties that affect signalling, unless careful controls are performed (Chakrabarti *et al*., 1995).

Functional measurements of μ*-opioid receptor desensitization*

In light of the above, what is known about μ-opioid receptor desensitization? In LC neurons, GIRK channel activation by met-enkephalin or DAMGO desensitizes with a time constant of 2–3 min, with a maximum decline to around 50% of the original response after about 5 min (Harris & Williams, 1991; Connor *et al*., 1996; Fiorello & Williams, 1996; Alvarez *et al*., 2002; Bailey *et al*., 2003). After removal of agonist, desensitization recovers in 10–20 min (Harris & Williams, 1991; Osborne & Williams, 1995; Fiorello & Williams, 1996). The desensitization observed in these studies was mostly homologous, although one group has reported that in immature rats most of the apparent μ-opioid receptor desensitization is heterologous (Blanchet & Luscher, 2002). The desensitization of μ-opioid responses in LC neurons represents a massive uncoupling of receptors, because, when concentration–response curves for μ-agonists were determined with or without a 5 min pre-treatment of a supramaximal concentration of met-enkephalin, it was estimated that more than 90% of receptors had been uncoupled, despite a decline in the maximal response to met-enkephalin of only 30–40% (Osborne & Williams, 1995). The apparent discrepancy between the extent of uncoupling and decline in the maximal response is explained by the presence of a substantial receptor reserve in LC for agonists such as met-enkephalin and DAMGO (Christie *et al*., 1987).

The notion that events related to receptor internalization may contribute to desensitization in the LC is supported by the observation that morphine, in contrast to DAMGO and methadone, does not produce desensitization of GIRK in LC cells (Alvarez *et al*., 2002; Bailey *et al*., 2003). Although the slow time course of drug equilibration of nonpeptide agonists in brain slices can make quantitative comparisons between peptides and drugs such as methadone and morphine problematic, morphine responses in LC are stable for 10's of minutes. The lack of morphine-induced desensitization in LC neurons is consistent with the apparently relatively poor ability of morphine to recruit arrestin binding to the μ-opioid receptor and promote subsequent receptor internalization, and there is strong correlation between a drug's capacity to produce μ-opioid receptor internalization in HEK 293 cells and desensitization in LC neurons (Alvarez *et al*., 2002). However, μ-opioid receptor internalization has not yet been measured in LC neurons, nor has the involvement of arrestin in μ-opioid receptor desensitization been tested.

A single study in nucleus raphe magnus neurons reported that short (<15 s) applications of DAMGO or morphine produced rapid desensitization during the initial application, as well as a reduction of the response to another application of drug 5 min later (Li & Wang, 2001). The desensitization was attenuated by intracellular application of a peptide corresponding to the G protein βγ subunit-binding domain of GRK2, leading the authors to suggest that GRK2 interaction with the μ-opioid receptor was mediating the reduced response. This study is somewhat difficult to place into context with other similar studies, because it is not known if the observed desensitization was homologous for the μ-opioid receptor, as agonists for other receptors were not examined. The extremely rapid initial decline in GIRK channel conductance is more consistent with events involving βγ subunit/GIRK channel interactions (Chuang *et al*., 1998; Leaney *et al*., 2004).

In contrast to results from the LC, experiments in the AtT-20 pituitary tumour cell line have indicated that morphine, methadone and DAMGO share a common mechanism of rapid desensitization. Activity of transfected μ-opioid receptors in AtT20 cells can be measured using inhibition of native P/Q-type I_{Ca} or activation of native GIRK channels, with the further advantage that receptor trafficking can be measured under the same conditions. While the inhibition of I_{Ca} produced by a high concentration of DAMGO, methadone or morphine does not decline significantly over 5 min, due to spare receptors, when desensitization during this time is probed with an EC_{50} concentration of DAMGO, there is a decline of about 75% in the response, indicating substantial receptor uncoupling (Borgland *et al*., 2003). This desensitization is entirely homologous (Borgland *et al*., 2003). Rapid desensitization of the inhibition of I_{Ca} in AtT20 cells (time constant 50 s) proceeds with a similar time

course to desensitization of agonist activation of GIRK channels in LC neurons, and is complete with a faster time course than receptor internalization (time constant approx. 10 min) in AtT20 cells (Borgland *et al*., 2003). Importantly, rapid desensitization in AtT20 cells displays a distinct profile of agonist sensitivity when compared with μ-opioid receptor internalization, or rapid desensitization in LC neurons. DAMGO, methadone and morphine all produced maximal rapid desensitization, but DAMGO and methadone produced three-fold more receptor internalization than morphine. Celver *et al*. (2004) also examined desensitization and internalization of μ-opioid receptors in AtT20 cells. They reported that a μ-opioid receptor mutant (T180A) internalized normally but did not desensitize after prolonged exposure to a low concentration of DAMGO, suggesting that a region of the second intracellular loop of the receptor is important for desensitization. Unfortunately, cells in this study were challenged with a supramaximal concentration of DAMGO, so the degree to which receptor desensitization was inhibited by the T180A mutation could not be assessed, as the number of spare receptors for DAMGO was not determined. Taken together, however, the studies of Borgland, Celver and colleagues suggest that rapid μ-opioid receptor desensitization in AtT20 cells is not a direct reflection of the internalization process, in contrast to the simplest interpretation of data from LC neurons. Most strikingly, morphine promotes rapid desensitization of μ-opioid receptor responses in AtT20 cells, but very little in LC neurons.

Expression of μ-opioid receptors with GIRK channels in *Xenopus laevis* oocytes has also provided a convenient system to examine agonist regulation of receptors and associated signalling proteins. μ-Opioid receptor-activated GIRK1 currents desensitize heterologously (Kovoor *et al*., 1995), but μ-agonists activate heteromeric GIRK1/GIRK4 channels with little desensitization (Kovoor *et al*., 1997). Co-expression of GRK3 and arrestin 3 promote desensitization to high-efficacy μ-opioid receptor agonists, although the time course for desensitization is slow, with approximately 20% desensitization after 8 min exposure (Kovoor *et al*., 1998; Lowe *et al*., 2002). This rate of desensitization only broadly approximates that found in neuronal cells or neuron-like cultures, and morphine produced very slow desensitization under these conditions. While these data show that μ-opioid receptor responses can be desensitized by a GRK/arrestin-dependent pathway in oocytes, it is not known whether GRK(s) mediate phosphorylation of μ-opioid receptors expressed in oocytes, nor has it been reported whether the desensitization of μ-opioid receptors facilitated by co-expression of GRK and arrestin is homologous or heterologous. Further, while *Xenopus* oocytes allow for controlled expression of exogenous proteins, the relative amounts of each expressed protein have not been determined; so the stoichiometry of the elements of the signalling cascade is unknown. While this is also true in many mammalian cell lines, successful attempts have been made to estimate the relative amounts of molecules important in μ-opioid receptor signalling, such as receptors and G proteins (Selley *et al*., 1997; Alt *et al*., 2001). The contribution of endogenous oocyte proteins to observed responses is also difficult to assess, because the effects of endogenous proteins may only be apparent when a mammalian partner is co-expressed, as originally demonstrated for the expression of GIRK channels in oocytes (see Kovoor *et al*., 1997).

In contrast to the relatively rapid desensitization of the μ-opioid receptor usually apparent when electrophysiological techniques are used to measure receptor activity, desensitization of μ-opioid receptor responses often happens quite slowly when measured using biochemical assays. In most studies, loss of agonist responsiveness does not become apparent until after 30–60 min, and is not maximal before 4–6 h (Chakrabarti *et al*., 1995; 1998; Elliot *et al*., 1997; Koch *et al*., 1997; 1998; 2001; 2004; El Kouhen *et al*., 1999; Law *et al*., 2000). In a few studies, significant desensitization of opioid modulation of AC has been reported at 20–30 min (Yu *et al*., 1997; Deng *et al*., 2000; Wang *et al*., 2002), but only Whistler and colleagues have found desensitization of μ-opioid receptor stimulation of GTPγS binding and inhibition of AC occurring with similar kinetics to the studies in neurons (Whistler & von Zastrow, 1998; Whistler *et al*., 1999). The rapid uncoupling of μ-opioid receptors from AC inhibition and G protein stimulation produced by DAMGO in these latter studies is consistent with desensitization being produced by receptor phosphorylation and arrestin binding, although the magnitude of the desensitization (100% at 5 min) is unusual (Whistler & von Zastrow, 1998; Whistler *et al*., 1999), and does not correlate well with the modest agonist-induced receptor phosphorylation detected in the same cells.

Relatively rapid desensitization of μ-opioid receptor-stimulated MAPK activation has also been reported (Li & Chang, 1996; Polakiewicz *et al*., 1998; Trapaidze *et al*., 2000) and, interestingly, morphine can be at least as effective as etorphine or DAMGO at producing this desensitization (Li & Chang, 1996). Unfortunately, it is not known whether the desensitization of μ-opioid receptor activation of MAPK reflects effects on the μ-opioid receptor or on one of the many components of the signalling cascade that leads to MAPK activation.

Significant differences in μ-opioid receptor desensitization produced by morphine and DAMGO, or etorphine, have been demonstrated in biochemical assays, but these differences are not consistent, and relating them to receptor regulatory events such as phosphorylation and internalization remains problematic. In HEK 293 cells, Whistler and colleagues have repeatedly shown that a 5 min pre-incubation with DAMGO (2 μM) produced complete desensitization of μ-opioid receptor stimulation of GTPγS binding and inhibition of AC, but a similar morphine (2 μM) pre-incubation had no effect at all (Whistler & von Zastrow, 1998; Whistler *et al*., 1999). By contrast, several other studies in HEK 293 cells have found that morphine (1 μM) and DAMGO (1 μM) produced similar rates and degrees of μ-opioid receptor desensitization, but this desensitization was not even 50% complete at 30 min (Koch *et al*., 2001; 2004). In CHO cells expressing human μ-opioid receptors, 20 min of DAMGO (1 μM) produced significantly greater desensitization than morphine (250 nM) (Yu *et al*., 1997), but in Neuro2A cells expressing μ-opioid receptors neither morphine nor DAMGO (500 nM each) produced any significant desensitization of AC activity until 8–10 h of treatment (Chakrabarti *et al*., 1998).

Is morphine really different from other μ*-receptor agonists?*

As outlined above, morphine differs most noticeably from DAMGO and other efficacious opioids in being disproportionally weak at internalizing receptors, which is consistent

across all cell types examined to date. By contrast, differences in receptor uncoupling and desensitization between morphine and DAMGO are not observed consistently, leading to uncertainty surrounding the relationship between agonist-induced μ-opioid receptor desensitization, receptor trafficking and long-term regulation of cellular opioid responses (cellular 'tolerance'). Two biochemical explanations have been advanced to explain differences between morphine- and DAMGO-induced receptor trafficking, differential agonist-induced phosphorylation of the receptor and differential activation of the enzyme PLD_2.

Firstly, it has been strongly suggested that morphine does not promote significant internalization of μ-opioid receptors because it fails to stimulate adequate receptor phosphorylation by GRKs, and thus subsequent arrestin binding and receptor recruitment to the clathrin-dependent endocytosis pathway is limited. There is significant circumstantial evidence in support of this supposition, because overexpression of GRKs promotes morphine phosphorylation and internalization of the receptor, and overexpression of arrestins also promotes morphine-induced μ-opioid receptor internalization. However, there is no detailed information about differential phosphorylation of identified residues of the μ-opioid receptor by morphine and DAMGO, and the specific role(s) of receptor phosphorylation and/or arrestin binding to the receptor in producing rapid uncoupling remain to be defined. When this information is available, we may be closer to understanding why morphine produces less efficient receptor internalization, and whether this has meaningful consequences for desensitization of opioid receptor signalling.

Secondly, it has been reported that DAMGO-stimulated activation of recombinant PLD_2 promotes μ-opioid receptor internalization, while morphine cannot stimulate μ-opioid receptor coupling to the enzyme and thus cannot promote receptor internalization. Koch *et al*. (2003) found that the carboxyl terminus of the μ-opioid receptor bound to the amino-terminus of PLD_2, and DAMGO, but not morphine, stimulated activity of the overexpressed enzyme. Furthermore, inhibiting PLD_2 activity with 1-butanol inhibited DAMGO-induced receptor endocytosis, as did overexpression of the amino-terminus of PLD_2 alone, suggesting that PLD_2 activation may be important for promoting μ-opioid receptor endocytosis. Koch and colleagues suggested that PLD_2 activation could lead to the accumulation of acidic phospholipids, which promote the correct membrane localization of AP2, a protein important in recruiting μ-opioid receptor/arrestin complexes to clathrin-coated pits. If PLD_2 activation does promote AP2 recruitment to receptor/arrestin/clathrin complexes, then the failure of morphine to activate this pathway may provide an explanation for the relative inability of morphine-activated μ-opioid receptors to internalize. However, the mechanism by which μ-opioid receptors activate recombinant PLD_2 is not known, nor has it been demonstrated that native receptors couple to native PLD_2.

Are there other possible explanations for the unique activity profile of morphine?

This review has focused on explanations for the perceived unique actions of morphine on μ-opioid receptor desensitization, which are largely derived from experiments aimed directly at elucidating the mechanisms underlying this process, that is, whether morphine binding promotes efficient receptor phosphorylation, receptor internalization and loss of receptor response. As stated repeatedly, the molecular basis for any differences that have been observed remain obscure, but a number of alternative mechanisms that may give rise to ligand-specific agonist properties have been suggested, largely based on insights derived from theoretical studies of ligand/receptor/G protein interactions. It is beyond the scope of this review to describe the background to these in detail, but careful consideration of the implications of these findings is warranted. Two fundamental properties of morphine that may be particularly important are μ-opioid receptor affinity and efficacy. Morphine has been described experimentally as an agonist of intermediate efficacy in many signalling systems, particularly when compared to enkephalins, β-endorphin, DAMGO, etorphine or fentanyl derivatives (e.g. Elliot *et al*., 1997; Ingram *et al*., 1997; Selley *et al*., 1997; Yu *et al*., 1997; Borgland *et al*., 2003). The difference in efficacy between commonly used agonists has several important implications for regulation of opioid receptor signalling. Firstly, agonists may have different efficacies for stimulating different cellular processes, and the rank order of efficacies for agonists at the same receptor may differ substantially for different processes. This may be reflected in a cellular environment as stimulus trafficking (Kenakin, 2002), with different agonists preferentially stimulating different signalling pathways. Ligand-specific stabilization of different conformations of the μ-opioid receptor have not been measured directly for the μ-opioid receptor, although the idea underlies the 'RAVE' hypothesis of Whistler and colleagues (Whistler *et al*., 1999; Waldhoer *et al*., 2004), and has been suggested many times as the reason underlying the 'unusual' inability of morphine to induce receptor internalization. Methods to directly measure ligand/receptor/G protein interactions at the intramolecular level are becoming available (see below); these may serve to identify significant differences between morphine and other agonists.

A second important consequence of differences in efficacy and affinity can emerge when ligand/receptor/effector interactions are modelled taking into account the kinetics of ligand binding at the receptor (Woolf & Linderman, 2003). Agonist off rate is an important determinant of the number of G proteins that can be activated by a ligand, because agonists that have faster off rates may be able to activate more G proteins by interacting with more receptors than ligands of equivalent efficacy but slower off rate (Stickle & Barber, 1983). If longer receptor occupancy is required for receptor regulatory events such as phosphorylation and arrestin binding, then purely kinetic considerations may be sufficient to explain an apparent efficacious agonist being relatively poor at recruiting receptor regulatory events (Woolf & Linderman, 2003). Unfortunately, there is little available data on the kinetics of opioid agonist binding in intact cellular systems.

The presence of other proteins that regulate elements of signalling, such as regulators of G protein signalling (RGS proteins, Zhong *et al*., 2003), can also have dramatic effects on the kinetics of agonist actions. For example, co-expression of RGS4 with α2 adrenoceptors promoted an apparent increase in G protein activity in the vicinity of the receptor, but a decrease in activity further away, essentially focusing on the agonist signal (Zhong *et al*., 2003). Further, expression of an RGS-insensitive Goα subunit in C6 glioma cells enhanced DAMGO signalling more than that of morphine (Clark *et al*.,

2003). Such activity may serve to further enhance the recruitment of receptor regulatory mechanisms by agonists that occupy receptors longer than morphine. The expression of different amounts or types of RGS proteins in cell types may also explain some of the differences in apparent receptor desensitization observed in studies of μ-opioid receptor regulation (e.g. Chuang *et al*., 1998).

Some of the uncertainties regarding the differential interaction of morphine and other agonists with the μ-opioid receptor may be resolved by emerging techniques such as fluorescent resonance energy transfer (FRET), which allow relatively direct measurements of conformational changes underlying receptor activation and, potentially, desensitization. Using fluorescent molecules attached to residues in the α_2-adrenoceptor and parathyroid hormone (PTH) receptors, Vilardaga *et al*. (2003) measured the changes in efficiency of the FRET between the molecules in response to agonist activation. FRET signals change as the distance between molecules changes, and a change in a FRET signal generated by two molecules in the same protein suggests a conformational change in the protein. Both noradrenaline and PTH produced rapid and sustained changes in FRET signal (<1 s) from their respective receptors, and, importantly, the partial agonist clonidine produced a FRET change different from the full agonist noradrenaline when applied to the α_2-adrenoceptor, indicating that it is apparently possible to distinguish agonists of different efficacy by the conformational changes they produce. Interestingly, by attaching a fluorescent molecule to arrestin, the authors also used FRET to measure arrestin recruitment to the vicinity of the PTH receptor, and they showed, as expected, that this occurred after receptor activation. A slightly different approach utilizes changes in the distribution of fluorescence lifetimes of reporter residues as they move in and out of different lipid/protein environments, presumably corresponding to different conformational states of the receptor. Utilizing the β_2-adrenoceptor, it was shown that agonists and partial agonists stabilize different sets of receptor conformation, and these different conformations could be correlated with the capacity of agonists to induce not only receptor signalling but also receptor internalization (Ghanouni *et al*., 2001; Swaminath *et al*., 2004). The usefulness of such techniques in exploring morphine and DAMGO regulation of μ-opioid receptors is obvious, although the techniques have potential limitations. These include the need to express relatively high densities of receptor in artificial systems, and the possibility that introduction of fluorescent reporter molecules into receptors will disrupt their native structure and function.

Despite the large amount of work exploring μ-opioid receptor regulation in isolated cells, the relevance of any of these processes to events *in vivo* or clinical management of opioid therapy remains to be established. Presumably, some continued signalling from μ-opioid receptors occupied by agonists is necessary to promote the cellular and synaptic adaptations that lead to clinical opioid tolerance and dependence, and, presumably, uncoupled receptors are of little use clinically. The amount of receptor uncoupling produced by chronic morphine treatment *in vivo* appears to be modest (Christie *et al*., 1987; Connor *et al*., 1999; Sim *et al*., 2000; Hack *et al*., 2003), while adaptations produced by similar regimens can be profound (Ingram *et al*., 1998; Hack *et al*., 2003). However, experiments comparing uncoupling and adaptation produced by chronic treatment with μ-opioid agonists that have similar signalling efficacies, but different efficacies for recruiting internalization have not been performed, and, crucially, the cellular adaptations produced by chronic morphine treatment of animals with genetic deletion of arrestin or a GRK remain largely unexplored. In animals with a deletion of arrestin 3, the acute antinociceptive action of morphine is prolonged, and functional measures of uncoupling of the μ-opioid receptor following chronic morphine treatment are abrogated, which suggests that arrestin-dependent regulatory processes are important for adaptations to both the acute and chronic effects of morphine (Bohn *et al*., 1999; 2000). These results may seem surprising in light of the experiments described above that find little evidence for an important interaction of morphine-activated μ-opioid receptors and arrestin. Unfortunately, the effects of opioids on single neurons from the arrestin knockout animals have not been reported, and, paradoxically, the antinociceptive effects of opioid drugs which do efficiently promote receptor internalization are apparently unchanged in either arrestin 2 or arrestin 3 knockout mice (Bohn *et al*., 2004). The potential implications of differential agonist-induced regulation of the μ-opioid receptor for the clinical management of chronic opioid therapies will remain very sketchy until the uncertainties outlined here are better resolved.

This work was supported by NH&MRC of Australia project grants #302002 (M.C.), #157158 (P.B.O.) and #211169 (M.J.C.). M.J.C. is also supported by NIDA grant DA12926-01.

References

ALT, A., MCFADYEN, I.J., FAN, C.D., WOODS, J.H. & TRAYNOR, J.R. (2001). Stimulation of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding in digitonin-permeabilized C6 rat glioma cells: evidence for an organized association of μ-opioid receptors and G protein. *J. Pharmacol. Exp. Ther.,* **298,** 116–121.

ALVAREZ, V.A., ARTTAMANGKUL, S., DANG, V., SALEM, A., WHISTLER, J., VON ZASTROW, M., GRANDY, D.K. & WILLIAMS, J.T. (2002). μ-Opioid receptors: ligand-dependent activation of potassium conductance, desensitization, and internalization. *J. Neurosci.,* **22,** 5769–5776.

ARDEN, J.R., SEGREDO, V., WANG, Z., LAMEH, J. & SADEE, W. (1995). Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged μ-opioid receptor expressed in HEK 293 cells. *J. Neurochem.,* **65,** 1636–1645.

AVIDOR-REISS, T., NEVO, I., SAYA, D., BAYEWITCH, M. & VOGEL, Z. (1997). Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J. Biol. Chem.,* **272,** 5040–5047.

BAILEY, C.P., COUCH, D., JOHNSON, E., GRIFFITHS, K., KELLY, E. & HENDERSON, G. (2003). μ-Opioid receptor desensitization in mature rat neurons: lack of interaction between DAMGO and morphine. *J. Neurosci.,* **23,** 10515–10520.

BLANCHET, C. & LUSCHER, C. (2002). Desensitization of μ-opioid receptor-evoked potassium currents: initiation at the receptor, expression at the effector. *Proc. Natl. Acad. Sci. U.S.A.,* **99,** 4674–4679.

BOHN, L.M., DYKSTRA, L.A., LEFKOWITZ, R.J., CARON, M.G. & BARAK, L.S. (2004). Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol. Pharmacol.,* **66,** 106–112.

BOHN, L.M., GAINETDINOV, R.R., LIN, F., LEFKOWITZ, R.J. & CARON, M.G. (2000). μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature,* **408,** 720–723.

BOHN, L.M., LEFKOWITZ, R.J., GAINETDINOV, R.R., PEPPEL, K, CARON, M.G. & LIN, F.T. (1999). Enhanced morphine analgesia in mice lacking β-arrestin 2. *Science,* **286,** 2495–2498.

BORGLAND, S.L., CONNOR, M., OSBORNE, P.B., FURNESS, J.B. & CHRISTIE, M.J. (2003). Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J. Biol. Chem.,* **278,** 18776–18784.

BUSHELL, T., ENDOH, T., SIMEN, A.A., REN, D., BINDOKAS, V.P. & MILLER, R.J. (2002). Molecular components of tolerance to opiates in single hippocampal neurons. *Mol. Pharmacol.,* **61,** 55–64.

CELVER, J., XU, M., JIN, W., LOWE, J. & CHAVKIN, C. (2004). Distinct domains on the μ-opioid receptor control uncoupling and internalization. *Mol. Pharmacol.,* **65,** 528–537.

CEN, B., XIONG, Y., MA, L. & PEI, G. (2001). Direct and differential interaction of β-arrestins with the intracellular domains of different opioid receptors. *Mol. Pharmacol.,* **59,** 758–764.

CHAKRABARTI, S., LAW, P.-Y. & LOW, H.H. (1998). Distinct differences between morphine- and [D-Ala^2,N-MePhe^4,Gly-ol^5]-enkephalin-μ-opioid receptor complexes demonstrated by cyclic AMP-dependent protein kinase phosphorylation. *J. Neurochem.,* **71,** 231–239.

CHAKRABARTI, S., PRATHER, P., YU, Y., LAW, P.-Y. & LOW, H.H. (1995). Expression of the μ-opioid receptor in CHO cells: ability of μ-opioid ligands to promote α-azidoanilido[^{32}P]GTP labeling of multiple G protein α subunits. *J. Neurochem.,* **64,** 2534–2543.

CHRISTIE, M.J., WILLIAMS, J.T. & NORTH, R.A. (1987). Cellular mechanisms of opioid tolerance: studies in single brain neurons. *Mol. Pharmacol.,* **32,** 633–638.

CHRISTOPOULOS, A. & EL-FAKAHANY, E.E. (1999). Qualitative and quantitative assessment of relative agonist efficacy. *Biochem. Pharmacol.,* **58,** 735–748.

CHUANG, H.H., YU, Y., JAN, Y.N. & JAN, L.Y. (1998). Evidence that the nucleotide exchange and hydrolysis cycle of G proteins causes acute desensitization of G protein-gated inward rectifier K^+ channels. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 11727–11732.

CLARK, M.J., HARRISON, C., ZHONG, H., NEUBIG, R.R. & TRAYNOR, J.R. (2003). Endogenous RGS protein action modulates μ-opioid signalling through Gαo. *J. Biol. Chem.,* **278,** 9418–9425.

CLARK, R.B., KNOLL, B.J. & BARBER, R. (1999). Partial agonists and G protein-coupled receptor desensitization. *Trends Pharmacol. Sci.,* **20,** 279–286.

CONNOR, M., BORGLAND, S.L. & CHRISTIE, M.J. (1999). Continued morphine modulation of calcium channel currents in acutely isolated locus coeruleus neurons from morphine-dependent rats. *Br. J. Pharmacol.,* **128,** 1561–1569.

CONNOR, M. & CHRISTIE, M.J. (1999). Opioid receptor signalling mechanisms. *Clin. Exp. Pharmacol. Physiol.,* **26,** 493–499.

CONNOR, M., VAUGHAN, C.W., CHIENG, B. & CHRISTIE, M.J. (1996). Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones *in vitro*. *Br. J. Pharmacol.,* **119,** 1614–1618.

DENG, H.B., YU, Y., PAK, Y., O'DOWD, B.F., GEORGE, S.R., SURRATT, C.K., UHL, G.R. & WANG, J.B. (2000). Role for the C-terminus in agonist-induced μ-opioid receptor phosphorylation and desensitization. *Biochemistry,* **39,** 5492–5499.

DENG, H.B., YU, Y., WANG, H., GUANG, W. & WANG, J.B. (2001). Agonist-induced μ opioid receptor phosphorylation and functional desensitization in rat thalamus. *Brain Res.,* **898,** 204–214.

EL KOUHEN, R., BURD, A.L., ERICKSON-HERBRANDSON, L.J., CHANG, C.-Y., LAW, P.-Y. & LOH, H.H. (2001). Phosphorylation of Ser^{363}, Thr^{370} and Ser^{375} residues within the carboxyl tail differentially regulates μ-opioid receptor internalization. *J. Biol. Chem.,* **276,** 12774–12780.

EL KOUHEN, R., MAESTRI-EL KOUHEN, O., LAW, P.-Y. & LOW, H.H. (1999). The absence of a direct correlation between the loss of [D-Ala^2,N-MePhe^4,Gly^5-ol]enkephalin inhibition of adenylyl cyclase activity and agonist-induced μ-opioid receptor phosphorylation. *J. Biol. Chem.,* **274,** 9207–9215.

ELLIOT, J., GUO, L. & TRAYNOR, J.R. (1997). Tolerance to μ-opioid agonists in human neuroblastoma SH-SY5Y cells as determined by changes in guanosine-5′-O-(3-[^{35}S]-thio)triphosphate binding. *Br. J. Pharmacol.,* **121,** 1422–1428.

FINN, A.K. & WHISTLER, J.L. (2001). Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of withdrawal. *Neuron,* **32,** 829–839.

FIORELLO, C.D. & WILLIAMS, J.T. (1996). Opioid desensitization: interactions with G-protein-coupled receptors in the locus coeruleus. *J. Neurosci.,* **16,** 1479–1485.

FREDERICKS, Z.L., PITCHER, J.L. & LEFKOWITZ, R.J. (1996). Identification of the G protein-coupled receptor kinase phosphorylation sites in the human β2-adrenergic receptor. *J. Biol. Chem.,* **271,** 13796–13803.

GHANOUNI, P., GRYCZYNSKI, Z., STEENHUIS, J.J., LEE, T.W., FARRENS, D.L., LAKOWICZ, J.R. & KOBILKA, B.K. (2001). Functionally different agonists induce distinct conformations in the G protein coupling domain of the β2 adrenergic receptor. *J. Biol. Chem.,* **276,** 24433–24436.

GUREVICH, V.V. & GUREVICH, E.V. (2004). The molecular acrobatics of arrestin activation. *Trends Pharmacol. Sci.,* **25,** 105–111.

HABERSTOCK-DEBIC, H., WEIN, M., BARROT, M., COLAGO, E.E.O., RAHMAN, Z., NEVE, R.L., PICKEL, V.M., NESTLER, E.J., VON ZASTROW, M. & SVINGOS, A.L. (2003). Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. *J. Neurosci.,* **23,** 4324–4332.

HACK, S.P., VAUGHAN, C.W. & CHRISTIE, M.J. (2003). Modulation of GABA release during morphine withdrawal in midbrain neurons *in vitro*. *Neuropharmacology,* **45,** 575–584.

HARRIS, G.C. & WILLIAMS, J.T. (1991). Transient, homologous μ-opioid receptor desensitization in rat locus coeruleus neurons. *J. Neurosci.,* **11,** 2574–2581.

HE, L., FONG, J., VON ZASTROW, M. & WHISTLER, J.L. (2002). Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell,* **108,** 271–282.

INGRAM, S., WILDING, T.J., MCCLESKEY, E.W. & WILLIAMS, J.T. (1997). Efficacy and kinetics of opioid action on acutely dissociated neurons. *Mol. Pharmacol.,* **52,** 136–143.

INGRAM, S.L., VAUGHAN, C.W., BAGLEY, E.E., CONNOR, M. & CHRISTIE, M.J. (1998). Enhanced opioid efficacy in opioid dependence is caused by an altered signal transduction pathway. *J. Neurosci.,* **18,** 10269–10276.

KEITH, D.E., ANTON, B., MURRAY, S.R., ZAKI, P.A., CHU, P.C., LISSIN, D.V., MONTEILLET-AGIUS, G., STEWART, P.L., EVANS, C.J. & VON ZASTROW, M. (1998). μ-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism *in vitro* and in the mammalian brain. *Mol. Pharmacol.,* **53,** 377–384.

KEITH, D.E., MURRAY, S.R., ZAKI, P.A., CHU, P.C., LISSIN, D.V., KANG, L., EVANS, C.J. & VON ZASTROW, M. (1996). Morphine activates opioid receptors without causing their rapid internalization. *J. Biol. Chem.,* **271,** 19021–19024.

KENAKIN, T. (2002). Drug efficacy at G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.,* **42,** 349–379.

KOCH, T., BRANDENBURG, L.-O., LIANG, Y., SCHULZ, S., BEYER, A., SCHRODER, H. & HOLLT, V. (2004). Phospholipase D_2 modulates agonist-induced μ-opioid receptor desensitization and resensitization. *J. Neurochem.,* **88,** 680–688.

KOCH, T., BRANDENBURG, L.-O., SCHULZ, S., LIANG, Y., KLEIN, J. & HOLLT, V. (2003). ADP-ribosylation factor-dependent phospholipase D_2 activation is required for agonist-induced μ-opioid receptor endocytosis. *J. Biol. Chem.,* **278,** 9979–9986.

KOCH, T., KROSLAK, T., MAYER, P., RAULF, E. & HOLLT, V. (1997). Site mutation in the rat μ-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein kinase II in agonist-mediated desensitization. *J. Neurochem.,* **69,** 1767–1770.

KOCH, T., SCHULZ, S., PFEIFFER, M., KLUZTNY, M., SCHRODER, H., KAHL, E. & HOLLT, V. (2001). C-terminal splice variants of the mouse μ-opioid receptor differ in morphine-induced internalization and receptor resensitization. *J. Biol. Chem.,* **276,** 31408–31414.

KOCH, T., SCHULZ, S., SCHRODER, H., WOLF, R., RAULF, E. & HOLLT, V. (1998). Carboxyl-terminal splicing of the rat μ opioid receptor modulates agonist-mediated internalization and receptor resensitization. *J. Biol. Chem.,* **273,** 13652–13667.

KOVOOR, A., CELVER, J.P., WU, A. & CHAVKIN, C. (1998). Agonist induced homologous desensitization of μ-opioid receptors is mediated by G protein-coupled receptor kinases and is dependent on agonist efficacy. *Mol. Pharmacol.,* **54,** 704–711.

KOVOOR, A., HENRY, D.J. & CHAVKIN, C. (1995). Agonist-induced desensitization of the mu opioid receptor-coupled potassium channel (GIRK1). *J. Biol. Chem.,* **270,** 589–595.

KOVOOR, A., NAPPEY, V., KIEFFER, B.L. & CHAVKIN, C. (1997). μ and δ opioid receptors are differentially desensitized by the co-expression of β-adrenergic receptor kinase 2 and β-arrestin 2 in *Xenopus* oocytes. *J. Biol. Chem.,* **272,** 27605–27611.

KRUEGER, K.M., DAAKA, Y., PITCHER, J.A. & LEFKOWITZ, R.J. (1997). The role of sequestration in G protein-coupled receptor resensitization. *J. Biol. Chem.,* **272,** 5–8.

LAW, P.-Y., ERICKSON, L.J., EL-KOUHEN, R., DICKER, L., SOLBERG, J., WANG, W., MILLER, E., BURD, A.L. & LOH, H.H. (2000). Receptor density and recycling affect the rate of agonist-induced desensitization of μ-opioid receptor. *Mol. Pharmacol.,* **58,** 388–398.

LEANEY, J.L., BENIANS, A., BROWN, S., NOBLES, M., KELLEY, D. & TINKER, A. (2004). Rapid desensitization of G protein-gated inwardly rectifying K^+ currents is determined by G protein cycle. *Am. J. Physiol. Cell Physiol.,* **287,** C182–C191.

LI, A.H. & WANG, H.-L. (2001). G protein-coupled receptor kinase 2 mediates μ-opioid receptor desensitization in GABAergic neurons of the nucleus raphe magnus. *J. Neurochem.,* **77,** 435–444.

LI, J., XIANG, B., SU, W., ZHANG, X., HAUNG, Y. & MA, L. (2003). Agonist-induced formation of opioid receptor-G protein-coupled receptor kinase (GRK)-G(βγ) complex on membrane is required for GRK2 function *in vivo*. *J. Biol. Chem.,* **278,** 30219–30226.

LI, L.Y. & CHANG, K.J. (1996). The stimulatory effect of opioids on mitogen-activated protein kinase in Chinese hamster ovary cells transfected to express mu-opioid receptors. *Mol. Pharmacol.,* **50,** 599–602.

LOHSE, M.J., BENOVIC, J.L., CARON, M.G & LEFKOWITZ, R.J. (1990). Multiple pathways of rapid β2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J. Biol. Chem.,* **265,** 3202–3211.

LOWE, J.D., CELVER, J.P., GUREVICH, V.V. & CHAVKIN, C.C. (2002). μ-Opioid receptors desensitise less rapidly than δ-opioid receptors due to less efficient activation of arrestin. *J. Biol. Chem.,* **277,** 15729–15735.

MASSOTTE, D., BRILLET, K., KIEFFER, B.L. & MILLIGAN, G. (2002). Agonists activate $G_{i1}\alpha$ or $G_{i2}\alpha$ fused to the human mu opioid receptor differently. *J. Neurochem.,* **82,** 1372–1382.

MCLAUGHLIN, J.P., MYERS, L.C., ZAREK, P.E., CARON, M.G., LEFKOWITZ, R.J., CZYZYK, T.A. & PINTAR, J.E. (2004). Prolonged kappa opioid receptor phosphorylation mediated by G protein receptor kinase underlies sustained analgesic tolerance. *J. Biol. Chem.,* **279,** 1810–1818.

MCQUAY, H. (1999). Opioids in pain management. *Lancet,* **353,** 2229–2232.

NOMURA, K., REUVENY, E. & NARAHASHI, T. (1994). Opioid inhibition and desensitization of calcium channel currents in rat dorsal root ganglion neurons. *J. Pharmacol. Exp. Ther.,* **270,** 466–474.

OAKLEY, R.H., LAPORTE, S.A., HOLT, J.A., CARON, M.G. & BARAK, L.S. (2000). Differential affinities of visual arrestin, βarrestin1, and βarrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J. Biol. Chem.,* **275,** 17201–17210.

OSBORNE, P.B. & WILLIAMS, J.T. (1995). Characterization of acute homologous desensitization of μ-opioid receptor-induced currents in locus coeruleus neurones. *Br. J. Pharmacol.,* **115,** 925–932.

PAK, Y., O'DOWD, B.F., WANG, J.B. & GEORGE, S.R. (1999). Agonist-induced, G protein-dependent and -independent down regulation of the μ opioid receptor. *J. Biol. Chem.,* **274,** 27610–27616.

POLAKIEWICZ, R.D., SCHIEFERL, S.M., DORNER, L.F., KANSRA, V. & COMB, M.J. (1998). A mitogen-activated protein kinase pathway is required for μ-opioid receptor desensitization. *J. Biol. Chem.,* **273,** 12402–12406.

SAMORISKI, G.M. & GROSS, R.A. (2000). Functional compartmentalization of opioid desensitization in primary sensory neurons. *J. Pharmacol. Exp. Ther.,* **294,** 500–509.

SCHULZ, R., WEHMEYER, A. & SCHULZ, K. (2002). Opioid receptor types selectively cointernalize with G protein-coupled receptor kinases 2 and 3. *J. Pharmacol. Exp. Ther.,* **300,** 376–384.

SELLEY, D.E., SIM, L.J., XIAO, R., LIU, Q. & CHILDERS, S.R. (1997). μ-Opioid-receptor stimulated guanosine-5′-*O*-(γ-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. *Mol. Pharmacol.,* **51,** 87–96.

SIM, L.J., SELLEY, D.E., DWORKIN, S.I. & CHILDERS, S.R. (2000). Effects of chronic morphine administration on mu opioid receptor-stimulated [^{35}S]GTPγS autoradiography in rat brain. *J. Neurosci.,* **16,** 2684–2692.

STERNINI, C., SPANN, M., ANTON, B., KEITH JR, D.E., BUNNET, N.W., VON ZASTROW, M., EVANS, C. & BRECHA, N.C. (1996). Agonist-selective endocytosis of μ opioid receptor by neurons *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 9421–9426.

STICKLE, D. & BARBER, R. (1983). Evidence for the role of epinephrine binding frequency in activation of adenylate cyclase. *Mol. Pharmacol.,* **36,** 437–445.

SWAMINATH, G., XIANG, Y., LEE, T.W., STEENHUIS, J., PARNOT, C. & KOBILKA, B.K. (2004). Sequential binding of agonists to the β_2-adrenoceptor. *J. Biol. Chem.,* **279,** 686–691.

TANOWITZ, M. & VON ZASTROW, M. (2003). A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J. Biol. Chem.,* **278,** 45978–45986.

TRAPAIDZE, N., GOMES, I., CVEJIC, S., BANSINATH, M. & DEVI, L.A. (2000). Opioid receptor endocytosis and activation of MAP kinase pathway. *Mol. Brain Res.,* **76,** 220–228.

TRZECIAKOWSKI, J.P. (1999). Stimulus amplification, efficacy, and the operational model. Part I – binary complex occupancy mechanisms. *J. Theor. Biol.,* **198,** 329–346.

VILARDAGA, J.P., BUNEMANN, M., KRASEL, C., CASTRO, M. & LOHSE, M.J. (2003). Measurement of the millisecond activation switch of G protein-coupled receptors in living cells. *Nat. Biotechnol.,* **21,** 807–812.

VON ZASTROW, M., SVINGOS, A., HABERSTOCK-DEBIC, H. & EVANS, C. (2003). Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. *Curr. Opin. Neurobiol.,* **13,** 348–353.

WALDHOER, M., BARTLETT, S.E. & WHISTLER, J.L. (2004). Opioid receptors. *Annu. Rev. Biochem.,* **73,** 953–990.

WANG, H.-L., CHANG, W.-T., HSU, C.-Y., HUANG, P.-C., CHOW, Y.-W. & LI, A.H. (2002). Identification of two C-terminal amino acids, Ser^{355} and Thr^{357}, required for short-term homologous desensitization of μ-opioid receptors. *Biochem. Pharmacol.,* **64,** 257–266.

WHISTLER, J.L., CHUANG, H.-H., CHU, P., JAN, L.Y. & VON ZASTROW, M. (1999). Functional dissociation of μ-opioid receptor signalling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron,* **23,** 737–746.

WHISTLER, J.L. & VON ZASTROW, M. (1998). Morphine activated opioid receptors elude desensitization by β-arrestin. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 9914–9919.

WILDING, T.J., WOMACK, M.D. & MCCLESKEY, E.W. (1995). Fast, local signal transduction between the mu opioid receptor and Ca^{2+} channels. *J. Neurosci.,* **15,** 4124–4132.

WOOLF, P.J. & LINDERMAN, J.J. (2003). Untangling ligand induced activation and desensitization of G protein-coupled receptors. *Biophys. J.,* **84,** 3–13.

YU, Y., ZHANG, L., YIN, X., SUN, H., UHL, G.R. & WANG, J.B. (1997). μ-Opioid receptor phosphorylation, desensitization and ligand efficacy. *J. Biol. Chem.,* **272,** 28869–28874.

ZHANG, J., FERGUSON, S.S.G., BARAK, L.S., BODDULURI, S.R., LAPORTE, S.A., LAW, P.-Y. & CARON, M.G. (1998). Role for G protein-coupled receptor kinase in agonist-specific regulation of μ-opioid receptor responsiveness. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 7157–7162.

ZHANG, L., YU, Y., MACKIN, S., WEIGHT, F.F., UHL, G.R. & WANG, J.B. (1996). Differential μ opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. *J. Biol. Chem.,* **271,** 11449–11454.

ZHONG, H., WADE, S.M., WOOLF, P.J., LINDERMAN, J.J., TRAYNOR, J.R. & NEUBIG, R.R. (2003). A spatial focusing model for G protein signals. *J. Biol. Chem.,* **278,** 7278–7284.

(Received April 15, 2004
Revised July 1, 2004
Accepted July 8, 2004)