

## PERSPECTIVE

# Agonists at $\mu$ -Opioid Receptors Spin the Wheels to Keep the Action Going

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### ABSTRACT

A study in this issue of *Molecular Pharmacology* on agonist-induced internalization of  $\mu$ -opioid receptors during long-term opiate drug exposure is discussed. The study demonstrates the

critical role of re-cycling of reactivated  $\mu$  receptors back to the plasma membrane for the maintenance of agonist signaling during long-term opiate exposure.

$\mu$ -Opioid receptors (MOP-r) are G protein-coupled receptors signaling primarily through Gi/o proteins to inhibit adenylyl cyclase and regulate the opening of K<sup>+</sup> and Ca<sup>2+</sup> ion channels. After activation of MOP-r by full agonists such as the enkephalin analog DAMGO or the synthetic drug sufentanil, the receptor is phosphorylated and then internalized. These processes are linked but can be separated (Clever et al., 2004; Cox and Crowder, 2004). Internalization of the receptor removes a significant fraction of functional MOP-r from the plasma membrane and thus may contribute in part to the loss of agonist signaling (tolerance) induced by high or sustained agonist exposures (Whistler et al., 1999). Receptor phosphorylation after exposure to morphine and some other clinically useful analgesics occurs at a much slower rate than after exposure to full agonists, and morphine induces little internalization of the receptor (Keith et al., 1996; Alvarez et al., 2002). Once internalized after full agonist exposure, a region of the MOP-r sequence directs the internalized receptors to a re-cycling pathway that ultimately returns de-phosphorylated receptors back to the plasma membrane with restored functionality, where they are immediately available to re-initiate agonist signaling (Tanowitz and von Zastrow, 2003). It has been unclear whether this recycling process is rapid enough to permit recovery of signaling during a single

exposure to high concentrations of full agonist. An article in the current issue of *Molecular Pharmacology* sheds further light on the role of agonist-induced internalization of MOP-r in maintaining agonist signaling (Koch et al., 2005).

Koch et al. (2005) have compared the loss of agonist signaling (desensitization) induced by 13 different opiate drugs and peptides whose abilities to induce receptor internalization differ considerably. Desensitization was measured as the loss of MOP-r agonist-induced inhibition of forskolin-stimulated adenylyl cyclase (AC) after 60-min exposure to a receptor-saturating concentration of each agonist in HEK 293 cells transfected with the rat MOP-r sequence containing a hemagglutinin (HA) epitope tag at the amino terminus to permit visualization and immunoquantitation. Receptor internalization was quantified by an enzyme-linked immunosorbent assay-based test measuring the loss of binding of a rabbit anti-HA antibody to HA-MOP-r on the plasma membrane of cells after 60 min of prior exposure to a wide range of concentrations of agonists. MOP-r ligands were selected to include drugs giving levels of desensitization from 10 to 70% loss of agonist inhibition of AC and a similarly broad range of extent of internalization, from 5% for morphine to 50% for DAMGO, the highest level of internalization observed in these studies. A highly significant inverse correlation ( $P < 0.0002$ ) was found between desensitization and internalization. Internalizing agonists such as DAMGO, etonitazene, and sufentanil induced little desensitization in 60 min. Agonists that failed to induce substantial internalization, such

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**ABBREVIATIONS:** MOP-r,  $\mu$ -opioid receptor; DAMGO, [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-oI<sup>5</sup>] enkephalin; AC, adenylyl cyclase; CB1, cannabinoid I receptor; HEK, human embryonic kidney; HA, hemagglutinin; DOP-r,  $\delta$ -opioid receptor.

TABLE 1

Cellular adaptations after MOP-r activation by internalizing agonists

Adaptive Process	Time Scale (after DAMGO treatment of HEK 293 cells expressing MOP-r)	Apparent Functional Consequences
MOP-r - effector functional un-coupling after receptor phosphorylation by GRKs, PKA, or PKC.	Within minutes. <sup>a</sup>	Rapid loss of agonist signaling (e.g., reduced inhibition of adenylyl cyclase) after receptor phosphorylation.
Endocytosis of MOP-r via clathrin-coated pits.	Within a few minutes.	Removal of desensitized receptor from plasma membrane.
Endosomal sorting and recycling of re-activated MOP-r to plasma membrane.	Within 1 h.	Recycled receptors restore agonist signaling.
Increased levels of cAMP (with increased PKA activity); other postreceptor adaptations.	Longer than 1 h, but less than 8 h.	Functional antagonism of MOP-r signaling; physical dependence.

GRK, G protein-coupled receptor kinase; PKA, protein kinase A; PKC, protein kinase C.

<sup>a</sup> From Alvarez et al. (2002). The biochemical techniques used by Koch et al. (2005) are not suited to an estimation of the initial rate of MOP-r desensitization.

as morphine, oxycodone, and pethidine (meperidine in the United States), showed the greatest loss of agonist response during a 60-min exposure.

These results strongly suggest that within the 60-min period of preincubation with agonists, a substantial fraction of internalized desensitized MOP-r are returned to the plasma membrane as reactivated receptors, whereas noninternalizing agonists induce an accumulation of nonfunctional desensitized receptors on the plasma membrane surface. However, interpretation of these results is complicated by the presence of spare receptors in this system, because the drugs that induced relatively little internalization of MOP-r are known from other studies to be partial agonists at MOP-r. It is possible that a high degree of desensitization might simply be a consequence of partial agonism. Koch et al. (2005) therefore reevaluated MOP-r desensitization when recycling of the internalized MOP-r to the plasma membrane was prevented by coincubation with monensin, an agent that raises endosome pH and thus prevents recycling of internalized receptors (Benzi et al., 1997; Baratti-Elbaz et al., 1999; Letierrier et al., 2004). In the presence of monensin, receptor-internalizing full agonists, such as DAMGO, or agonists with intermediate ability to internalize MOP-r, such as methadone, produced a significantly greater desensitization than in its absence, a level of desensitization that was similar to that observed with morphine, a partial agonist, in the absence of monensin. Thus, prevention of receptor recycling after full agonist exposure results in a level of desensitization comparable with that observed with noninternalizing partial agonists.

Another possible explanation of the inverse correlation between desensitization and internalization observed by Koch et al. (2005) is that the reduction of agonist signaling after long-term morphine treatment might result simply from the well established increase in cAMP production (called superactivation) after sustained opiate drug exposure (Avidor-Reiss et al., 1995). However, there was no increase in forskolin-stimulated cAMP production in the MOP-r-transfected HEK 293 cells after 60 min of full or partial agonist exposure followed by washout, indicating that cAMP superactivation cannot explain the observed desensitization induced by either type of MOP-r agonist at 60 min. After 8 h of agonist treatment, however, an up-regulation of cAMP production was clearly apparent, with the receptor-internalizing agonist DAMGO giving a significantly greater increase in cAMP levels than the noninternalizing agonist morphine. Coincident treatment with DAMGO together with monensin for 8 h to prevent receptor recycling reduced the level of

superactivation of cAMP production by DAMGO back to the level of observed with morphine alone, confirming that the extent of cAMP superactivation is related to the degree of MOP-r internalization and re-cycling, and not to the nature of the specific agonist inducing superactivation. Cotreatment with monensin and morphine did not alter the lower degree of cAMP superactivation induced by morphine because morphine does not induce receptor re-cycling.

Changes can be made in the MOP-r sequence that facilitate more rapid morphine-induced receptor phosphorylation and a greater degree of internalization than morphine can induce in the wild-type receptor. Finn and Whistler (2001) made a MOP-r construct in which the cytoplasmic tail of the wild-type MOP-r was replaced by the cytoplasmic tail of the  $\delta$ -opioid receptor (DOP-r); morphine treatment induced significant endocytosis of this chimeric receptor. Long-term exposure (14 h) of this receptor to morphine induced significantly less superactivation of cAMP production than equivalent exposure of the wild-type MOP-r. Finn and Whistler (2001) interpreted this result to indicate that endocytosis of MOP-r reduces cAMP superactivation. However, the cytoplasmic tail of DOP-r directs the internalized receptor to a lysosomal degradative pathway precluding recycling of the chimeric receptor to the plasma membrane. In contrast, endocytosis of wild-type MOP-r leads to rapid re-cycling of functional receptors to the plasma membrane (Tsao and von Zastrow, 2000; Wang et al., 2003). Koch et al. (2001) prepared a different splice-variant form of MOP-r, MOR1D, that permits morphine-induced endocytosis while directing the variant receptor to the re-cycling pathway. Koch et al. (2005) now show that in an HEK 293 cell line transfected with the MOR1D splice-variant form of MOP-r, exposure to morphine for 8 h produces a degree of cAMP superactivation that is significantly greater than that induced by morphine acting on wild-type receptors and closely comparable with that induced by DAMGO. Thus, the morphine-internalized chimeric MOP/DOP-r of Finn and Whistler (2001) is targeted to a degradation pathway resulting in a sustained loss of agonist signaling and less cAMP superactivation, whereas the morphine-internalized MOR1D-MOP-r of Koch et al. (2001, 2005) is rapidly re-cycled to the plasma membrane to restore agonist signaling, inducing a greater cAMP superactivation. Together, these results demonstrate unambiguously that recycling of wild-type MOP-r to the cell membrane after internalization by agonist results in a restoration of agonist inhibition of AC activity, and a delayed (8 h or more) up-regulation of cAMP production.

A critical question is whether these results can be transferred to the regulation of endogenous MOP-r in normal neurons. Each of the adaptive processes studied by Koch et al. (2005), including agonist-induced desensitization of MOP-r, receptor internalization, recycling of reactivated MOP-r to the plasma membrane, and agonist-induced up-regulation of cAMP pathways, have been observed in MOP-r expressing neurons in the central nervous system of rat and other species (Trafton et al., 2000; Alvarez et al., 2002; Trafton and Basbaum, 2004). Thus, the detailed events documented in the model system used by Koch et al. (2005) are probably relevant to the actions of opiate drugs acting on MOP-r in neurons in vivo. The recycling pathway for MOP-r seems similar to that used by several other G protein-coupled receptors, including  $\beta_2$ -adrenergic receptors (Tsao and von Zastrow, 2000), the TSH receptor (Baratti-Elbaz et al., 1999), and CB1 cannabinoid receptors (Leterrier et al., 2004).

The functional significance of the rapid re-cycling of activated MOP-r with respect to the actions of endogenous peptide ligands (enkephalins,  $\beta$ -endorphin) or to the therapeutic use of opiate analgesics is unclear. It is possible that transiently released endogenous opioids induce receptor internalization with recycling of receptor maintaining agonist function. Transient activation of MOP-r does not induce cAMP superactivation or other longer-term adaptive responses. Clinical exposures to opiate drugs are usually of longer duration, and many of the drugs used in this study are clinically useful agonists at MOP-r. The reduced loss of agonist signaling observed after treatment with DAMGO or etonitazene (relative to morphine) might suggest that these drugs would be more effective as analgesic agents than the rapidly desensitizing agents morphine, hydromorphone, oxycodone, or pethidine. In practice, however, morphine and related drugs retain a clinically useful degree of agonist potency as analgesics over days or weeks despite the high level of desensitization of MOP-r that they can induce. This may be related to the presence of an excess of MOP-r (spare receptors) in opiate-regulated pain pathways (Zernig et al., 1995, 1997). Therapeutic doses of morphine can apparently activate a sufficient percentage of MOP-r at strategic sites on nociceptive pathways to provide effective modulation of pain perception even though a significant fraction of receptors activated by morphine are desensitized. Internalizing agonists, such as DAMGO, sufentanil, or etonitazene, may give better retention of agonist signaling in the short term, but may also induce more marked superactivation of compensatory pathways, including enhanced AC activity, that would at later time points indirectly reduce the effectiveness of MOP-r activation and also induce increased physical dependence. DAMGO, a peptide, does not readily enter the CNS, limiting its usefulness for therapeutic purposes. Etonitazene, a potent analgesic in rodents, is also not used clinically. Sufentanil and closely related drugs are used in the clinic primarily as potent anesthetic agents for relatively short procedures and for regional analgesia in child-birth, not as systemic analgesics for pain relief over periods of many days. This is largely

because of their rapid elimination in vivo, although a recent study has shown that continuously administered sufentanil can provide moderate pain relief over periods as long as 16 h (Reynolds et al., 2004). Any possible clinical relevance of the interesting results reported by Koch et al. (2005) will require further study in more complex neuronal systems where the additional modulating influences of *trans*-synaptic adaptations can also be evaluated.

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