Agonists at mu opioid receptors spin the wheels to keep the action going.

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Running Title:
Recycling of mu opioid receptors sustains agonist signaling
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosylmonophosphate</td>
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<tr>
<td>CB1</td>
<td>cannabinoid 1 receptor</td>
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<tr>
<td>DAMGO</td>
<td>[D-Ala², NMePhe⁴,Gly-ol⁵]enkephalin</td>
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<tr>
<td>DOP-r</td>
<td>delta opioid receptor</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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<tr>
<td>GRK</td>
<td>G protein coupled receptor kinases</td>
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<tr>
<td>HA-MOP-r</td>
<td>hemagglutinin tagged mu opioid receptor</td>
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<tr>
<td>HEK293</td>
<td>human embryonic kidney cell line 293</td>
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<tr>
<td>MOP-r</td>
<td>mu opioid receptor</td>
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<tr>
<td>MOR1D</td>
<td>splice variant of mu opioid receptor</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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</table>
ABSTRACT:

A study in this issue of *Molecular Pharmacology* on agonist-induced internalization of mu-opioid receptors during chronic 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 chronic opiate exposure.
Mu-opioid receptors (MOP-r) are G protein coupled receptors (GPCR) signaling primarily through Gi/o proteins to inhibit adenylyl cyclase and regulate the opening of K+ and Ca2+ ion channels. Following 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 (Celver et al, 2004; see Perspective by Cox & 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; Alvares et al, 2002). Once internalized after full agonist exposure, a region of the MOP-r sequence directs the internalized receptors to a recycling 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 & 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. A paper in the current issue of *Molecular Pharmacology* now sheds further light on the role of agonist-induced internalization of MOP-r in maintaining agonist signaling (Koch et al, 2004).

Koch et al (2004) have compared the loss of agonist signaling (desensitization) induced by thirteen 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 immuno-quantitation. Receptor internalization was quantified by an ELISA-based assay 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, etonitazine and sufentanil, induced little desensitization in 60 min. Agonists that failed to induce substantial internalization, such as morphine, oxycodone and pethidine (meperidine in USA), showed the greatest loss of agonist response during a 60 min exposure.

These results strongly suggest that within the 60 min period of pre-incubation with agonists, a substantial fraction of internalized desensitized MOP-r are returned to the plasma membrane as reactivated receptors, while non-internalizing agonists induce an accumulation of non-functional desensitized receptors on the plasma membrane surface. However interpretation of these results is complicated by the presence of spare receptors in this system since 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 (2004) therefore re-evaluated MOP-r desensitization when recycling of the internalized MOP-r to the plasma membrane was prevented by co-incubation with monensin, an agent that raises endosome pH and thus prevents recycling of internalized receptors (Benzi et al, 1997; Baratti-Elbaz et al, 1999; Leterrier 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 to that observed with non-internalizing partial agonists.

Another possible explanation of the inverse correlation between desensitization and internalization observed by Koch et al (2004) is that the reduction of agonist
signaling after chronic 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 hr 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 non-internalizing agonist, morphine. Coincident treatment with DAMGO together with monensin for 8 hr 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. Co-treatment with monensin and morphine did not alter the lower degree of cAMP superactivation induced by morphine since 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 & 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 hr) of this receptor to morphine induced significantly less superactivation of cAMP production than equivalent exposure of the wild-type MOP-r. Finn & 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 & 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 (2004) now show that in an HEK 293 cell line transfected with the MOR1D splice-variant form of MOP-r, exposure to morphine for 8 hr produces a degree of cAMP superactivation that is significantly greater than that induced by morphine acting on wild-type receptors, and closely comparable to that induced by DAMGO. Thus, the morphine-internalized chimeric MOP/DOP-r of Finn & Whistler (2001) is targeted to a degradation pathway resulting in a sustained loss of agonist signaling and less cAMP superactivation, while the morphine-internalized MOR1D-MOP-r of Koch et al (2001; 2004) is rapidly re-cycled to the plasma membrane to restore agonist signaling, inducing a greater cAMP superactivation. Collectively 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 hr 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 (2004), 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; 2004; Alvarez et al, 2002). Thus the detailed events documented in the model system used by Koch et al (2004) are probably relevant to the actions of opiate drugs acting on MOP-r in neurons in vivo. The recycling pathway for MOP-r appears similar to that utilized by several other GPCR, including β2-adrenergic receptors (Tsao & 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, β-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 etonitazine (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 etonitazine, 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. Etonitazine, 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 hrs (Reynolds et al, 2004). Any possible clinical relevance of the interesting results reported by Koch et al (2004) will require further study in more complex neuronal systems where the additional modulating influences of trans-synaptic adaptations can also be evaluated.
REFERENCES:


Table 1. Cellular Adaptations Following MOP-r Activation by Internalizing Agonists

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