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PERSPECTIVE

Receptor Domains Regulating μ Opioid Receptor Uncoupling and Internalization: Relevance to Opioid Tolerance

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Opiate drugs produce analgesia by activation of μ opioid receptors (MOP-r), a class of G protein-coupled receptor (GPCR). Receptor-mediated activation of G proteins results in opening of K_{ir} channels with consequent hyperpolarization of neuronal membranes, and/or inhibition of Ca^{2+} currents. Both actions reduce efficiency of transmission at regulated synapses. Sustained or repeated activation of MOP-r leads to a rapid desensitization with reduction of agonist response followed by internalization of receptors, making them unavailable for activation by agonists. Functionally, extended exposure to opiate results in reduced analgesic effects (see review by Williams et al., 2001). Despite many years of study, the detailed mechanisms underlying opiate drug tolerance remain unclear. Several recent studies have brought some clarification but also revealed hitherto unexpected complexity in the many adaptive processes that seem to play a role in opiate tolerance. Careful electrophysiological studies measuring ion channel conductances have revealed that opiate agonist exposure for only a few minutes can induce a rapid loss of opioid response (Alvarez et al., 2002, Borgland et al., 2003). This is associated with internalization of the agonist-bound receptor and sorting of the receptor either to a recycling pathway returning active receptor to the plasma membrane or to a degradative pathway trafficking receptors to lysosomes for proteolytic degradation (Tanowitz and von Zastrow, 2003; von Zastrow et al., 2003). These events contribute to a selective loss of response to agonists acting through the MOP-r. Because other receptors regulating the same ion channels remain functional, the desensitization is described as homologous. In many opioid-sensitive systems, MOP-r activation is also followed by post-receptor adaptations in effector system functions that may further reduce agonist effectiveness and may also modify the responses to agonists acting through other receptor types (generating a heterologous tolerance). These post-receptor adaptations may persist after withdrawal of the opiate drug, leading to exposure of a drug-dependent state (Williams et al., 2001). Each of these multiple actions is probably important in the adaptive responses induced by long-term opiate drug exposure in vivo.

The apparent simplicity of homologous desensitization of opioid receptors has raised hopes that this component of the adaptive responses to opiate drug exposure might be readily understood. Studies on β-adrenergic receptors by Lefkowitz and colleagues (Bouvier et al., 1998), among others, later extended to many types of GPCR (see review by von Zastrow et al., 2003), have shown the critical role of receptor phosphorylation in desensitization. Agonist-induced activation of GPCR specifically activates G protein coupled-receptor kinases (GRKs), probably through liberated $G\beta\gamma$ subunits. GRKs phosphorylate Ser/Thr residues on the C terminus of the MOP-r, facilitating the recruitment of β-arrestins. Clusters of receptor-β-arrestin complexes segregate on the cell surface before being internalized by a clathrin- and dynamin-dependent process (Zastrow et al., 2003). Elimination of some of these phosphorylation sites by C-terminal truncation of the receptor or by mutation of specific C-terminal amino acids prevents MOP-r internalization (Chavkin et al., 2001). In the simplest GRK-β-arrestin model of desensitization, β-arrestin recruitment by receptor phosphorylation terminates effector signaling and triggers the internalization process, removing receptors from the plasma membrane. However, the specific events that terminate agonist signaling to effectors have remained ill defined. In this issue of *Molecular Pharmacology*, Celver et al. (2004) offer evidence suggesting that the domains of the receptor implicated in the termination of agonist signaling during desensitization are different from those directing receptor internalization.

Internalization and Recycling. Several studies have shown that important targets for GRK phosphorylation reside in the C-terminal tail of MOP-r (Chavkin et al., 2001; Qiu et al., 2003). C-terminal truncation or mutation of selected phosphorylation sites in the C terminus of MOP-r

ABBREVIATIONS: MOP-r, μ opioid receptor; GPCR, G protein-coupled receptor; GRK, G protein coupled-receptor kinase; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; HEK, human embryonic kidney.

prevents agonist-induced internalization. It is presumed that phosphorylation of critical C-terminal residues leads to interaction with β-arrestin, triggering receptor clustering in clathrin- coated pits in the plasma membrane and translocation of the agonist-receptor complexes to the interior of the cell. Tanowitz and von Zastrow (2003) report that a sequence in the C terminus of MOP-r provides a specific signal for recycling of the MOP-r to the plasma membrane after internalization. Not all MOP-r agonists are equally effective at inducing internalization (Keith et al., 1996). Several groups have now shown that full agonists induce rapid internalization, whereas agonists with lower efficacy generally do not induce internalization (Keith et al., 1996; Celver et al., 2001; Alvarez et al., 2002). However, in a recent study of the cellular location of MOP-r after agonist exposure of AtT20 cells (a pituitary cell line) stably transfected to express an epitope-tagged MOP-r (FLAG-MOP), Borgland et al. (2003) found significant internalization of MOP-r after morphine exposure without coaddition of any full agonist, although morphine-induced internalization was slower and less complete than that induced by the stable enkephalin analog [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) or methadone (which exhibited relatively high efficacy in these studies).

The rapid internalization induced by full agonists is proposed to be important in ensuring that internalized receptors are rapidly returned to the cell surface in an active form, where they immediately become available for reactivation by agonist, thus leading to a reinitiation of agonist action. In this model, low-efficacy agonists such as morphine that do not internalize receptors will cause an accumulation of desensitized, noninternalized receptors in the plasma membrane; an agonist response cannot be sustained and tolerance ensues (Whistler et al., 1999). This model has recently been extended to include a role for receptor dimerization (Rios et al., 2001). In recent studies in MOP-r–transfected HEK293 cells, He at al (2002) reported that low concentrations of a full agonist such as DAMGO can facilitate the internalization of MOP-r co-occupied by a low-efficacy agonist such as morphine. It is suggested that when DAMGO and morphine both activate homo-oligomers of MOP-r; the complex is efficiently internalized. These results, if confirmed, have obvious implications for analgesic therapy; full agonists given alone or in combination with a partial agonist should induce less tolerance than partial agonists given alone. In fact, however, a recent study failed to confirm a facilitating effect of DAMGO on morphine-induced internalization (Bailey et al., 2003); these authors also found that coapplication of low concentrations of DAMGO neither enhanced nor reduced the relatively low rate of morphine-induced desensitization at MOP-r in rat locus ceruleus neurons in brainstem slice preparations.

An important question concerning MOP-r internalization is what happens in vivo. Trafton et al. (2000) have shown that intraspinal administration of DAMGO or high doses of [Met^5]enkephalin to rats increased the amount of endogenous immunoreactive MOP-r in the cytoplasm of dorsal horn neurons of rat spinal cord. The demonstration by Bohn et al. (1999, 2000, 2002) that the analgesic potency of morphine is increased in mice with genetic deletion of β-arrestin-2 and that analgesic tolerance development is significantly slowed in these mice suggests that β-arrestin-dependent processes contribute to the analgesic effect of morphine in vivo and also to the rate at which tolerance develops to these actions, despite the fact that morphine does not readily internalize MOP-r. In fact, deletion of β-arrestin-2 (thereby presumably reducing MOP-r internalization), enhanced the analgesic potency of morphine in Bohn's studies. If internalization and recycling were essential for maintenance of a pool of active plasma membrane receptors after morphine treatment, β-arrestin depletion would be expected to accelerate morphine-induced receptor desensitization and loss of analgesic response by producing progressive depletion of the pool of available receptors. However, the role of β-arrestin in MOP-r signaling may not be limited to initiating internalization of receptors (see below).

MOP-r are located in the spinal cord and in brain in the vicinity of neurons and other cells that can release endogenous opioids (e.g., enkephalins, β-endorphin) with full agonist activity at MOP-r. It is probable that some level of receptor occupancy by endogenous peptide full agonists released in response to the noxious stimulus will occur after exposure to all but trivial painful stimuli. However, Trafton et al. (2000) failed to observe measurable MOP-r internalization in spinal cord neurons after application of various short-term noxious stimuli or after induction of peripheral inflammation, stimuli known to cause release of enkephalins or other endogenous opioids. An intriguing study by Haberstock-Debic et al. (2003) on exogenous agonist-induced MOP-r internalization in GABA neurons of nucleus accumbens of rats subjected to virally mediated transfection of epitope-tagged MOP-r found that morphine treatment did not induce receptor internalization in neuronal soma but seemed to produce substantial internalization in dendritic processes of the same neurons. At first sight, this suggests that morphine can internalize receptors in an optimal membrane environment (dendrites) but not in a different cellular location (soma, the cellular region studies in many tissue culture models). A role for endogenous opioids in nucleus accumbens facilitating internalization at dendritic but not somatic sites cannot be ruled out in these studies. However, other explanations relating to altered receptor trafficking from soma to dendrites after drug treatment might also explain these results. The apparent differences in internalization efficiency in different transfected cell lines, and between cell soma and dendritic process, suggest that the environment of the receptor is a critical determinant of the extent of internalization. It seems likely that local factors, including the local receptor concentration (the presence of spare receptors), in addition to agonist efficacy, are critical determinants of the extent of internalization that will be induced by partial agonists.

Possible differences in kinetics for the various components of the receptor recycling process also require consideration. Agonist-induced MOP-r internalization seems to occur rapidly relative to the rate of return of recycled receptors to the plasma membrane. Studies with epitope-tagged receptors show that internalization results in a net loss of receptors from the plasma membrane within a time frame of minutes; significant transfer of receptors to the cytoplasm occurs within 5 min of DAMGO exposure, and most receptors may be in the cytoplasm after 30 min of exposure (He et al., 2002; Borgland et al., 2003; Celver et al., 2004). Significant return of epitope-tagged MOP-r to the plasma may take 60 min or more in transfected HEK293 or mouse neuro2A cells (Qiu et al., 2003; Tanowitz and von Zastrow, 2003). These results

seem to suggest that exposure to a concentration of full agonist giving a high level of receptor occupancy would leave the plasma membrane depleted of functional receptors from the point where the majority of receptors are initially internalized until they could be re-cycled as active receptors as much as an hour later. However, if enough spare receptors are present in an in vivo system, fully effective analgesic doses may not need to occupy the majority of the large excess of spare MOP-r receptors that seem to exist in pain processing pathways (Zernig et al., 1995). The level of MOP-r reserve will vary between different functional systems in brain (Zernig et al., 1997). In some systems, it is likely that agonist effects will be transiently limited by agonist-induced depletion of functional receptors from the plasma membrane.

Desensitization. Different authors have used this term to mean different things. Here, it is used to refer specifically to the loss of agonist signaling to effector systems—a functional uncoupling of receptor and effector systems. In many studies, loss of signaling cannot be separated from internalization of receptors. In a study reported in this issue of *Molecular Pharmacology*, Celver et al. (2004) show that these phenomena can occur independently. We now know from measures of K^+ or Ca^{2+} conductances that receptor uncoupling occurs almost coincidently with the initiation of opiate agonist action by a relatively high concentration of agonist, with a significant loss of agonist action evident within minutes of exposure to full agonists (Celver et al., 2001; 2004; Alvarez et al., 2002; Borgland et al., 2003). In MOP-r transfected HEK293 cells, administration of the MOP-r agonists DAMGO, met-enkephalin, etorphine, or methadone all induced desensitization whereas morphine and morphine-6-glucuronide were ineffective (Alvarez et al., 2002). Others have noted that some desensitization can be achieved with morphine (Borgland et al., 2003), but all agree that morphine is much less effective in inducing desensitization than the full agonists listed above. Thus, agonist efficacy seems to be an important determinant of MOP-r desensitization. Most studies have found that little desensitization is observed with concentrations of full agonists giving low levels of fractional occupancy of the receptor; both desensitization and internalization require concentrations giving high levels of receptor occupancy and activation (Borgland et al., 2003; Celver et al., 2004). The inefficiency of morphine and partial agonists in inducing desensitization probably results from their inability to activate and induce GRK phosphorylation of a sufficiently high fraction of receptors to trigger desensitization and internalization mechanisms (GRK activation and β-arrestin recruitment).

The mechanism by which termination of agonist signaling occurs in receptor desensitization is unclear. Borgland et al. (2003) report that the relative potencies of a series of agonists for receptor activation and desensitization differ from their relative potencies as inducers of internalization. Recent studies suggest MOP-r signaling can also be reduced in the absence of detectable internalization of receptors. In *Xenopus laevis* oocytes injected with cRNAs for receptors, K^+ channels, GRK, and/or β-arrestin, the loss of agonist signaling induced by a full MOP-r agonist (the enkephalin analog DAMGO) was shown to be dependent on both GRK and β-arrestin, suggesting that both are required for termination of agonist signaling (Celver et al., 2001). However, in oocytes, receptor internalization is not observed, clearly indicating that loss of agonist response can occur without removal of receptor from the plasma membrane. Furthermore, Celver et al. (2001) found that a single mutation of Thr-180 to Ala in the second transmembrane loop of the MOP-r completely prevented DAMGO-induced desensitization in oocytes. When the same mutated MOP-r (T180A)-r was transfected into AtT20 cells, which exhibit more neuron-like properties than the oocyte expression system and also permits agonist-induced internalization of GPCR, Celver et al. (2004) confirmed that the rapid DAMGO-induced desensitization of effector signaling is completely lost after the T180A substitution. This mutation, however, had no effect on the rate or extent of DAMGO-induced internalization; MOP(T180A)-r was internalized by DAMGO as readily as the wild-type MOP-r. This surprising result indicates that the MOP-r domain through which agonist signaling to effectors is terminated in homologous desensitization (uncoupling) is probably found within the second intracellular loop. This domain is clearly different from the C-terminal domain of MOP-r critical for agonist-induced MOP-r internalization.

The functional role of the second intracellular loop of MOP-r, and of T180 in particular, in receptor-G protein signaling is not clear. Studies of other GPCRs indicate that the second intracellular loop region is critically involved in activating G proteins (Burstein et al., 1998; Chung et al., 2002). The role of T180 in MOP-r is less clear. Mutation of T180 did not affect agonist signaling—it prevented agonist-induced desensitization (Celver et al., 2001, 2004). In the wild-type receptor, this desensitization was GRK- and β-arrestin–dependent (Celver et al., 2001). Possible roles for T180 in desensitization mechanisms remain a subject for speculation. It is possible that T180 is a substrate for GRK. Phosphorylation of MOP-T180 may facilitate β-arrestin recruitment, and the association of β-arrestin with MOP-r may be the signal preventing further activation of G proteins by MOP-r, but this remains to be demonstrated. The consequence of β-arrestin recruitment to the receptor may also not be limited to termination of agonist signaling through G proteins and initiation of MOP-r internalization and recycling. Other signaling systems might also be activated, including *src* and MAP kinases, with modulation of function of other signaling pathways (Luttrell and Lefkowitz, 2002; Belcheva et al., 2003).

Relevance of Desensitization and Internalization to Opiate Action in Vivo. The importance of these results, if confirmed in other MOP-r–regulated systems, is that agonist-induced uncoupling of receptor-effector systems and receptor internalization can now be seen to be independent events regulated by different domains of MOP-r (Fig. 1), although both events are GRK- and β-arrestin–dependent and both are more readily induced by full agonists than partial agonists. However, the role of receptor uncoupling per se as a contributor to loss of MOP-r agonist effect in vivo remains unclear. Desensitization occurs rapidly and also reverses within minutes; high levels of receptor occupancy also seem to be required (Alvarez et al., 2002). It is not clear whether in vivo analgesic doses of full agonists achieve these levels of occupancy. Desensitization also seems to be limited in extent; most studies show that only about 50% of agonist effect is lost after exposure to quite high doses of agonist. It remains to be seen whether this is a major determinant of agonist signaling in in vivo systems, where a high level of receptor reserve exists. The roles of MOP-r internalization in

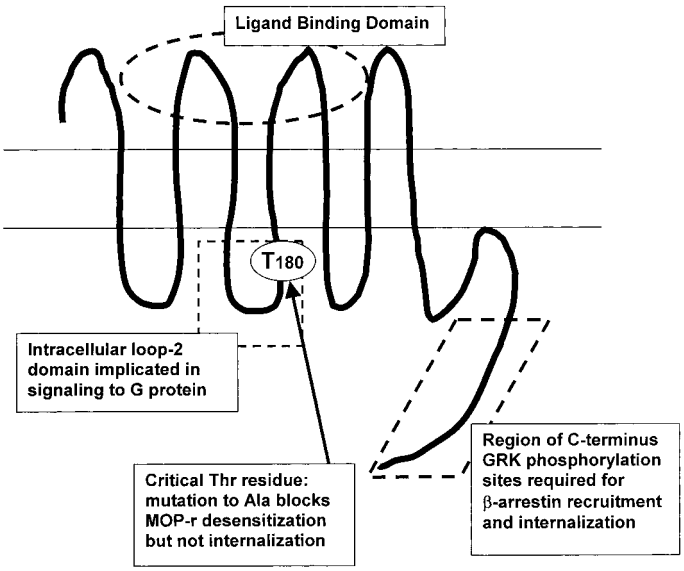

Fig. 1. Functional domains in MOP-r implicated in desensitization and internalization, following Celver et al. (2004).

reducing opiate response in vivo, and of receptor recycling in replenishing plasma membrane pools of receptor for maintenance of agonist action, also require further study. Post-receptor adaptations, which are beyond the scope of this *Perspective* but may be induced by the signaling mechanisms considered here, are almost certainly additional critical determinants of the actions of opiate drugs in long-term treatment regimens. Tolerance to opiates is a complex phenomenon with many facets.

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