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MOLECULAR MECHANISMS AND REGULATION OF OPIOID RECEPTOR SIGNALING

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■ **Abstract** Cloning of multiple opioid receptors has presented opportunities to investigate the mechanisms of multiple opioid receptor signaling and the regulation of these signals. The subsequent identification of receptor gene structures has also provided opportunities to study the regulation of receptor gene expression and to manipulate the concentration of the gene products in vivo. Thus, in the current review, we examine recent advances in the delineation basis for the multiple opioid receptor signaling, and their regulation at multiple levels. We discuss the use of receptor knockout animals to investigate the function and the pharmacology of these multiple opioid receptors. The reasons and basis for the multiple opioid receptor are addressed.

INTRODUCTION

The cloning of the δ-, μ-, and κ-opioid receptors (1–7) have led to further understanding of the molecular mechanism of opioid receptor function. From the sequence analysis of these cloned opioid receptors, it is unequivocal that the opioid receptors belong to the superfamily of G protein–coupled receptor (GPCR) and the subfamily of rhodopsin receptor. These opioid receptors all have the putative structure of seven transmembrane domains, extracellular N terminus with multiple glycosylation sites, third intracellular loop with multiple amphiphatic α-helixes, and fourth intracellular loop formed by the putative palmitoylation sites at the carboxyl tails (1–7). On the whole, these receptors are about 60% identical to each other, with the greatest identity found in the transmembrane domains (73–76%) and intracellular loops (86–100%). The greatest divergent areas were found in the N terminus (9–10%), extracellular loops (14–72%), and C terminus (14–20%) (8). These opioid receptors could regulate the same spectrum of effectors,

which include adenylyl cyclase (1–7), the N-type (9) and L-type (10, 11) Ca^{2+} channels, phospholipase C (12, 13), inward rectifying K^+ channels (14), and mitogen-activated protein kinases ERK1 and ERK2 (15, 16).

With these multiple effectors being regulated by the opioid receptors, the question of the molecular basis for the pharmacology of opioid agonists needs to be addressed. In order to address such an issue, the structural requirement for the receptor activation of these effectors, the G proteins, and other cellular proteins that are involved must be evaluated. The identity of the opioid receptor that mediates the specific pharmacological function of the agonist, such as analgesia, must be identified. How these receptors are being regulated cellularly must be addressed. Thus, in this review, we examine the differential regulation of the effector systems by various opioid agonists, and the subsequent cellular regulation of the receptor activities. We also review the current status of using receptor knockout mice to address the pharmacological activities of the opioid peptides and opiate alkaloids.

REGULATION OF EFFECTORS BY OPIOID RECEPTORS

Opioid receptors are prototypical ''G_i/G_o-coupled'' receptors because opioid signals are efficiently blocked by pertussis toxin (PTX), a bacterial toxin produced by *Bordetella pertussis* that ADP-ribosylates and inactivates the α subunits of G_i/G_o proteins ($G\alpha_{i/o}$ subunits). Like many receptors that utilize G_i subfamily members for signal transduction, the opioid receptors have long been known to inhibit adenylyl cyclases (17) and Ca^{2+} channels (18, 19), as well as to stimulate K^+ channels (20) and to increase intracellular Ca^{2+} levels (21). More recently, the opioid receptors have been shown to regulate the mitogen-activated protein (MAP) kinase cascade (15, 16, 22). One of the major advances in understanding opioid-mediated signal transduction is the unraveling of the regulatory mechanisms for these effectors.

New Insights on the Regulation of Adenylyl Cyclase Activity by Opioids

Early studies on opioid-induced inhibition of adenylyl cyclase in brain membranes and neuroblastoma cells were interpreted on the basis of our limited understanding of the complexity of the G protein–-adenylyl cyclase pathway. As PTX abolished opioid inhibition of adenylyl cyclase in the neuroblastoma x glioma NG108–15 cells (23), and rat-brain opioid receptors were solubilized as a tight complex with PTX-sensitive G proteins (24), it was generally believed that opioid receptors inhibit adenylyl cyclase only via the G_i proteins. Studies with $G\alpha$-specific antibodies suggested that G_{i2} mediates the δ-opioid receptor inhibition of adenylyl cyclase activity in NG108–15 cells (25) whereas G_o mediates the μ-

opioid receptor inhibition of the adenylyl cyclase activity in SHSY5Y cells and brain membrane (26). The promiscuity of the opioid receptor was demonstrated by the ability of the receptor to induce GTP binding to all the G_i/G_o α-subunits. By using either ^{32}P-azidoanilido GTP to photoaffinity label the Gα subunits or cholera toxin to ADP-ribosylate the $G\alpha_{i/o}$ α-subunits after their dissociation from the βγ subunits, authors of several reports have indicated that the μ-, δ- and κ-opioid receptors could activate the G_i/G_o proteins with equal potency (27–31, 191, 264). All three $G\alpha_i$ subtypes ($G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$) were shown to inhibit the adenylyl cyclase activity (32, 33). The discovery that G_z, a PTX-insensitive member of the G_i subfamily, can also potently inhibit cAMP accumulation upon receptor activation (33) provided new perspectives on opioid signaling. The functional and structural (~66% homology) similarities between $G\alpha_z$ and $G\alpha_i$ subunits strongly suggest that $G\alpha_z$ may substitute for $G\alpha_{i/o}$ to mediate opioid-induced signals. Detailed examination of opioid-induced inhibition of adenylyl cyclase in NG108–15 cells, which are known to coexpress the δ-opioid receptor and G_z, revealed a small but significant inhibitory component that cannot be abolished by PTX (34). When G_z was coexpressed with any one of the three cloned opioid receptors in transfected mammalian cells, opioid-mediated inhibition of adenylyl cyclase became PTX-resistant (35, 36, 37). Physical association between G_z and the δ-opioid receptor was demonstrated by coimmunoprecipitation of the recombinant proteins (38). These studies lend staunch support to the notion that opioid receptors can utilize PTX-insensitive G proteins for signal transduction. There is evidence to indicate the involvement of G_z in μ-opioid receptor-induced supraspinal antinociception in mice by immunological (39), antisense (40, 41), and biochemical (42) approaches. Because G_z is primarily expressed in neuronal tissues, it may play other roles in coupling brain opioid receptors to their corresponding effectors. The recent discovery of G_z's ability to link GPCRs to inhibition of N-type Ca^{2+} channels and stimulation of G protein gated inward rectifying potassium channels (GIRK) channels in superior cervical ganglion neurons (43) suggests that G_z may indeed serve additional roles in the propagation of opioid signals.

One of the intriguing observations in opioid signaling is that opioids and opiates can stimulate adenylyl cyclase in brain membranes (44), F-11 neuroblastoma-sensory neuron hybrid cells (45), olfactory bulb (46), and spinal cord–ganglion explants (47). At least nine isoforms of mammalian adenylyl cyclases have been cloned, and they exhibit diverse sensitivities to regulators such as G proteins, Ca^{2+}, and kinases (48). Of particular relevance here is the ability of the G-protein βγ complex (Gβγ) to stimulate type 2, 4, and 7 adenylyl cyclases. Many classical inhibitory receptors (e.g. α_2-adrenergic and dopamine-D_2 receptors) stimulate the type 2 adenylyl cyclase through the Gβγ released from activated PTX-sensitive G_i proteins (49, 50). In order for the Gβγ subunits to stimulate type 2 adenylyl cyclase, GTP-bound $G\alpha_s$ must also be present (49, 51). This requirement allows the cell to integrate extracellular signals generated through G_s and other G-protein pathways into the intracellular messenger cAMP. Provision of activated $G\alpha_s$ can

indeed permit all three forms of opioid receptors to stimulate cAMP accumulation in transfected cells coexpressing the type 2 adenylyl cyclase (35–37). Under such conditions, the opioid-induced stimulation of cAMP formation is PTX-sensitive. However, if G_z is also present in the heterologous expression system, the opioid-induced stimulatory response becomes PTX-resistant (35–37). Because both type 2 and 4 adenylyl cyclases are expressed in the brain, it is conceivable that some of the central actions of opioids are mediated by these adenylyl cyclases, which act as molecular switches for the detection of coincident signals. The δ-opioid agonist-induced potentiation of the behavioral responses elicited by dopamine D_1 receptor agonists in mice may involve these kinds of coincident signals (52). An interesting twist in the tale of Gβγ-mediated stimulation of type 2 adenylyl cyclase is the replacement of the activated $G\alpha_s$ preconditioning by protein kinase C (PKC)-mediated phosphorylation (53). Activation of PKC by G_q-coupled receptors or phorbol esters leads to the phosphorylation of type 2 adenylyl cyclase, and this modification in turn allows the Gβγ interacting domain of the enzyme to become responsive. Hence, costimulation of a G_q-coupled receptor and an opioid receptor may activate type 2 adenylyl cyclase in a synergistic fashion. Endogenous cholecystokinin has been reported to enhance the analgesic potentials of opioids in the CNS (54). It remains to be determined if these synergistic actions are, in fact, processed through the type 2 adenylyl cyclase.

An alternative explanation for opioid-induced elevation of cAMP levels is a direct coupling between opioid receptors and G_s. The α_2-adrenergic receptor is typically considered a G_i-coupled receptor, yet it is capable of eliciting a weak stimulation of adenylyl cyclase via G_s when the G_i proteins are ADP-ribosylated by PTX (49). Using the Gβγ-mediated stimulation of type 2 adenylyl cyclase as an index of G-protein activation, functional interactions between the μ-opioid receptor and various G proteins have been examined (35). The μ-opioid receptor can couple to six members of the G_i subfamily ($G_{i1–3}$, $G_{o1–2}$, and G_z), but there is no evidence of its association with G_s. A conversion step may be required, as in the case for other GPCRs. The G_s-coupled vasoactive intestinal peptide and β_2-adrenergic receptors can acquire the ability to interact with G_i proteins when they are phosphorylated by cAMP-dependent protein kinases (PKA) (55). For opioid receptors, the conversion may be regulated by the GM1 ganglioside (56). In dorsal root ganglion (DRG) neurons, the action potential duration is modulated by morphine in a bimodal fashion (57) where the cAMP-dependent excitatory effects are mediated by G_s-coupled opioid receptors. Treatment with GM1 ganglioside, but not with other gangliosides, rapidly converts the opioid receptors from an inhibitory to an excitatory mode in the DRG neurons. Similar treatments with GM1 ganglioside allow the δ-opioid receptor to stimulate cAMP formation in NG108–15 (58) and CHO (59) cells. It is noteworthy that gangliosides are abundantly distributed on the surface of most neurons, and by altering the coupling specificity of opioid receptors, GM1 may modulate opioid analgesia, tolerance, and dependence (56).

The complexity and versatility of the mammalian adenylyl cyclase system allow other routes for opioids to stimulate rather than inhibit cAMP production. For instance, type 1 and 8 adenylyl cyclases are activated by Ca^{2+}/calmodulin, whereas the basal activities of type 2, 4, and 7 adenylyl cyclases are elevated upon phosphorylation of the enzyme by PKC. Given that opioid receptors are capable of stimulating phospholipase C (PLC) and mobilizing intracellular Ca^{2+} (as discussed below), it is not surprising to note that the opioid-induced elevation of basal cAMP level in SK-N-SH cells involves Ca^{2+} entry and calmodulin activation (60). With the existence of nine different adenylyl cyclases, the regulation of intracellular cAMP by opioid receptors is far more complicated than first envisaged two decades ago. Under different cellular environments, the μ-opioid receptor can inhibit the activity of type 5 adenylyl cyclase but stimulate that of the type 7 enzyme (61). Adenylyl cyclase superactivation induced by chronic exposure of cells to opioids also appears to be isozyme specific (62). Future attempts to map opioid-induced cAMP signals will undoubtedly require the identification of cell-specific molecular components in order to fully understand the functions of opioid receptors.

Opioid Receptors and Ion Channels

Opioid receptors are known to suppress the release of neurotransmitters in many pharmacological preparations by preventing Ca^{2+} influx. All three opioid receptors share the ability to inhibit different types of Ca^{2+} channels in many regions of the mammalian brain. For example, μ- and κ-opioid receptors inhibit N- and P/Q-type Ca^{2+} channels in the nucleus tractus solitarius of the rat (63), whereas Christie and co-workers (64) showed that μ-opioid receptors, but not δ- or κ-opioid receptors, are responsible for the modulation of Ca^{2+} channel currents in mouse periaqueductal grey neurons. The cloning of multiple Ca^{2+} channel subunits provided the molecular basis for a variety of voltage-gated Ca^{2+} channels (e.g. L-, N-, P/Q-, R-, and T-type), and soon it became apparent that functional regulation of Ca^{2+} channels is no less complicated than the adenylyl cyclase system (65). The number of possible subunit combinations of Ca^{2+} channels is staggering, and the subunit composition of each channel may dictate its regulatory profiles. The availability of cloned opioid receptors together with modern electrophysiological techniques provided unique opportunities to study the modulation of different Ca^{2+} channels by opioid agonists. The first step toward this endeavor involves coexpression of cloned opioid receptors and Ca^{2+} channel subunits in cellular environments amenable to electrophysiological recordings. When expressed in NG108–15 cells, the cloned rat μ-opioid receptor is functionally coupled to the ω-conotoxin-sensitive N-type Ca^{2+} channels (66). On the other hand, the cloned μ- and δ-opioid receptors inhibit voltage-activated L-type Ca^{2+} channels via G_i/G_o proteins in GH3 pituitary cells (10, 11). Functional coupling of the κ-opioid receptor to Ca^{2+} channels has also been reported. Coexpression of neuronal Ca^{2+} channel subunits with the κ-opioid receptor in *Xenopus*

oocytes allows the κ-agonist, U50488H, to inhibit the depolarization-evoked Ba^{2+} current (67). At least those Ca^{2+} channels containing the α1A, α1B, or α1E subunits have been shown to be inhibited by the μ-opioid receptor (68). It is not known if Ca^{2+} channels composed of other α1 subunits can be similarly regulated by opioid receptors.

The involvement of G_o proteins in the mediation of opioid-induced inhibition of Ca^{2+} channels has been demonstrated more than a decade ago (18) and was later confirmed by the use of $G\alpha_o$-specific antiserum (69). It is now realized that the Ca^{2+} channel is inhibited by the Gβγ rather than the $G\alpha_o$ subunit. Expression of Gβγ in rat sympathetic neurons mimicked GPCR-induced inhibition of Ca^{2+} currents (70), and similar results were observed when Gβγ was coexpressed with Ca^{2+} channel subunits in a heterologous expression system (71). Although the Gβγ subunits are responsible for mediating the inhibition of Ca^{2+} channels, the $G\alpha_o$ subunit is indispensable for coupling the opioid receptors to this Gβγ-dependent effect. The most convincing evidence comes from $G\alpha_o$ knockout studies. In DRG neurons obtained from $G\alpha_o$ knockout mice, the ability of opioid agonists to inhibit Ca^{2+} channels is significantly impaired (72). It is interesting that the G_o-deficient mice are also hyperalgesic.

The regulation of Ca^{2+} channel activity is subject to multiple inputs. In neurons, activation of PKC results in the phosphorylation and stimulation of N-type Ca^{2+} channel activity and antagonizes G protein–mediated inhibition (73). Indeed, in rat DRG neurons, activation of κ- and μ-opioid receptors decreases N-type Ca^{2+} current whereas activation of PKC produces an opposite effect (74). However, inhibition of neuronal Ca^{2+} channel subunits by the κ-opioid receptor is unaffected by the actions of PKA and PKC (67). It seems reasonable to speculate that additional crosstalk between G proteins and other signaling molecules may exist. If so, they may explain why κ-agonists stimulate rather than inhibit L-type Ca^{2+} channels in the human placenta (75).

At the postsynaptic membrane, many GPCRs produce hyperpolarization by activating K^+ channels, thereby preventing excitation or propagation of the action potentials. Electrophysiological studies in the rat locus coeruleus have shown that both μ- and δ-opioid receptors can activate K_G channels via PTX-sensitive G proteins (20). However, κ-agonists are without effect in the same preparation. Intracellular recordings made from substantia gelatinosa neurons indicate that all three types of opioid receptors are capable of activating K_G currents (76, 77). Indeed, coexpression studies in *Xenopus* oocytes confirmed that the κ-opioid receptor can activate an inward rectifying K^+ channel via PTX-sensitive G proteins (14, 78). In terms of physiological relevance of the regulation of K_G channels by opioid agonists, activation of δ_1-opioid receptors is involved in the cardioprotective effect of ischemic preconditioning. This is supported by the observation that a δ_1-selective agonist, TAN-67, can significantly reduce infarct size in rats with coronary artery occlusion by activating K_G channels via G_i proteins (79). It should be noted that K_G channels can also be inhibited by opioid receptors, as seen with the κ-subtype in a catecholaminergic neuronal cell line (80).

Recent progress in the cloning of K_G channels has enabled researchers to study the structure and function relationship of these channels at the molecular level, and to elucidate the mechanism of activation by Gβγ subunits (81). At least 12 distinct channel subunits are responsible for the complexity and diversity of inward rectifying K^+ channels. One of the major subunits of K_G is GIRK1. There is evidence to indicate that different types of Gβ interacted with GIRK1 with distinct efficacies (82). The differential abilities of different opioid receptors to activate K_G channels may therefore be due to their association with distinct G-protein heterotrimers containing different Gβ subunits. However, the specificity of interaction with K_G is lost when Gβ is bound to Gγ, because K_G channel currents in *Xenopus* oocytes expressing GIRK1 can be activated by different combinations of Gβγ (83). A recent study proposed that phosphatidyl-D-myo-inositol-4,5-bisphosphate (PIP_2) is critically involved in Gβγ-induced activation of K_G channels (84) and opens the possibility for opioid receptors to regulate K^+ channel activities indirectly via the metabolism of PIP_2.

Apart from Ca^{2+} and K^+ channels, opioid receptors may regulate the functions of other ion channels. For example, excitatory postsynaptic currents evoked by N-methyl-D-aspartate (NMDA) receptors in the hippocampal dentate gyrus are inhibited by μ-opioid agonists (85). Since intracellular application of PTX as well as activators and inhibitors of PKA can prevent and reverse the μ-opioid–induced reduction in NMDA currents, G_i/G_o proteins and PKA may be involved. It is interesting that the opioid receptors can be reciprocally modulated by NMDA. Acute incubation of NG108–15 cells with NMDA significantly attenuated the ability of the δ-opioid receptor agonist DPDPE to inhibit forskolin-stimulated cAMP production (86). The ability of DPDPE to stimulate [^{35}S]GTPγS binding in NG108–15 cells is also significantly suppressed by NMDA in a dose-dependent manner, and the mechanism of regulation may in part involve PKC-mediated phosphorylation of $G\alpha_{i2}$ (87). The inhibitory effect of NMDA is also observed with μ- and κ-opioid receptors in primary cultured neurons. The ability of NMDA to attenuate acute opioid-induced inhibition of adenylyl cyclase (86) suggests that other effectors of opioid receptors may be similarly affected by NMDA.

Stimulation of Phospholipase C and Ca^{2+} Mobilization

The ability of opioid receptors to regulate the phospholipase Cβ (PLCβ) pathway and Ca^{2+} mobilization was not examined seriously until it was realized that many G_i-coupled receptors can regulate these effectors without activating G_q proteins. Sure enough, activation of δ-opioid receptors in NG108–15 cells stimulates myo-inositol 1,4,5-triphosphate (IP_3) formation and subsequent Ca^{2+} mobilization (21, 88). Opioid stimulation of PLCβ and the generation of IP_3 have also been reported in the human neuroblastoma SH-SY5Y cells (89). Similar observations were noted with the cloned opioid receptors. The cloned δ-opioid receptor stimulates IP_3 production in transfected Ltk^- cells (37) whereas the rat μ-opioid receptor activates PLCβ in transfected CHO cells (90). Both systems require the partici-

pation of PTX-sensitive G proteins. Since none of the PTX-sensitive Gα subunits can activate PLCβ by themselves (35), the opioid-induced stimulation of PLCβ appears to be mediated via the Gβγ subunits. It is well established that the activities of PLCβ1–3 are potentiated upon binding Gβγ subunits. The relatively high EC_{50} of opioids required to stimulate IP_3 formation is in fact consistent with such a concept. However, other mechanisms are available for opioids to regulate the PLCβ pathway. The mechanism of activation of PLC by μ-opioids in SH-SY5Y cells does not appear to involve Gβγ subunits; instead, Ca^{2+} influx via the L-type Ca^{2+} channel may be involved (91). Intriguingly, a subtype of κ-opioid receptor is found to inhibit PLCβ activity in the guinea pig cerebellum via G_{i1} (92). The significance of this observation is unclear, but it should be noted that the κ-opioid receptor can also produce atypical responses in the regulation of Ca^{2+} (75) as well as K^+ (80) channels.

The ability of different opioid receptors to stimulate PLCβ is determined in part by the availability of complementary G proteins in any particular cell type. Using antisense oligodeoxynucleotides against specific Gα subunits, it was shown (100) that the opioid-induced Ca^{2+} mobilization in ND8–47 neuroblastoma x DRG hybrid cells is specifically mediated by G_{i2}. In contrast, activation of PLCβ by μ- and κ-opioid appears to utilize $G\alpha_{i1}$ because coinjection of $G\alpha_{i1}$ RNA into *Xenopus* oocytes is required for the detection of opioid-induced Ca^{2+}-dependent chloride currents (94), whereas all three types of opioid receptors activate PLC-β3 via Gβγ released from G_{i2} or G_o in intestinal smooth muscle (95). In a human neuroblastoma cell line, SK-N-BE, δ-opioid receptors mobilize Ca^{2+} from intracellular ryanodine-sensitive stores, and the mechanism involved is independent of the PTX-sensitive G_i/G_o proteins (96). The possibility that opioid receptors can utilize PTX-insensitive G proteins to regulate PLCβ and Ca^{2+} mobilization was demonstrated by coexpressing the μ-opioid receptor with $G\alpha_{16}$ in COS-7 cells (97). Linkage to this promiscuous G protein allowed the μ-opioid receptor to stimulate PLCβ in a PTX-insensitive manner. Both δ- and κ-opioid receptors were subsequently shown to activate G_{16} more efficiently than the μ-opioid receptor (98). Albeit the opioid receptors were able to stimulate PLCβ via $G\alpha_{16}$, the EC_{50} values for their respective agonists were ~50-fold higher than those observed for G_i-mediated inhibition of adenylyl cyclase (98). This lower efficacy in opioid receptor coupling to G_{16} versus G_i may provide a mechanism to differentially activate the two systems by controlling the agonist concentration.

The physiological relevance of opioid-induced stimulation of PLCβ is not immediately apparent. Elevation of intracellular IP_3 has been associated with only a few opioid effects. PLCβ1 is implicated in the supraspinal antinociceptive effects of δ-agonists because mice treated with antisense oligodeoxynucleotides against $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, $G\alpha_{11}$, or PLCβ1 exhibit impaired antinociceptive response to δ-agonists (99). Another example is the arrhythmogenic effect of κ-agonists, which is mediated via a PTX-sensitive, G protein–regulated PLC pathway in the isolated rat heart (100). Even though the mere coexpression

of opioid receptors with $G\alpha_{16}$ can hardly be taken as evidence of their functional association, several reports do in fact support such a notion. In T cells, activation of the δ-opioid receptor stimulates Ca^{2+} mobilization (101) and enhances interleukin (IL)-2 secretion (102). On the contrary, both Ca^{2+} mobilization and IL-2 secretion are reduced in T cells expressing a function-deficient mutant of $G\alpha_{16}$ (103). Stimulation of PKC and Ca^{2+}-dependent protein kinases usually occur after the activation of PLCβ. PKC-mediated phosphorylation of PLCβ3 has been demonstrated to rapidly attenuate opioid-induced phosphoinositide turnover in NG108–15 cells (104). This feedback mechanism may limit the involvement of PLCβ in the chronic actions of opioids. With regard to Ca^{2+}-dependent protein kinases, the activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) in the rat hippocampus is stimulated by morphine (55). Chronic morphine treatment appears to down-regulate CaMK II, whereas naloxone-induced precipitation of morphine withdrawal leads to the up-regulation of CaMK II, in particular the β isoform. It has been reported that suppression of PLC can block G_i-mediated inhibition of adenylyl cyclase activity in NG108–15 and SK-N-SH cells (105). Such an observation might be explained by the presence of the neurospecific type 1 adenylyl cyclase in these neuroblastoma cells. In the presence of Ca^{2+}/calmodulin, type 1 adenylyl cyclase is inhibited by $G\alpha_o$ as well as $G\beta\gamma$ (106). Inhibition of PLCβ activity will invariably lead to a decrease in the level of Ca^{2+}/calmodulin and thus attenuate the ability of opioids to inhibit adenylyl cyclase via $G\alpha_o$ and $G\beta\gamma$.

Links to MAPK Cascades

A large number of GPCRs regulate cellular events such as growth and differentiation by stimulating the MAP kinase cascades. There are at least three sets of mammalian MAP kinase modules. They are the extracellular-signal–regulated kinases (ERKs), the Jun N-terminal kinases (JNKs), and the p38 kinases. Mitogenic signals from GPCRs are often transmitted along the ERK pathway. Stimulation of the ERK1 and ERK2 by opioids was first demonstrated with the μ-opioid receptor in recombinant CHO cells (15). The stimulation showed ligand selectivity, agonist dose-dependency, and PTX sensitivity. Likewise, when expressed in Rat-1 fibroblasts, the δ-opioid receptor can stimulate the phosphorylation and activation of ERK1/2 (22). The involvement of G_i/G_o proteins in the activation of MAP kinase is again implicated by the ability of PTX to block this response. In fact, all three types of opioid receptors have been shown to stimulate ERK1/2 in a heterologous expression system (16), and the activation of ERKs occur through the $G\beta\gamma$ subunits in a Ras-dependent manner. An interesting twist in the tale is the recent discovery that GPCRs can stimulate ERKs via focal adhesion complexes as well as by the process of GPCR desensitization and sequestration (107). Although μ-opioid agonists have been shown to activate the focal adhesion kinase in chick embryo cortical neurons (108) and internalization of the δ-opioid receptor is required for opioid stimulation of MAP kinase (109), κ-opioid receptor

internalization does not appear to be necessary for MAP kinase activation (110). Apart from linking opioid receptor activation to mitogenesis, stimulation of the MAP kinase cascade may be required for other aspects of opioid signaling. For instance, the immunomodulatory and immunosuppressive effects of morphine on human lymphocytes may be mediated in part by the activation of the MAP kinase cascade (111). Desensitization as well as internalization of μ-opioid receptors may also involve MAP kinase (112). MAP kinase activities in cortical neurons (layers II/III), median eminence, and amygdaloid and hypothalamic nuclei are diminished in rats with chronic morphine treatment (113). Acute morphine treatment has no effect on the ERK activity in these brain regions. On the other hand, morphine withdrawal produces a dramatic increase in ERK MAP kinase phosphorylation in somata and fibers of locus coeruleus, solitary tract and hypothalamic neurons. The relationship between the observed differential regulation of ERK and opioid tolerance and dependence is unclear but certainly warrants further investigation.

In the complex signaling network of MAP kinases, there are plenty of opportunities for opioids to modulate the activities of disparate pathways through the actions of Gβγ subunits. One of the many capabilities of Gβγ is the stimulation of the γ-isoform of phosphoinositide 3-kinase (PI3K) (114). Agonists of the μ-opioid receptor have been shown to stimulate three different effectors of a PI3K-dependent signaling cascade in recombinant CHO cells (115). [D-Ala^2,MePhe4,Gly^5-01]-enkephalin (DAMGO) stimulates the activity of Akt (also known as protein kinase B), a serine/threonine protein kinase downstream of PI3K, which inhibits apoptosis in neurons. Two other effectors of PI3K, the p70 S6 kinase and the repressors of mRNA translation, 4E-BP1 and 4E-BP2, are also phosphorylated upon stimulation by DAMGO. Hence, opioids may regulate neuronal development and synaptic plasticity by modulating neuronal survival and translational control. The opioid receptors can also modulate signals generated by classical growth factors. For example, chronic activation of μ- or κ-opioid receptors has been shown to attenuate the epidermal growth factor–induced stimulation of ERKs (116). Furthermore, tyrosine kinase activity appears to be stimulated upon activation of the opioid receptor in SK-N-SH cells (117), where a 58 kDa protein is phosphorylated on tyrosine residues following treatment with morphine. The tyrosine phosphorylation can be blocked by PTX treatment. In Rat-1 fibroblasts stably expressing the δ-opioid receptor, [D-Ala^2,D-Leu^5]-enkephalin (DADLE) significantly stimulates the tyrosine phosphorylation of p52 Shc adaptor protein in a PTX-sensitive manner (118). DADLE can also concentration-dependently activate the p70 and p85 S6 kinases in these Rat-1 fibroblasts (119). Collectively, the activation of MAP kinase, S6 kinase, PI3K, and Shc proteins provides a strong mitogenic signal for opioids to regulate cell growth. The μ- and δ-opioid receptors possess different abilities to potentiate growth factor–induced cell proliferation in various cell types (120). Such differences may be related to the different capacities of the opioid receptors to regulate specific mitogenic signals. Little is known with regard to the involvement of JNK or p38 kinase in

opioid signaling. The ability of deltorphin to enhance the activity of NF-AT/AP-1 transcription factor in Jurkat T cells (102) suggests that at least the δ-opioid receptor may possess the capability to regulate JNK in addition to activating the ERKs. Given that all three forms of opioid receptors are capable of stimulating G_{16} (98) and that G_{16} has been shown to activate JNK (121), such an assumption is perfectly plausible.

REGULATION OF RECEPTOR ACTIVITY BY PHOSPHORYLATION

Receptor Phosphorylation and Desensitization

Being a member of the GPCR, the opioid receptor activities could be regulated similarly as those of other GPCRs. A model in which the β_2-adrenergic receptor activities can be regulated has been proposed by Lefkowitz (122). In this model, agonist binding to the receptor results in the rapid phosphorylation of the receptor by protein kinases including the G protein–coupled receptor kinases (GRKs), thereby promoting the association of the cellular protein arrestin. Not only did the association of arrestin with the receptor uncouple the receptor from the respective G protein that transduces the signal and thus blunt the receptor signaling (receptor desensitization), the arrestin also is involved in the agonist-induced, clathrin-coated vesicles–mediated receptor internalization. Arrestin itself also serves as an adapter molecule in the β_2-adrenergic receptor signaling such that a receptor-src kinase complex is formed through which activation of the MAP kinases ERK1/2 by the β_2-adrenergic receptor is accomplished (123).

In the proposed model for GPCR desensitization, the initiation step involves the phosphorylation of the receptor. Concrete demonstration of opioid receptor phosphorylation was first demonstrated by Pei et al (124) with the δ-opioid receptor and by Arden et al (125) with the μ-opioid receptor. Agonist-induced phosphorylation of the κ-opioid receptor was also reported (126). A variety of biochemical approaches have revealed a rapid, agonist-dependent phosphorylation of the receptor protein. Studies with the δ-opioid (124) and μ-opioid (127, 128) receptors suggested that the agonist-induced phosphorylation is mediated via GRKs and not by protein kinase C. Predictably, the ability of opioid ligand to induce receptor phosphorylation correlated to its efficacy (129). With the exception of morphine, agonists such as DAMGO or etorphine were all reported to induce μ-opioid receptor phosphorylation. Wang and coworkers (129) reported that morphine induces μ-opioid receptor phosphorylation in CHO cells, whereas Arden et al (125) and Zhang et al (130) reported morphine does not induce receptor phosphorylation in HEK293 cells. The fact that overexpression of GRK2 in HEK293 cells resulted in the morphine-induced phosphorylation of the μ-opioid receptor (130) suggests that the morphine-receptor complex is a poor substrate for the GRKs. Thus, the discrepancy in the ability of morphine to induce receptor

phosphorylation could be due to the differences in the level of protein kinases in the CHO and HEK293 cell lines. The difference between the morphine-receptor complex and other agonist-receptor complexes was further illustrated by the ability of in vitro PKA catalytic subunit to phosphorylate the morphine-receptor complex and not the DAMGO-receptor complex (131).

Though the sites of agonist-dependent receptor phosphorylation have not yet been identified, it is apparent that the major phosphorylation sites are at the carboxyl tails of the opioid receptors. Deletion of the last 31 amino acids of the δ-opioid receptor resulted in the abolition of both GRK- and PKC-mediated agonist-dependent phosphorylation of the receptor (132). Truncation of the mouse δ-opioid receptor after Thr^{344} also blocked the ability of DPDPE to induce phosphorylation of the receptor (133). Since there are seven putative phosphorylation sites within the carboxyl tail sequence of the δ-opioid receptor, such observations suggested that the agonist must induce phosphorylation of either Thr^{352}, Thr^{353}, Thr^{358}, Thr^{361} or Ser^{363}. As for the μ- and κ-opioid receptor, the phosphorylation sites are less well defined. Our studies have indicated that the carboxyl tail is the target for the kinases. Mutation of all the Ser and Thr within the carboxyl tail to Ala resulted in a mutant μ-opioid receptor that was not phosphorylated in the presence of DAMGO (Maestri-El-Kouhen, PY Law, HH Loh, unpublished data). The exact residues that are being phosphorylated within the carboxyl tail of the μ-opioid receptor remain to be identified.

The protein kinases that participate in the agonist-induced receptor phosphorylation are most likely members of GRKs. Expression of the dominant negative mutant of GRK or overexpression of GRK5 resulted in the attenuation or potentiation of agonist-dependent phosphorylation of the δ-opioid receptor (124). Though overexpression of GRK2 in HEK293 cells could potentiate etorphine- or morphine-induced phosphorylation of the μ-opioid receptor (130), the same overexpression of GRK2 had minimal effect on the DAMGO-induced receptor phosphorylation (128). Probably, the discrepancy between these studies lies within the level of receptor being expressed in the HEK293 cells, or the morphine- or etorphine-receptor complexes represent a better substrate for the GRK2 than the DAMGO-receptor complex, or the DAMGO-receptor complex is an excellent substrate for the endogenous GRKs. Whether these ligands induced the phosphorylation of the same residues in the presence or absence of overexpressed GRK2 needs to be examined.

Some reports suggested that other protein kinases might be involved in the phosphorylation of the receptor. The candidates are Ca^{2+}/calmodulin-dependent protein kinase II, PKA, and the ERK1/ERK2. Though the agonist-dependent phosphorylation of the μ- and δ-opioid receptor is not mediated by the PKC (124, 127, 128), basal phosphorylation of the μ-opioid receptor appears to involve the CaM kinase, as indicated by CaM kinase inhibitor studies (134). Koch et al (135) reported that by mutating the two putative consensus sites (Ser^{261} and Ser^{266}) for CaM kinase II of the μ-opioid receptor to Ala, the increase in rate of receptor desensitization when the CaM kinase II is overexpressed can be blocked either

in HEK293 cells or in *Xenopus* oocytes. Unfortunately, no direct phosphorylation experiment was carried out; therefore, whether the μ-opioid receptor is being phosphorylated by CaM kinase II could not be evaluated. Nevertheless, in view of the observation that the amino terminus domain of GRK5 could interact with calmodulin while the carboxyl terminus domain interacts with the Gβγ subunit (136), it is possible that CaM kinase II could be recruited to the vicinity of the μ-opioid receptor and affect the receptor activities.

Indirect evidence suggested that PKA might phosphorylate the μ-opioid receptor during chronic treatment. By carrying out back-phosphorylation studies, both Chakrabarti et al (131) and Berstein & Welch (137) reported that the treatment of neuroblastoma cells or animals with morphine resulted in a decrease in PKA-induced phosphorylation of the μ-opioid receptor. This reduction in the PKA-mediated back-phosphorylation does not indicate that during chronic morphine treatment, there is PKA phosphorylation of the receptor. Besides, chronic DAMGO treatment did not alter the ability of in vitro morphine-dependent PKA-mediated phosphorylation of the receptor (131). Since chronic treatment of the neuroblastoma cells with either DAMGO or morphine resulted in the loss of response, the lack of DAMGO effect on the back-phosphorylation studies further suggested that PKA did not participate in the agonist-induced receptor phosphorylation.

Intriguing probable kinases that might phosphorylate the μ-opioid receptor are the ERK1/2 of the MAP kinase family. Blockade of this MAP kinase pathway activation by the MAP kinase kinase (MEK) inhibitor PD98059, or the PI3K inhibitors wortmannin or LY294002, resulted in the inability of a 2-h DAMGO pretreatment to desensitize or to down-regulate the μ-opioid receptor stably expressed in CHO cells (112). Though the direct phosphorylation of the receptor was not determined in this study, the ability of MAP kinase to phosphorylate other GPCRs, such as the angiotensin AT1 receptor, has been reported (138). The μ- or κ-opioid receptor carboxyl tail sequences do not contain a consensus phosphorylation sequence recognized by MAP kinase. However, the Thr^{361} residue of the δ-opioid receptor is a putative MAP kinase phosphorylation site. ERK1/2 could probably mediate the agonist-dependent phosphorylation of the δ-opioid receptor.

There appears to be a casual relationship between δ-opioid receptor phosphorylation and desensitization. Desensitization of the δ-opioid receptor was reported to correlate with the phosphorylation of the receptor protein in the SK-N-BE cells (139). The strongest evidence in this report in support of the hypothesis is the inhibitor studies in which heparin or Zn^{2+}, inhibitors of GRKs, could block the desensitization, whereas the PKA/PKC inhibitor H7 could not. Pei et al (124) demonstrated with the dominant negative mutants of GRKs that DPDPE-induced receptor desensitization can be blocked. Overexpression of GRK2 in HEK293 cells could accelerate the DPDPE-induced δ-opioid receptor desensitization (128). Mutation of the last four Thr and Ser residues at the C terminus of the δ-opioid receptor to Ala would block the GRK- and arrestin-mediated desen-

sitization (140). However, the δ-opioid receptor lacking the C-terminal 31 amino acids, the sites for agonist-induced phosphorylation, can be rapidly desensitized by pretreating the CHO cells with DPDPE for 10 min (141). In the same studies, the authors reported that staurosporin could block the DPDPE-induced desensitization, suggesting the involvement of protein phosphorylation by PKC.

The question of whether there is a direct correlation between μ-opioid receptor phosphorylation and desensitization has not been adequately addressed. Zhang et al (127) used two separate models to demonstrate a time course for DAMGO-induced μ-opioid receptor regulation of the GIRK1 channels' fast desensitization, as measured in *Xenopus* oocytes, and receptor phosphorylation, as measured in CHO cells. Pak et al (142) reported the T394A mutant of the μ-opioid receptor could not be desensitized by a 1-h DAMGO pretreatment. Since the mutation of the glutamic acid residues preceding the Thr^{394} also eliminated the DAMGO desensitization, these data implicated the phosphorylation of Thr^{394} by the GRKs, which are acidokinases (143). The importance of Thr^{394} in receptor desensitization was partially supported by the findings with the splice variant of μ-opioid receptor, MOR-1B. MOR-1B has sequence homology with the wild-type MOR-1 receptor up to Glu^{386}, where the sequence then differs by five amino acids and is ultimately seven amino acids shorter than MOR-1 (144). This mutant is more resistant to agonist-induced receptor desensitization than is the wild-type receptor, and the rate of desensitization could be enhanced if the inhibitor of endosome acidification, monensin, was used (145). This apparent decrease in the rate of desensitization could be attributed to the faster internalization and resensitization rates of the splice variant (146). However, the increase in the internalization rate should accelerate the fast desensitization of the MOR-1B variant. Pak et al (147) demonstrated that the agonist-induced desensitization of the μ-opioid receptor was mediated by the loss of membrane receptors. Studies by Whistler & von Zastrow (148) indicated that the overexpression of arrestin could enhance the morphine-induced receptor internalization and the fast desensitization of the μ-opioid receptor. Whether phosphorylation of Thr^{394} is a requisite for such regulation of the opioid receptor activities remains to be demonstrated.

Rapid desensitization of the μ-opioid receptor was reported only with the GTPγS binding assays, or with membrane adenylyl cyclase assays (142, 148). Even with coexpression of the μ-opioid receptor with GRK3 (β-ARK2) and β-arrestin 2 in *Xenopus* oocytes, the desensitization of the receptor required more than 2 h (140). These data are in agreement with the lack of effect on μ-opioid receptor–mediated inhibition of adenylyl cyclase activity in HEK293 cells overexpressing β-arrestin 1 (149). Taken together, these data suggested that the phosphorylation of the μ-opioid receptor might not lead to the uncoupling from the G protein by arrestin and, subsequently, receptor desensitization.

Other cellular events in addition to receptor phosphorylation might play an important role in μ-opioid receptor desensitization. This hypothesis is supported by the observation that the complete mutation of all Ser and Thr residues within the third intracellular loop and the C terminus of the μ-opioid receptor did not

prevent the slow desensitization induced by DAMGO (150). Though prolonged morphine treatment could elicit a loss of response (151), morphine normally does not induce receptor phosphorylation. Thus, it is interesting to note that Koover et al (152), in a later study, reported rapid μ-opioid receptor desensitization (fewer than 20 min) in oocytes expressing GRK3 or GRK5 with β-arrestin 2 where the regulation of GIRK1/GIRK4 channel activities were measured. DAMGO fentanyl or sufentanyl but not morphine could induce such rapid desensitization. These data, in contrast to data from one of their earlier reports (140), suggested that phosphorylation of the μ-opioid receptor resulted in the rapid uncoupling to the regulation of K^+ channel. Because GRK could phosphorylate substrates other than GPCRs, and because the rapid uncoupling of the μ-opioid receptor from GIRK1 channels might not involve phosphorylation (153), it is imperative that the actual phosphorylation of the μ-opioid receptor by the exogenously expressed GRKs be demonstrated.

Role of Receptor Phosphorylation in Receptor Internalization and Down-Regulation

Agonist-induced receptor internalization and down-regulation were initially demonstrated in clonal or recombinant cell lines expressing the δ-opioid receptor (154–157). Agonist-induced internalization of the receptor via the endocytic pathway was first demonstrated in NG108–15 cells (158) and later with cell lines expressing the cloned δ-opioid receptor either with antibodies (159–161) or with fluorescent opioid peptides (162). Only agonists could induce down-regulation of the receptor; partial agonists and antagonists could not (159, 163). Morphine could not induce δ-opioid receptor down-regulation except in the presence of μ-opioid receptor (164, 165). This effect of morphine can be blocked by the μ-opioid receptor selective antagonist β-funaltrexamine (165).

Agonist-induced μ-opioid receptor down-regulation was demonstrated with the 7315C pituitary tumor cells (166), human neuroblastoma SHSY5Y cells (164, 167, 168), human neuroblastoma SK-N-SH cells (165), and human neuroblastoma NMB cells (169). Similar agonist-induced receptor down-regulation was reported with the cloned μ-opioid receptor expressed in neuroblastoma neuro2A cells (151), C6 glioma cells (170), or fibroblasts (125, 130, 159, 171). Though morphine could not induce the rapid receptor internalization (125, 130, 148, 159), it could induce μ-opioid receptor down-regulation in clonal cell lines (164, 165, 167) or in cell lines heterologously expressing the cloned μ-opioid receptor (151, 170). The mechanisms by which morphine could induce receptor down-regulation but could not promote receptor internalization remain to be determined.

Similarly, the κ-opioid receptor can be down-regulated upon agonist treatment. The down-regulation of the receptor was demonstrated in the mouse R1.1 thymoma cell line (169, 172) or in CHO cells expressing the cloned human κ-opioid receptor (173). Though the pretreatment of these two cell lines with U50,488 resulted in the decrease in antagonist binding, the agonist treatment resulted in

receptor desensitization in CHO cells, as measured by the GTPγS binding assay (173), but not in R1.1 thymoma cells, as measured by agonist inhibition of the adenylyl cyclase activity (172). In the same studies, a 50% reduction in the receptor number by pretreating with the irreversible opioid antagonist, β-chlornaltrexamine, resulted in a six-fold increase in the U50,488 IC_{50} value to inhibit adenylyl cyclase activity in the R1.1 thymoma cells. Thus, it is intriguing that a similar reduction in receptor number would not alter the agonist potency in the same cell.

Trafficking of the opioid receptor in the agonist-dependent receptor internalization and down-regulation is probably mediated by the clathrin coated pits of the endocytic pathway. This is concluded from observations in which opioid receptors co-localize with the transferrin receptor after internalization (159, 161, 174) and in which this receptor internalization can be blocked by the dominant negative mutant of arrestin or dynamin (110, 130, 133, 148, 175). However, the functioning of this endocytic pathway appears to be agonist and receptor type-dependent. DAMGO, but not morphine, induced the μ-opioid receptor internalization (125, 130, 148, 159). The inability of morphine to induce receptor internalization can be rescued by the overexpression of GRK2 (130) or β-arrestin (148). This suggested that the morphine-receptor complex could be promoted into the endocytic pathway by increasing the arrestin binding to the complex either by the increase in receptor phoshorylation or by the increase in arrestin concentration. However, the inability of morphine to induce δ-opioid receptor internalization cannot be rescued by overexpression of GRK2 (176). The absolute requirement for receptor phosphorylation in the agonist-induced receptor endocytosis has not been established. Thr^{353} of the δ-opioid receptor has been initially reported to be required for agonist-induced receptor internalization and down-regulation in CHO cells (157, 160). However, though the agonist-induced receptor phosphorylation of the δ-opioid receptor truncated mutant (DOR344T) was blocked in HEK293 cells, the agonist-induced receptor endocytosis between the wild-type and mutant receptors was similar in HEK293 cells but was attenuated in CHO cells by the truncation (133). Mutation of Ser^{356} and Ser^{363} of the μ-opioid receptor could block etorphine-induced receptor down-regulation without significantly altering the agonist-induced receptor phosphorylation (177). These data suggested that mutation of putative phosphorylation sites might not alter the agonist-induced phosphorylation, but rather the interaction between receptor and cellular molecules that are involved in receptor endocytosis, such as arrestin. Moreover, there appear to be differences in the cellular regulation of the opioid receptors. The rate of the agonist-induced receptor endocytosis appears to be different, with δ- >μ- >κ-opioid receptor. Etorphine induced a rapid internalization (175) and down-regulation (178) of the δ-opioid receptor, whereas the internalization of the κ-opioid receptor expressed in the same cell (175), or the down-regulation of the μ-opioid receptor (178), was slow. The carboxyl tail of these receptors apparently was involved in these processes. Receptor chimeras containing the carboxyl tails of the δ-opioid receptors exhibited an increased rate of internalization or down-regulation (175, 178). However, caution must be used

in the interpretation of such data. In view of the facts that mutation of Thr^{394} of the μ-opioid receptor to Ala can enhance the recycling of the receptor (146) and truncation mutation such as the MOR354T can result in a constitutively internalizing and recycling receptor (174), the increase in the apparent rate of receptor internalization could be due to a change in the recycling rate.

In addition to being dependent on β-arrestin and dynamin, the opioid agonist-induced receptor endocytosis also appears to depend on the ability of receptor to form a high-affinity complex with G proteins independent of receptor activation. Pretreatment of cell lines with PTX resulted in the uncoupling of the δ-opioid receptor from G_i/G_o and the abolition of effector signals, but not the agonist-induced receptor down-regulation (163, 179, 180). A similar observation was reported with the ability of agonists to induce down-regulation but not activation of the receptor mutant in which Asp^{95} was mutated to Ala (180). Under these conditions, a high percentage of the δ-opioid receptor remained in the high-affinity, G protein–coupled states (179, 180). The requirement of a high-affinity state was demonstrated with similar experiments with the μ-opioid receptor. Mutation of Asp^{114} of the μ-opioid receptor, or pretreatment of the cells with PTX resulted in the complete uncoupling of the μ-opioid from the G proteins and blockade of the agonist-induced receptor down-regulation (180). Yabaluri & Medzihradsky (170) reported that PTX pretreatment did not block the agonist-induced receptor down-regulation of C6 glioma cells expressing the μ-opioid receptor. However, the percentage of receptors in the high-affinity state was not measured in this study. Aggregation or association of the receptor with each other or with other cellular proteins prior to internalization was supported by the clustering or capping of the receptors in the presence of agonists, but not antagonists (162, 181). Conversely, Cvejic & Devi (182) reported that the δ-opioid receptor existed as a dimer, and upon agonist binding, monomers are formed. However, receptor mutants with the last 15 amino acid residues deleted that did not exhibit agonist-induced receptor internalization did not exist as dimers. Hence, the formation of monomers appeared to be the prerequisite for agonist-induced receptor internalization. Such behavior of the δ-opioid receptor in the presence of agonist is in contrast with other GPCRs in which agonist induced the dimerization of the receptors (183–185). The role of receptor dimerization in opioid receptor endocytosis remains to be demonstrated.

REGULATION OF OPIOID RECEPTOR ACTIVITIES AT THE TRANSCRIPTIONAL LEVEL

Regulation of Opioid Receptor mRNA Levels

In addition to the phosphorylation and uncoupling of the receptor from G proteins, the activities of the opioid receptors can be regulated by the transcription of the receptor genes and subsequently the receptor levels. Though the general principle

of ''spare'' receptor applies in the opioid receptor regulation of the second messenger systems, the agonist potency and the effector that it regulates are receptor density-dependent (22, 155). Thus the control of the expression of the opioid receptor will determine the agonist activities.

The reduction of the receptor protein during chronic agonist treatment as a probable mechanism for tolerance development has been widely reported by several laboratories. Thus, it is logical to hypothesize that the observed reduction in receptor protein is due to the inhibition of the receptor gene transcription and hence the steady state levels of the opioid receptor mRNAs. However, the majority of the studies reported the decrease in the receptor level was not accompanied by a decrease in the receptor mRNA level. Intracerebroventricular injection of [D-Ala^2]deltorphin II for 5 days resulted in the development of tolerance to the peptide without alteration in the δ-opioid receptor mRNA levels (186). Though chronic morphine or antagonist treatment could up-regulate the receptor level, such treatment did not alter the opioid receptor mRNA levels (187–189). A report with female guinea pigs suggested that morphine treatment could decrease the μ-opioid receptor mRNA levels minimally (15%) in the basal hypothalamus (190). Such action of morphine on the receptor level in the female guinea pig might be related to the estrogen regulation of the μ-opioid receptor mRNA levels in the forebrain of female rats (191). Nevertheless, one could argue that the lack of alteration in the receptor mRNA levels after chronic agonist treatment is the result of the receptor-specific agonist not being used or a relatively high steady state level of the agonist could not be maintained with the animals. Treatment of NG108–15 cells with etorphine (192) or cortical astrocytes primary cell cultures with DPDPE (193) resulted respectively in the down-regulation or up-regulation of the δ-opioid receptor mRNA. However, the ability to down-regulate the δ-opioid receptor mRNA levels in NG108–15 by etorphine treatment was not mimicked by treating the same cells with the peptide agonist, DSLET (188). Whether such discrepancy is due to the agonist used or treatment paradigm remains to be resolved.

In contrast to chronic opioid agonist treatment, the opioid receptor gene expression can be altered dramatically by the exposure to pharmacological agents such as alcohol or cocaine. In neuroblastoma cell lines, ethanol has been reported to increase δ-opioid receptor transcripts, which can be blocked by the activation of PKA activity (194–196). Because it is difficult to maintain in vivo alcohol concentration at $>$100 mM, it is not surprising that the induction of the δ-opioid receptor gene was not observed in animals (197, 198). But in experiments in which alcohol-preferring mice, C57BL/6, and alcohol-avoiding mice, DBA/2, were used, differential alteration of the δ- and μ-opioid receptor mRNA levels were observed in distinct brain areas such as striatum and hypothalamus (199). Cocaine treatment, either under the chronic or ''binge'' paradigm, resulted in the up-regulation of the μ-opioid mRNA in the nucleus accumbens (200, 201) or decrease in κ-opioid receptor mRNA in the substantia nigra (202), that might be related to the activation of the dopamine receptor (203). This alteration in the

receptor mRNA levels could be caused by the alteration in the levels of growth factors or second messengers that regulate protein kinase activity. The nerve growth factor (NGF) has been reported to increase both mRNA and protein levels of the δ-opioid receptor in the rat pheochromocytoma PC12 cells (204). The κ-opioid receptor mRNA level increased in oligodendrocytes in the presence of bFGF and PDGF-BB (205). Immunocytokines such as interleukin-1, IL-1b, were reported to increase the μ-opioid receptor mRNA in the astrocytes-enriched striatal, cerebellar, and hippocampal primary cultures, but not in cultures derived from the cortex or hypothalamus (206, 207). IL-1 also has been observed to induce the expression of μ-opioid receptor mRNA in the neural microvascular endothelial cells (208). Opioid receptor transcription could also be induced with agents such as retinoic acid in NG108–15 cells (209) or concanavalin A in $CD4^+$ T cells from murine splenocytes (210). Despite a contradictory report from another group in which concanavalin A apparently reduced the δ-opioid receptor level (211), the control of the opioid receptor level by extracellular signals is unmistakable. Elevation in the intracellular cAMP level in NG108–15 cells has been reported to consistently decrease the opioid receptor mRNA levels, which is probably not due to increase in the degradation of the mRNA (212, 213). Increase in the intracellular cAMP level in astrocytes also decreased the κ-opioid receptor mRNA level (214). Hence, changes in the intracellular cAMP level during acute and chronic agonist treatment could account for the changes in the opioid receptor mRNA levels (192, 193). Increase in the PKC activity with the phorbol ester phorbol-12-myristate-13-acetate (TPA) resulted in the decrease in the μ-opioid receptor mRNA level in SHSY5Y cells (214), whereas activation of the Ca^{2+}/calmodulin-dependent kinase by membrane depolarization in NG108–15 cells resulted in the increase in the δ-opioid receptor mRNA level (215). Because the measurement of δ-opioid receptor mRNA level was not determined simultaneously with the SHSY5Y studies, it is unclear whether the increase in the various Ca^{2+}-dependent protein kinases' activities would result in differential regulation of the transcription of these two opioid receptor genes.

Opioid Receptor Gene Structures

If the regulation of the opioid receptor mRNA levels is due to the alteration in the transcriptional activities of the receptor genes, then the structure of the opioid receptor genes and the *cis*- and *trans*-elements that regulate the transcriptional activities must be determined. Though the three cloned opioid receptor genes are distributed in different chromosomes [distal part of the short arm of the chromosome 1 for DOR, distal part of the long arm chromosome 6 for MOR, and the proximal long arm of chromosome 8 for KOR (216, 217)], the three receptors all have multiple introns and they span large distances in the chromosomal DNA. The MOR-1 gene is more than 53k bp long, with exon splice junctions at the first intracellular loop (Arg^{95}), the second extracellular loop (Glu^{213}), and the cytoplasmic C-terminal region (Glu^{386}/Leu^{387}) (218), and with a splice variant at the

cytoplasmic C-terminal region (219). Similarly, the DOR gene spans 32 kbp with multiple intronic structure (220). The splice junctions of the DOR gene are located at the corresponding amino acids in the first intracellular and second extracellular loop, with the exception that the splice junction at the carboxyl tail of MOR is absent in DOR. However, the multiple-exons structure of the mouse κ-opioid receptor gene is different from those of MOR and DOR. The KOR gene spans more than 16 kbp in the chromosome and has at least four exons (221). Exon I of the KOR encodes the major portion of the 5′-untranslated region and spans a distance of 334, 340, or 716 nucleotides, depending on the sites of transcription initiation. The first intron spans a distance of 371 nucleotides. Exon II of the KOR gene contains 271 nucleotides, including 14 nucleotides of the 5′-untranslated sequence and a splice site at Arg^{86}. Exon III contains 353 nucleotides and has the splice site at Val^{204}. Exon IV begins at Val^{204} and encodes the rest of the 3′ end sequence of the mouse κ-opioid receptor cDNA. The fact that the exon splice junctions of these three opioid receptor genes are at the same amino acids of the coding region suggests that they evolved from a single ancestral gene.

Using 5′RACE and RNase protection assays or primer extension studies, the multiple transcriptional start sites of these opioid receptor genes have been identified. They all have distal and proximal promoters (218, 220, 222). In most cases, transcription of the opioid receptor mRNAs is initiated from the proximal promoters. In rodent brain, the μ-opioid receptor transcripts originated from the proximal promoter (223). Reporter gene assays indicated that both μ- and δ-opioid receptor gene transcriptions are controlled by the proximal promoters (223, 224). However, the KOR gene is transcribed by both proximal and distal promoters, with the transcripts from the distal promoter being the dominant ones (222). The role of these distinct promoter regions in the transcription of the opioid receptor gene is unknown. However, it can be demonstrated that the distal promoter, as reported by Liang et al (225), is the transcriptional start site for MOR-1 and is under inhibitory control. Removal of an inhibitory regulatory region (-775 to -444 from the ATG start site) restored the distal promoter activity in a reporter gene assay (226). The distal promoter regulation sequence can be defined to center around a 34-bp negative *cis*-acting element that was demonstrated to be position- and promoter-dependent (226). Hence, the regulation of the distal promoter activity by such negative element can affect the transcription of the opioid receptor gene.

Similar to other GPCR genes, the opioid receptor genes contain no consensus TATA box within the promoter regions. Comparing the nucleotide sequences of receptor genes upstream from the ATG initiation codon with those in the Transcription Factors Database, it could be demonstrated that several putative binding sites for known transcriptional factors are present. In the 5′ upstream region of MOR-1, consensus binding sites for Sp1, AP2, AP1, glucocorticoid/mineralcorticoid response element, immune-cell-specific element Pu-1, cytokine response elements NF-IL6 and NF-GMb, and the cAMP response elements are found (218). Similarly, consensus binding sites for AP2, NF-κB, NGF–induced transcriptional activator NGFI-B, and NF-IL6 are found in the 5′ upstream regions of DOR

(220). The presence of these transcriptional binding sites could explain the observed cytokine-induced increase in the μ-opioid receptor mRNA levels in the astrocyte-enriched primary culture (206, 207), NGF induced increase in the δ-opioid receptor mRNA levels in PC12 cells (204), and the cAMP induced decrease of the opioid receptor gene transcripts (205, 212, 213). However, the direct demonstration of the involvement of these *cis*-acting elements with reporter gene assays could not be established. The putative NF-IL6 binding site of the opioid receptor genes was demonstrated to be nonfunctional, as determined by reporter gene assays in several immune cell line models (227). Reporter gene assays with the 5′ upstream sequence could not demonstrate that the transcriptional factors were involved in either the cAMP- or NGF-dependent regulation of the δ-opioid receptor mRNA levels (228). Such lack of effects could have several explanations: (*a*) The cytokines' effect on μ-opioid receptor mRNA levels with mixed cell cultures may suggest involvement of multiple cytokines. (*b*) The changes in the steady state levels of mRNAs as detected by RT-PCR could reflect the stability of the mRNAs and not the de novo transcription of the receptor gene. (*c*) The regulation of receptor gene transcription involves elements other than those that are within the proximity of the promoter regions. However, the most likely explanation is that the receptor gene is under the control of multiple transcription factors and *cis*-elements.

An excellent example of the interaction among transcriptional factors could be demonstrated with the analysis of the promoter activities of these receptor genes. Electrophoretic mobility shift assays have indicated that the nuclear proteins immunologically related to Sp1 and Sp3 are specifically bound to the iGA motif of the MOR promoter region (229). Mutation of the binding sites and the use of *Drosophila* SL2 cells which do not express Sp1 or Sp1-like proteins demonstrated that these Sp proteins have a major role in MOR promoter activity. Furthermore, the transactivation of Sp1 and Sp3 are additive (229). Hence, the ratio of Sp1 and Sp3 molecules in the cells can contribute to the μ-opioid receptor gene transcription. A similar situation is also observed with the δ-opioid receptor gene. The presence of the E box and GC box within the promoter region of the DOR gene allows the transactivation of the DOR promoter by the upstream stimulating factor (USF) and Sp families of transcriptional factors (224). Functional and physical interactions between the USF and Sp transcriptional factors can be demonstrated. Such interactions are critical for the activity of the DOR promoter. By regulating the cellular content or composition of the complex, the opioid receptor gene transcription can be controlled. The significance of these transcriptional factors' interaction on the overall spatial and temporal control of the receptor gene expression remains to be investigated.

Receptor Gene Concentration and Pharmacological Activities

The isolation of the receptor genes and the identification of their structures provided an opportunity to address the fundamental question of how a receptor's level affects the pharmacological activities of drugs. By disrupting the transcrip-

tion of a specific receptor gene, the involvement of a specific opioid receptor in the in vivo activities of a drug can be determined. Using the homologous recombination method to disrupt receptor transcription, several groups have successfully generated strains of mice in which the μ-opioid receptor was ''knocked-out'' (230–234). A similar approach was also used to generate mice in which the κ- (235) or the δ-opioid receptor (236) was knocked out. Either by radioactive ligand binding studies, immunoflourescence studies, or quantitative autoradiographic studies (237), these receptor knockout animals exhibited the specific reduction in the receptor protein levels without the alteration of other opioid receptor types. Furthermore, the reduction in the level of receptor was proportional to the gene dosage, demonstrated by the heterozygotic mice that had 50% of the receptor level.

The overall behavior of the receptor knockout animals remains similar to that of the wild-type, with some minor behavioral changes. Changes in the locomotive activity were described in the MOR knockout animals (230, 232) but not in KOR-deficient mice (235). The KOR and MOR knockout mice did not exhibit any changes in the anxiety tests (open-field and O-maze tests). One strain of MOR knockout mice appeared to have changes in their sexual function, as shown by reduced mating activity (232). With the exception of one report with the MOR knockout (231), the lack of a single opioid receptor did not alter the nociceptive threshold after the application of thermal stimuli, either the tail-flick, tail immersion, or the hot plate tests (230, 232, 233, 235, 236), or by mechanical stimuli (238). It is interesting to note that the KOR knockout animals exhibited an increase in the writhing response with the injection of acetic acid that indicated a decrease in the nociceptive response (235). Apparently, the κ-opioid receptor is linked to the control of chemical visceral pain, as suggested by the earlier pharmacological studies.

The absence of an opioid receptor type in these receptor knockout animals enabled investigators to address the issue of which opioid receptor mediates the specific functions of the opioid agonists. In every strain of MOR knockout mice, morphine did not exhibit any antinociceptive effect after thermal stimuli, or produced lethality at high doses (230–234). Though in one report, lethality was observed with an extremely high dose of morphine (233), the cause of death was not due to the normal respiratory suppression effect of the drug. Depending on the method of generating the mutant animals, there were conflicting reports on the ability of the metabolite of morphine, morphine-6β-glucuronide (M6G), and heroin to elicit antinociceptive responses in the knockout animals. M6G and heroin did not produce antinociceptive responses in the μ-opioid receptor knockout mice generated by the deletion of exons 2 and 3 (233, 239) but retained antinociceptive activities in mice generated by an exon 1 deletion (234). Significant levels of M6G binding and the presence of mRNAs detected with the exon 2 and 3 primers were demonstrated in mutant mice with an MOR exon 1 deletion (234). These observations and others suggest the existence of a splice variant of the μ-opioid receptor that is specific for the M6G pharmacological actions. However,

active receptors can be formed from two separate fragments of the rhodopsin and muscarinic receptor. In addition, formation of a putative heterodimer between δ- and κ-opioid receptors could result in a receptor complex that exhibits different pharmacological responses to the receptor selective ligands (240). Hence it is probable that the exon 1 truncated μ-opioid receptor could either scavenge or dimerize with other GPCRs to form a functional M6G receptor. The role of M6G and heroin function in the exon 1 deleted MOR knockout animals remains unclear.

The κ-opioid agonist activities were retained in the MOR knockout animals. However, there appears to be a discrepancy in the δ-opioid agonist activity among different strains of mice. The DPDPE antinociceptive effect was not altered in MOR knockout mice when the peptide was injected intracerebroventricularly (233). However, this effect was attenuated when the peptide was injected intrathecally in the μ-opioid receptor–deficient mice (230, 241). This apparent discrepancy can probably be partially explained by the involvement of multiple opioid receptors in the DPDPE-induced analgesia. In DOR-1–deficient mice, Zhu et al (236) reported that the spinal analgesia of DPDPE was greatly attenuated whereas the supraspinal analgesia was not affected by the absence of δ-opioid receptor. The supraspinal analgesic activity of DPDPE was eliminated in the MOR and DOR double-knockout mice (J Pintar, personal communication). Such observed decrease in the DPDPE analgesic response in the MOR knockout animal could be held as genetic evidence for the existence of a μ/δ-opioid receptor complex (242). However, even with the reduction in the DPDPE analgesic activity, the in vitro activities of DPDPE were not altered in the MOR knockout (243). Thus, whether the DPDPE analgesic activity depends on the physical interaction between the μ- and δ-opioid receptor remains to be investigated.

In addition to identifying whether the μ-opioid receptor is responsible for the morphine-induced antinociceptive responses in the animals, it could be demonstrated that MOR is involved in the morphine-induced decrease in the gastrointestinal transit time but not in the basal transit time (244). The effect of morphine on lymphoid organ atrophy was not observed in the MOR knockout animals (245). Similarly, the regulation of macrophage phagocytosis and secretion of TNFα by morphine was absent in μ-opioid receptor–deficient animals (246). In both of these two studies, the immune cells' functions, such as regulating splenic and thymic cell number and mitogen-induced proliferation, or the inhibition of IL1 and IL6 secretion by macrophages, were not different between the wild-type and mutant mice. However, increase in the proliferation of granulocyte-macrophage and in erythroid and multipotential progenitor cells in both bone marrow and spleen were observed in the MOR knockout animals (232). These data indicated a probable link between hematopoiesis and the μ-opioid receptor.

As expected, the KOR-deficient mice demonstrated that KOR is critical for the U50,488-induced hypolocomotor, analgesic, and aversive activities in the animals (235). KOR was shown to be not involved in the morphine analgesia and reward. This finding substantiated the observation with the MOR knockout in which the morphine pharmacological actions were completely eliminated. Inter-

estingly, KOR was shown to participate in the manifestation of morphine abstinence. The naloxone-precipitated morphine withdrawal syndrome was less severe in the knockout animals as compared to that in the litter mate wild-type control (235). Because the degree of tolerance to morphine was not investigated in the reported studies, it is not certain whether the diminished naloxone-precipitated withdrawal syndrome has a parallel in the tolerance development.

The role of δ-opioid receptor in the pharmacology of the receptor-selective ligands such as DPDPE or BW873U86 was not clearly defined by the DOR-deficient mice. As discussed earlier, the ability of DPDPE or [D-Ala^2,D-Glu^4]deltorphin to elicit a supraspinal analgesic response was not altered in the DOR knockout animals (236). Though the DPDPE spinal analgesic response was reduced by sixfold, the peptide can elicit maximal antinociceptive activity when injected intrathecally into the knockout animals. The complete absence of the δ-opioid receptor in these animals suggested that the DPDPE spinal analgesic response could be mediated by receptors other than the classical δ-opioid receptor. To compound the problem, the antinociceptive activity of BW873U86 was greatly potentiated in the DOR-deficient mice (236). These data suggested that a secondary analgesic pathway, unmasked in the DOR knockout mice, was responsible for the observed antinociceptive activities of these compounds.

The definite role of DOR in the development of morphine tolerance was established with the knockout studies. In previous reports with the selective δ-opioid receptor antagonist naltrindole (247) or with antisense oligonucleotides to DOR (248), partial blockade of the tolerance development to morphine was observed with the reduction of δ-opioid receptor activity. In the DOR-deficient mutant mice, chronic treatment with morphine for 10 days did not change the potency of morphine (236). The DPDPE-induced supraspinal analgesia in these knockout animals also did not develop tolerance during chronic DPDPE treatment. These data suggested not only the role of DOR in the development of morphine tolerance, but also that the receptor that manifest the supraspinal DPDPE effect in the knockout mice is regulated similarly to that of the μ-opioid receptor.

PERSPECTIVE

The opioid receptors are unique among all the GPCRs. The number of multiple receptor subtypes as defined by pharmacological or biochemical binding studies appears to far exceed the number of cloned receptors and their genes. The existence of μ_1-, μ_2-, μ_3-, δ_1-, δ_2-, κ_1-, κ_2-, and κ_3-opioid receptors has long been postulated. The presence of introns within the receptor genes allows for the generation of splice variants, and probable subtypes of the receptors. Though a splice variant of the μ-opioid receptor cDNA was isolated (144), the pharmacology of this splice variant resembled that of the wild-type MOR. With extensive low-stringency hybridization procedures, no opioid receptor type other than the cloned μ-, δ-, and κ-opioid receptors could be isolated. At best, the orphanin FQ or

nociceptin receptor with high homology to the opioid receptors was cloned using this approach (249–254). One could argue that the other opioid receptor subtypes are structurally dissimilar to the cloned opioid receptors, as in the case of GABA or histamine receptor types. However, this argument was not supported by the studies with the receptor knockout animals. The disruption of an opioid receptor gene normally did not result in residual binding that could account for the existence of receptor subtypes. Though with the mice that have the first intron of MOR deleted, M6G and heroin remained active. The multiple possibilities that could cause such a phenomenon, as discussed previously, suggested that the conclusion of the existence of unique M6G binding sites may be too premature. The vast literature on the existence of multiple δ-opioid receptor subtypes was not supported by the DOR knockout. The complete absence of DPDPE binding in the DOR-deficient mice and the retention of the DPDPE-induced supraspinal analgesia in these animals suggested the observed effect of DPDPE is mediated by other opioid receptors. It is also probable that this DPDPE effect is mediated by a yet-unidentified receptor.

In the absence of identified proteins, alternative explanations must be used to account for the reported multiple opioid receptors. It is still possible that a single amino acid difference among the receptors will generate the subtype pharmacology, as it was clearly demonstrated with the single amino acid mutation in the putative fourth transmembrane serine residue could result in the phenotype of antagonist activating the receptor (255). However, the human opioid receptor polymorphism studies have not revealed such a situation. At best, the human μ-opioid receptor with a single nucleotide polymorphism binds β-endorphin with higher affinity (256). A different polymorphism in δ-opioid receptor did not reveal any pharmacological phenotype (257). Thus it is possible that the multiple opioid receptor subtypes could be generated from single nucleotide polymorphism that has eluded detection by conventional means. This could be accomplished only by detailed nucleotide sequencing of the receptor mRNAs in regions where the pharmacology of the receptor subtypes was reported. With the current sequencing technology, such a goal can be reached. Another intriguing possibility has been reported on the alteration of the pharmacological activities of the opioid receptors when they are heterodimerized (240). The inability of the receptor-selective ligands such as DPDPE or U69593 to compete for diprenorphine binding to the putative κ/δ-opioid receptors dimer provided another level of regulation of the opioid receptor activities. It is possible that the opioid receptor subtypes reported are the results of the heterodimerization of various opioid receptors. Whether the opioid receptors actually dimerized and whether receptor dimerization could generate the pharmacology reported for the various subtypes should be investigated.

With all the molecular biological tools, the fundamental question on the molecular mechanism for opioid tolerance and dependence remains to be adequately addressed. It is attractive to suggest that receptor phosphorylation is the fundamental basis for opioid tolerance. No doubt, opioid agonists could induce the phosphorylation of all the opioid receptor types. However, it is even doubtful that

the receptor phosphorylation is the sole mechanism for the observed homologous desensitization to various effector systems. The noncorrelation between the time course of receptor phosphorylation and desensitization, as reported by some laboratories, could be reconciled by the dephosphorylation and resensitization of the internalized receptor. Depending on the efficacy of the receptor coupling to individual effector and the rate of receptor recycling, it is probable that the receptor activity is not altered even with a robust phosphorylation of the receptor. Phosphorylation and subsequent inactivation of the effector system could also cause homologous desensitization. Phosphorylation of the type II adenylyl cyclase in the guinea pig ileum longitudinal muscle myenteric plexus (258) and PLCβ_3 in NG108–15 cells (104) after opioid receptor activation have been reported. In NG108–15 cells, the transient activation and inactivation of PLCβ_3 by δ-opioid receptors correlated to the phosphorylation of the enzyme itself (104). In the case of PLCβ_3 phosphorylation, the ability of other GPCR agonists, such as LPA, to induce phosphorylation of the same proteins suggested probable compartmentalization of the receptor with the PLCβ_3, thus allowing homologous desensitization. Hence, homologous desensitization could occur with multiple mechanisms.

The uncoupling of the receptor from the effector, or the inactivation of a specific pool of effector, may not be the basis for opioid tolerance and dependence either. The involvement of transcriptional responses for the adaptation to long-term exposure to the drug has long been proposed. This was clearly demonstrated in mice that had the α and δ isoforms of the cAMP-responsive element binding protein (CREB) disrupted. The symptoms of morphine withdrawal were greatly attenuated in these animals (259). However, the uncoupling of the opioid receptor, CREB, and the immediate early genes might be three of the many factors that contribute to the chronic opioid responses. The involvement of other receptors in the modulation of opioid tolerance has been demonstrated. The DOR-deficient mice provided the genetic evidence for the involvement of δ-opioid receptor for morphine tolerance (236). Additionally, the participation of NMDA receptor in morphine tolerance has been established. By the concurrent administration of the NMDA antagonists, LY274614 and MK-801, morphine tolerance can be attenuated (260, 261). Thus, the regulation of multiple neuronal activities by multiple receptors could then manifest the overall response to chronic opioid treatment. The manifestation of the chronic response might be a probable reason for the evolution of the multiple opioid receptors.

The complexity in the signal transduction of the opioid receptor goes beyond the simple involvement of receptor-Gi/Go proteins and the effectors. As discussed in this review, a universal mechanism cannot be applied to all receptor agonists. The two μ-opioid receptor agonists, morphine and DAMGO, definitely elicited differential cellular responses, and their receptor complexes can be distinguished from each other. Further, the overall response to the activation of Gi/Go by opioid agonists will depend on the composition of the neurons expressing the receptor. This is best illustrated by the differential responses exhibited by the multiple adenylyl cyclase subtypes to the activation of the receptor. One can imagine that the identity and the concentration of the proteins participating in the signaling

within the membrane microdomains of the receptor will greatly affect the opioid receptor signaling. The signaling through scaffold, anchoring, and adaptor proteins has been well established with many membrane receptors, in particular those of the tyrosine kinase family (262). Recognition of the phosphorylated tyrosine by proteins such as Grb2 that contain both the SH2 and SH3 domains will allow Grb2 to serve as an adaptor that recruits other cellular proteins, such as Sos, to the vicinity of the receptor and participates in the signal cascades. The recruitment of other proteins with an adaptor that has multiple docking sites will allow the amplification or modulation of the signals. An excellent example is the *Drosophila* InaD gene that codes for a protein with 5 PDZ domains (263). InaD associates through these PDZ domains with a light-activated Ca^{2+} channel (TRP), PLCβ, and PKC. The organization of these effectors by InaD allows for the efficient activation of TRP by PLCβ in response to the stimulation of rhodopsin and $G\alpha_q$, and the inactivation by the phosphorylation of TRP by PKC. If a similar scenario exists for the opioid receptor, the recruitment of molecules such as $PLC\beta_3$ and PKC to the vicinity of the receptor could provide a rapid control mechanism for the opioid receptor signaling. The local increase in the intracellular Ca^{2+} level due to $PLC\beta_3$ activation would active the PKC, which in turn could phosphorylate and inactivate the $PLC\beta_3$, and thus turn off the signal. Depending on the kinetics of such a feedback cycle, the magnitude of the signals can be regulated. Hence, the immediate emphasis for the understanding of the receptor signaling should be the identification of cellular proteins that participate in the opioid receptor signaling. With the possibility that opioid receptor dimerization could affect the activity also, the formation of the signaling complexes within a microdomain could greatly affect the cellular responses to a specific pharmacological agent targeted for a particular type of opioid receptor. These signaling complexes might be the true distinction between the multiple opioid receptors.

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