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Perspectives in Pharmacology

Unifying Perspectives of the Mechanisms Underlying the Development of Tolerance and Physical Dependence to Opioids

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ABSTRACT

The cellular basis of tolerance to, and dependence upon, many types of drugs, including opioids, has long defied identification. Tolerance to opioids cannot be explained solely on the basis of modification of opioid receptors or altered metabolism or disposition of the opioid. The development of tolerance following chronic exposure to opioids presents at least three different types of change in cellular responsiveness, each of which has been suggested to represent some type of adaptive modification in cellular responsiveness. These different forms of tolerance are distinguishable on the basis of their time course and whether or not the tolerance is specific for opioid receptor agonists (homologous) or extends to agonists of other systems (heterologous). The adaptive modulation of responsiveness via regulation of cellular proteins has been proposed to be the basis for both longer-term forms of tolerance. The divergent signaling pathways activated by G-protein-coupled receptors like the μ-opioid receptor provide multiple downstream targets for both short- and long-term regulation of cell function that is associated with the development of tolerance and/or dependence. Since the magnitude of receptor activation is an important determinant of the degree to which various signaling pathways are activated, the expressed characteristics of tolerance and/or dependence may be functionally related to which of these diverse pathways are stimulated to the greatest degree. Thus, the possibility that different signaling events are activated either sequentially or concurrently offers the possibility to explain the interaction between these different forms of tolerance and/or dependence.

The chronic use of opioids is often accompanied by the development of tolerance and/or dependence upon these agents due to adaptive changes in the response of the subject to the agent. Tolerance may be defined as a reduction in sensitivity to an agent following repeated exposure, while dependence is generally thought of as the absolute requirement for the agent to maintain normal physiological function. A complication in identifying cellular mechanisms of the adaptation is the presence of multiple forms of tolerance and dependence that include both homologous and heterologous changes in responsiveness. Dependence also presents in different forms that are defined by the presence of a withdrawal reaction (physical dependence) and/or the presence of a "drug-craving" component (psychic dependence). The existence of multiple forms of these phenomena raises the possibility that each component may possess different cellular mechanisms and, furthermore, that multiple mechanisms could be concurrently operating to produce the complex behavior normally associated with drug dependence and tolerance in humans. Indeed, a number of potential mechanisms for these states of altered responsiveness have been suggested from animal and isolated tissue studies. Providing a perspective from which multiple mechanisms, each based upon sound scientific modeling, could interact to produce these effects on cell responsiveness is a challenging goal. To

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ABBREVIATIONS: AC, adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1]; CREB, cyclic AMP response element-binding protein; GPCR, G-protein coupled receptor; GRK, G-protein coupled receptor kinase; GIRK, inwardly rectifying K^+ channel(s); LM/MP, longitudinal muscle/myenteric plexus; LC, locus ceruleus; nTS, nucleus tractus solitarius; RGS, regulators of G-protein signaling; PKA, protein kinase A; PKC, protein kinase C; E_k, potassium equilibrium potential; MAP kinase, mitogen-activated protein kinase; GABA, γ-aminobutyric acid.

achieve such a goal within this limited format requires the liberal use of review citations from which the reader can retrieve the original references discussed and a limited number of individual citations for each observation.

To avoid the complications of the phenomena that are created through the impact on neuronal networks, attention will be devoted to the mechanisms that have been described at the level of the individual neuron or can reasonably be inferred to that level. It is obvious that adaptation to opioid responses in one neuron within the framework of a network can induce adaptations in secondary neurons that may not possess opioid receptors, for example, those that follow chronic opioid action involving a compensatory elevation in the activity of glutamatergic neurons whose actions are mediated through *N*-methyl-D-aspartate (NMDA) receptors (Mao, 1999). Such "network" adaptations are clearly beyond the limits of this analysis. Clearly, psychic dependence is a phenomenon of complexity well beyond the adaptation of individual neurons and has been reviewed by Koob et al. (1998).

There is clear evidence that the expression of tolerance in individual neurons occurs with different characteristics that can be distinguished by the specificity of change in responsiveness and the temporal development. One form (i.e., "desensitization") is highly specific for opioids (homologous), develops rapidly following receptor occupation (seconds to minutes), is due to uncoupling of the receptor from its cognate G-protein (with or without internalization), and is often produced by exposure to high concentrations of agonist (Johnson and Fleming, 1989; Law and Loh, 1999). Another form is also homologous but develops on a somewhat slower time course (hours) and has been proposed to be due to changes in the adenylyl cyclase (AC) cascade (Nestler et al., 1994; Nestler and Aghajanian, 1997). A third form develops and decays even more slowly (days), exhibits nonspecific changes in responsiveness (heterologous), and has been suggested to be due to a partial depolarization resulting from down-regulation of the sodium pump and a reduction in its electrogenic contribution to membrane potential (Fleming and Taylor, 1995; Fleming, 1999). Interestingly, the expression of these different types of "tolerance" can be observed in a variety of individual neurons and may develop separately or concurrently, depending upon the method used to induce tolerance (Johnson and Fleming, 1989).

The concept that tolerance and physical dependence upon opioids are expressions of individual neuronal adaptation has been the subject of several reviews (Johnson and Fleming, 1989; Nestler et al., 1994; Fleming and Taylor, 1995; Christie et al., 1997; Nestler and Aghajanian, 1997), although no single mechanism has been clearly identified to underlie the development of the phenomena. Increasing emphasis has been placed on the regulation of cell protein levels as a general mechanism by which long-term adaptations in cellular responsiveness occur (Nestler et al., 1994; Fleming and Taylor, 1995; Nestler and Aghajanian, 1997). Certain criteria must be met for any proposed cellular mechanism of adaptation to be confidently established as being responsible for the change in responsiveness. The proposed cellular change must: 1) be induced by experimental procedures identical to those that induce tolerance and/or dependence; 2) follow a similar time course as the tolerance and/or dependence in that tissue; 3) quantitatively account for the tolerance and/or dependence; 4) account for the qualitative characteristics of the tolerance and/or dependence; and 5) occur in the very cells upon which the opioid is acting. It is also assumed that the cellular changes develop as a consequence of activation of the acute signaling pathway by the opioid.

These criteria were first explicitly expressed in the review by Fleming and Taylor (1995). However, they have been implicitly applied for decades in studies of adaptation in non-neuronal cells. For example, all of the criteria have been met in identifying the spread of cholinoceptors outward from the end-plate as the cause of the highly specific supersensitivity of denervated skeletal muscle (Thesleff, 1960; Fleming and Westfall, 1988). Similarly, all of the criteria have been met for the role of partial depolarization and reduced Na^+, K^+ pump function underlying the nonspecific supersensitivity to excitatory agonists in the rabbit aorta (Abel et al., 1981) and the guinea pig vas deferens (see *Discussion* in Hershman et al., 1995), which follow chronic interruption of adrenoceptor activation. These criteria have not been established fully for any proposed mechanism of opioid tolerance to date, although the collective data supporting a role for membrane depolarization and reduced function of the Na^+, K^+ pump in myenteric neurons come close to meeting all of the criteria (see *Effects of Chronic Exposure to Opioids* below). The criteria generally lacking for other mechanisms are numbers 3 and 4 and sometimes 2.

It is particularly intriguing to consider the possibility that multiple mechanisms could be concurrently activated to affect the adaptation associated with chronic exposure to opioids, as discussed later. Since activation of the receptor-mediated signal transduction pathway is an obligatory component in the development of all types of tolerance, examination of the acute effects of opioids should identify potential molecular targets that could underlie the long-term modification in responsiveness.

Cellular Mechanisms of Acute Opioid Action

The actions of opioids in any biological system are a combination of multiple independent components acting in concert. The first source of diversity is the receptor where at least three major types of opioid receptors (i.e., μ, κ, and δ) mediate the response to exogenous or endogenous opioid ligands (di Chiara and North, 1992). Each of these receptors is a member of the superfamily of G-protein-coupled receptors (GPCR) characterized by seven transmembrane-spanning tertiary structures with a large intracellular loop between TM5 and TM6 where the presumed contact with the cognate G-protein α-subunit resides. Since the μ-opioid receptor subtype (hereafter termed the μ-receptor) is predominately responsible for the production of both analgesia and euphoria by the more common opioids, consideration of the mechanisms of development of tolerance/dependence will focus primarily on this receptor subtype.

The G-proteins that couple these receptors to the intracellular effectors exist as heterotrimers derived from three different primary classes of subunits: G_α [18 subunit isoforms from four families (**G**$_s$, **G**$_{i/o}$, **G**$_q$, and **G**$_{12}$)]; G_β (5 subunit isoforms), and G_γ (11 subunit isoforms) (Hildebrandt, 1997). Each heterotrimer, consisting of a single α-subunit isoform combined with a dimer of $\beta\gamma$-subunits, can link a different GPCR with a specific downstream effector system. Substan-

tial mechanistic diversity can be achieved through this type of combinatorial cell signaling involving only two partners, a GPCR and a G-protein heterotrimer, as depicted in Fig. 1. As illustrated, the μ-receptor is activated by agonist forcing the replacement of GDP bound to the α-subunit by GTP, which then forces the dissociation of α- and $\beta\gamma$-subunits. The activated α-subunit (i.e., G_α * GTP) and $\beta\gamma$-subunits can each mediate downstream effects providing a locus for the two entities to couple to multiple cellular effectors like enzymes and ion channels (Fig. 1). According to this scheme, opioid receptor activation could not only change ion conductance but also act upon other cellular effectors simultaneously through the combined actions of the α- and $\beta\gamma$-subunits (see activities in Table 1). Through this type of coupling between a single ligand-receptor-transducer complex and multiple effectors, the potential for activating pathways whose primary targets are separated between short- and long-term regulation of cellular function is markedly enhanced. Given that adaptive processes underlying tolerance and/or dependence utilize multiple signaling systems, the possibility that one pathway may be predominately activated over another by varying the conditions of agonist exposure must be considered. Thus, when high concentrations of agonist are used, the specific pathway that exerts the primary effect could change from that used in the normal acute situation or under conditions of chronic low-concentration agonist exposure.

The inactivation of G-protein-linked signaling through GTP hydrolysis offers another unique site for regulation that may be dependent upon the level of receptor activation. Since the α-subunits possess intrinsic GTPase activity (see Dohlman and Thorner, 1997), the potential for signal regulation to occur through inactivation prompted efforts to identify other GTPase or GTPase-activating proteins. A family of 20 known protein isoforms called **R**egulators of **G**-protein **S**ignaling (RGS proteins) have been identified that exhibit high specificity as GTPase-activating proteins that function to accelerate the exchange of GTP for GDP on the α-subunits of G_i, G_o, and G_q (see Dohlman and Thorner, 1997). The mRNAs encoding these different proteins are differentially distributed throughout the rat brain (Gold et al., 1997). The mRNA for three of these proteins (i.e., RGS4, -7, and -8) has been found in high abundance in the rat locus ceruleus (LC), while two other areas heavily studied with respect to acute and chronic opioid effects, the nucleus accumbens and ventral tegmental area, express RGS8 mRNA in high abundance with moderate amounts of RGS4 mRNA also present in the nucleus accumbens. As illustrated in Fig. 1, these RGS proteins would increase the rate of GTP hydrolysis forcing the reassociation of the $\beta\gamma$-subunits with an α-subunit and thereby turning off the signal mediated by both G-protein components. This process has been suggested to participate in acute desensitization of μ-receptor-mediated activation of inwardly rectifying K^+ channels (GIRK) (Doupnik et al., 1997; Chuang et al., 1998).

Another mechanism by which GPCR signaling is acutely down-regulated involves receptor phosphorylation and subsequent internalization or sequestration of the receptor into caveolae or clathrin-coated pits. Phosphorylation of the receptor occurs through the action of a family of six different protein isoforms known as **G**-protein-coupled **R**eceptor **K**inases (GRK, also known as βARK). These GRK proteins are serine/threonine-phosphorylating proteins that are differentially distributed and regulated in a wide variety of tissues (see Krupnick and Benovic, 1998). Simplistically, the GRK-phosphorylated receptor is bound to one of a family of cytosolic proteins, β-arrestins, that uncouples the receptor from its cognate G-protein and targets the complex for sequestration in clathrin-coated pits (see Krupnick and Benovic, 1998). Thus, the cellular machinery exists to regulate the responsiveness of the system in the short term via receptor availability rather than receptor abundance, and recent studies have demonstrated that the activation of G-proteins by opioids and subsequent activation of GRK proteins plays an important role in this process (Zaki et al., 2000). Receptor uncoupling and sequestration is an example of a mechanism that clearly associates with tolerance. However, it is more

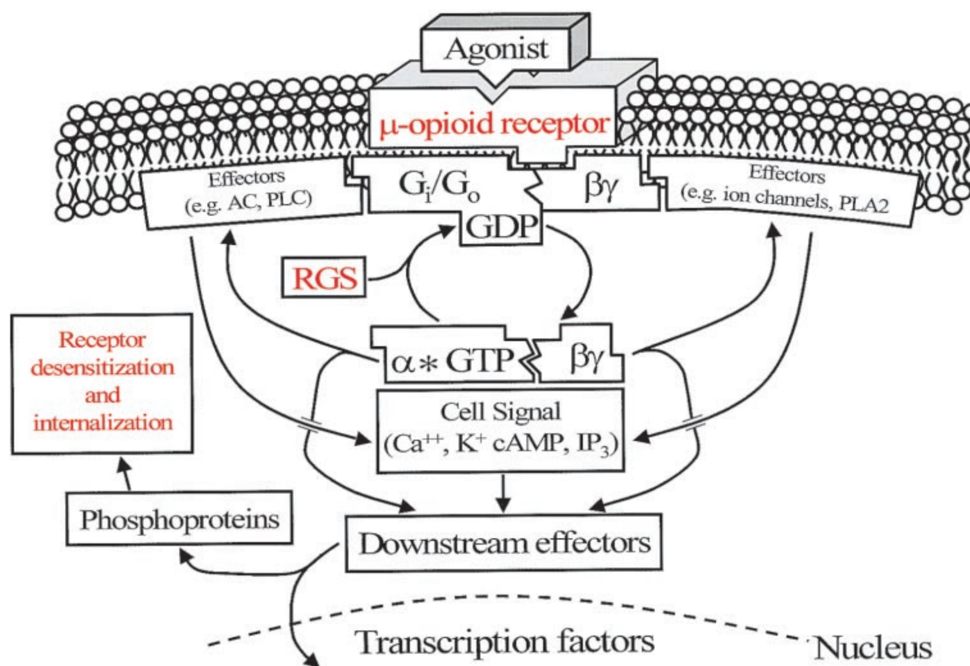

Fig. 1. Schematic representation of the signaling pathways activated acutely by μ-opioid receptor activation. The G-protein-coupled receptor can modify the activity of a variety of cellular effectors through the combined action of the dissociated α- (i.e., GTP-bound) and $\beta\gamma$-subunits. The action of the cellular effectors are amplified further by the capacity to simultaneously produce multiple cell signals that can then interact with multiple downstream effectors within the cell. The cellular effectors that have been identified to be modified acutely by μ-opioid receptor agonists are presented in Table 1. The phosphoproteins component actually represents another potential site of regulation through modification of the phosphorylation/dephosphorylation state. The cellular loci highlighted in red represent those entities that have been proposed or demonstrated to be modified during the production of acute changes in responsiveness (i.e., desensitization through receptor uncoupling or receptor sequestration and internalization).

TABLE 1
Impact of μ-opioid receptor activation on various cellular elements that may be involved in production of tolerance and/or dependence upon opioids

Cellular Effector/Signal	Acute Effect of Opioids	Chronic Effect of Opioids
μ-Opioid receptor	Activation; phosphorylation; sequestration (internalization)	Decrease; no change in number/affinity
G-proteins		
G_i	Activation	Increase; decrease; no change
G_o	Activation	Increased abundance
G_s	No effect	Increased abundance
RGS proteins	Activation via $\beta\gamma$-subunits	Not determined to date
Adenylyl cyclase	Inhibition via G_i	Activation via G_s; increased abundance: AC types I, II, IV, and VIII
Phospholipase A_2	Activation leading to increased PKC and intracellular Ca^{2+}	Switched transduction path to adenylyl cyclase-dependent pathway
Protein kinases	Inhibition (PKA); activation (PKC; MAP kinase); calcium/calmodulin-dependent activation; translocation (PKC)	Elevated abundance: PKA; increased PKC and MAP kinase activation; translocation (PKC)
Phosphoproteins	Decreased levels: CREB; MARPPs	Increased abundance: CREB; MARPPs
G-protein receptor kinase; tyrosine receptor kinase	Activation	Increased abundance GRK2
Transcription factors	Activation (Ras, fos, CREB)	Increased production and long-term gene regulation
Sodium pump	No direct effect	Decreased abundance reduced electrogenic activity
Calcium channels	Inhibition of N-type via G_o activation of L-type via increased $[Ca^{2+}]_i$	Decrease in dihydropyridine binding; no effect on function or expression of L-, N-, or P/Q-type channels
K^+ channel (inward rectifier; *GIRK*)	Activation via $\beta\gamma$-subunits	No change in function or expression
Cyclic AMP-dependent nonspecific cation channel	Inhibition via decreased PKA	Activation via increased PKA

MARPPs, morphine- and cyclic AMP-regulated phosphoproteins.

difficult to explain physical dependence by such a mechanism.

An additional component of the GPCR signaling pathways impacting upon the levels of available receptors is the family of receptor tyrosine kinases. This signaling pathway is activated by cellular processes that produce free $G_{\beta\gamma}$ and/or G_α subunits or elevate intracellular Ca^{2+} (Luttrell et al., 1997) and eventually lead to the phosphorylation of mitogen-activated protein kinases (MAP kinases) (van Biesen et al., 1995). This protein family includes the **E**xtracellular signal-**R**egulated protein **K**inases (ERKs) and the **Ju**n protein **K**inases (JNKs), which phosphorylate different transcription factors and serve as central intermediates in signal transduction from the plasma membrane to the nucleus (see Segal and Greenberg, 1996). The participation of this series of proteins in a signaling cascade can also be directly influenced through the intracellular regulation of Ca^{2+} concentration and/or activation of protein kinase C (PKC). Both chronic and acute exposure to morphine elevates the levels of specific ERKs in the rat brain (Berhow et al., 1996) and transfected cell lines (Gutstein et al., 1997; Belcheva et al., 1998). Confocal imaging studies have also suggested a role for receptor internalization following μ-receptor stimulation in the activation of the MAP kinase pathway (Ignatova et al., 1999) that involves free $G_{\beta\gamma}$ subunits and Ras (Belcheva et al., 1998).

Understanding the acute effects that follow occupation of μ-receptors provides signaling cascades that could participate in either the short- or long-term regulation of responsiveness to opioids. One prominent acute action mediated by the μ-receptor that is responsible for reduced neuronal excitability is the hyperpolarization of the cell membrane due to activation of GIRK. The activation of GIRK by μ-receptors has been demonstrated in guinea pig myenteric neurons (Cherubini et al., 1984), guinea pig and rat LC neurons (see Christie, 1991), rat nodose ganglion neurons (Ingram and Williams, 1994), rat and guinea pig spinal trigeminal substantia gelatinosa neurons (Grudt and Williams, 1994), rat parabrachial neurons (Christie and North, 1988), rat anterior cingulate cortical neurons (Tanaka and North, 1994), rat periaqueductal gray neurons (Chieng and Christie, 1994), neurons in the hippocampal formation (Wimpey and Chavkin, 1991), as well as in expression systems transfected with μ-receptor and GIRK1 (Chuang et al., 1998). In particular, in both guinea pig myenteric neurons and rat LC neurons, the activation of GIRK has been demonstrated to occur without the requirement for production of any diffusable substance like cyclic AMP (Miyake et al., 1989; Johnson and Pillai, 1990).

In many brain regions, however, opioid receptor activation uses other mechanisms to regulate neuronal responsiveness including the regulation of transmitter release and/or postsynaptic membrane responsiveness. One process activated by opioid receptor subtypes, especially κ, that could account for the presynaptic actions is the interaction with calcium channels seen in several neuronal populations (see di Chiara and North, 1992; Moises et al., 1994). The μ-receptor-mediated activation of GIRK likely involves the $G_{\beta\gamma}$ subunits released from G_i (Doupnik et al., 1997; Chuang et al., 1998). One additional electrophysiological effect that has been described for μ-receptor activation is inhibition of a cAMP-dependent resting cation conductance in LC neurons (see Nestler and Aghajanian, 1997). However, the physiological relevance and functional significance of these changes in LC neurons in the production and propagation of the withdrawal response has been questioned (Christie et al., 1997). Furthermore, the concentrations of agonist required to activate this conductance are 100 to 200 times greater than the concen-

tration required to increase K^+ conductance (see Fleming and Taylor, 1995). The latter effect clearly is important in the modulation of membrane potential by μ-receptor agonists (see di Chiara and North, 1992; Christie et al., 1997).

Other acute effects of μ-receptor activation have been identified that could be important in the signaling pathways that ultimately lead to the adaptation. Particularly significant among these effects is the inhibition of AC that has been described in a number of neuronal populations (see Nestler et al., 1994; Fleming and Taylor, 1995; Wang and Gintzler, 1997). The interaction of μ-receptors with the AC cascade introduces an opportunity for the agonists to impact on downstream effectors of the action of cAMP including protein kinase A (PKA) and the cyclic AMP response element-binding protein (CREB). This latter protein provides access of the cascade to the nucleus for activation of transcription factors as suggested in Fig. 1. However, μ-receptor activation has also been demonstrated to activate and/or translocate PKC in guinea pig longitudinal muscle/myenteric plexus (LM/MP) (Wang et al., 1996), rat central neurons (Mayer et al., 1995; Ventayol et al., 1997), and in vitro expression systems (Kramer and Simon, 1999a,b). In periaqueductal gray neurons, acute μ-receptor-mediated responses involve activation of a voltage-dependent K^+ conductance through an action on phospholipase A_2 (Ingram et al., 1998) thereby offering another cell signaling pathway that also is a site for the synergy that exists between the opioids and nonsteroidal anti-inflammatory agents. Finally, μ-receptors have also been shown to acutely activate the MAP kinase cascade through a Ras-dependent mechanism in expression systems (Gutstein et al., 1997; Belcheva et al., 1998; Ignatova et al., 1999) as well as the immediate early genes (e.g., *fos*) in brain tissue, permitting communication between the extracellular signal and the nucleus (see Nestler et al., 1994; Nestler and Aghajanian, 1997). Thus, the divergent acute effects of μ-receptor activation can account for both the acute electrical events that regulate neuronal responsiveness in the short term as well as permit sufficient cross-talk among different signaling cascades between the cell surface and the nucleus to provide a scaffold for regulation of cellular protein abundance and, ultimately, neuronal sensitivity in the long term.

Effects of Chronic Exposure to Opioids

Following chronic exposure to opioids, Cox (1978) identified two components of subsensitivity to opioids that differed in characteristics and temporal relationships (see Johnson and Fleming, 1989). One component decayed rapidly after drug removal and was apparently dependent on the presence of the tolerance-inducing drug, while the second component outlasted the presence of the drug and was associated with long-lasting changes in the properties of opioid-sensitive neurons. A similar two-phase response has been described during morphine withdrawal in rat behavior and in LC neurons (Rasmussen et al., 1990). Marked changes in behavior and increases in LC firing rate occurred with a rapid decline over 4 h, followed by a lesser, prolonged effect that was undiminished for 24 h with complete recovery requiring 72 h. In the guinea pig ileum, three components of subsensitivity have been identified. The first is the rapidly decaying homologous tolerance, probably resulting from receptor uncoupling and internalization (Johnson and Fleming, 1989). Second, chronic treatment with opioid leads to sustained changes in G-proteins (Ammer et al., 1991) and the AC system (Wang et al., 1996; Wang and Gintzler, 1997) as indicated in Table 1. Third, there is a slowly disappearing, heterologous tolerance that results from changes in resting membrane potential due to an alteration in electrogenic sodium pumping (see Table 1 and Fleming, 1999).

The long-term nonspecific tolerance, membrane depolarization, and pump protein changes 1) are produced by the same procedure (i.e., morphine pellet implantation); 2) occur at the same time (i.e., 6–7 days after morphine pellet implantation, although a complete time course has yet to be completed); 3) account for the qualitative (nonspecific) characteristics of tolerance, including the subsensitivity to inhibitory agents and supersensitivity to excitatory agents; 4) account for the magnitude of the subsensitivity (tolerance); and 5) occur in the *same* cells (the S-type myenteric neurons). Compare to the five criteria presented earlier. Number 4 above requires some elaboration. The half-maximum concentrations of morphine and 2-chloroadenosine for twitch inhibition were determined by Taylor et al. (1988). In that same study, maximum effect (i.e., a doubling of the half-maximum effect) was achieved by concentrations of each agonist approximately 10-fold greater. The depolarization associated with tolerance is 7 to 9 mV (Leedham et al., 1992). The approximate half-maximum concentrations of morphine and 2-chloroadenosine (from Taylor et al., 1988) hyperpolarized the control and tolerant S neurons by 6 to 8 mV (Meng et al., 1997). Because of the offsetting depolarized state, to achieve the same twitch inhibition, twice as much hyperpolarizing effect is required (see analysis by Fleming, 1999). Doubling the half-maximum effect is achieved by a 10-fold increase in concentration of each agonist (Taylor et al., 1988). Thus, a depolarized state should lead to a 10-fold subsensitivity, which is what is observed for twitch inhibition by either drug in tolerant preparations (Taylor et al., 1988). Since the low-efficacy agonist clonidine can only achieve 50% inhibition (Taylor et al., 1988), it cannot double its hyperpolarizing effect of 6 to 8 mV (Meng et al., 1997) to overcome the equal depolarized state in tolerant neurons. Thus, it is predicted that clonidine's twitch inhibition should virtually disappear with tolerance; this is what is observed (Taylor et al., 1988). For the higher efficacy agonists, the maximum hyperpolarizing effect is limited by the potassium equilibrium potential (E_k). Thus, depolarization, which increases the difference from E_k, allows for increases in the maximum opioid-mediated hyperpolarizations. In contrast, with clonidine, the low maximum effect is limited by its receptors and associated signal transduction to ion channels, which do not change (Meng et al., 1997).

The mechanism involves a global change in excitability with no evidence of altered receptor or signal transduction processes of only those neurons upon which the opioid acts (see Fleming, 1999). Since this altered state of excitability is responsible both for subsensitivity to inhibitory substances and supersensitivity to excitatory substances, this mechanism is a clear example of one that can explain both tolerance and physical dependence.

Studies from this laboratory in the LM/MP (Leedham et al., 1989) have provided circumstantial evidence that homologous and heterologous tolerance may occur concomitantly. Analyzing the time course over which tolerance develops and

decays in the LM/MP, the concentration-response curve for morphine is shifted to the right nearly 2 times greater 4 days after pellet implantation as the shift observed for 2-chloro-adenosine. However, by 7 days after implantation, the concentration-response curves are shifted to the right by similar magnitudes, suggesting that both types of tolerance may develop concomitantly in the same animal but with different onset and/or decay rates.

A schematic summary of the major changes that have been documented in cell signaling proteins after chronic exposure to opioids is presented in Fig. 2 and detailed in Table 1. In particular, Fig. 2 illustrates the fact that changes in a variety of signaling proteins and electrical excitability have been identified after chronic treatment with opioid. In the rat LC and nucleus accumbens, chronic administration of opioids increases the abundance of AC and cyclic AMP-dependent protein kinase and decreases the abundance of CREB immunoreactivity, all of which are consistent with subsensitivity to opioid-mediated inhibition of cAMP systems (Nestler et al., 1994; Nestler and Aghajanian, 1997). In addition, increases in the levels of several cyclic AMP-dependent phosphoproteins, G-protein-coupled receptor kinases (βARK1), and gene expression have been observed in the rat LC (see Nestler et al., 1994; Nestler and Aghajanian, 1997). In the rat LC, chronic morphine increases the abundance of the α-subunits of G_i and G_o, while similar treatment decreases the levels of G_i in the rat nucleus accumbens. It is particularly intriguing, however, that the elevated levels of G_i and G_o associated with tolerance and dependence in the rat LC disappear over a short time course similar to the rapid phase associated with behavioral changes and changes in LC firing observed in homologous tolerance (Rasmussen et al., 1990). In the rat periaqueductal gray, chronic treatment with morphine induced a shift in coupling of μ-receptors from a transduction process involving phospholipase A_2 to one that was dependent upon AC and protein kinase A (Ingram et al., 1998). In addition, involvement of cAMP-dependent mechanisms has been identified as a potential common locus to account for long-term changes in GABAergic synaptic transmission in the ventral tegmental area following chronic cocaine or morphine treatment (Bonci and Williams, 1996).

Changes in the AC system similar to those in the LC have been observed in the guinea pig LM/MP following chronic treatment with morphine. Interestingly, modulation of the AC cascade in the LM/MP has been observed as a switch from a G_i-dependent inhibition to a G_s-mediated stimulation of AC, phosphorylation of AC type II (Chakrabarti et al., 1998), and an increase in AC type IV mRNA (Rivera and Gintzler, 1998). Furthermore, while there is a significant increase in G_s (Wang and Gintzler, 1997) as seen in the rat LC, there was no significant change in G_i in these preparations. The meaning of these observations, however, is still unclear since electrophysiological data clearly indicate that modulation of intracellular cAMP levels does not directly impact upon the electrical properties of LM/MP neurons (Johnson and Pillai, 1990) and the altered electrical properties adequately explain the tolerance, as discussed earlier in this review. These observations reinforce the concept that multiple signaling pathways may be differentially involved in the short- and long-term effects of opioids in myenteric neurons. It is important to emphasize that little attempt has been made, thus far, to determine whether multiple pathways are altered in other neuronal populations.

Studies from this laboratory evaluated the sensitivity of guinea pig nucleus tractus solitarius (nTS) and LC neurons 1 week after implantation of morphine or placebo pellets. Neurons of the nTS displayed a significant disinhibitory response to morphine that was mediated by GABA (Malanga et al., 1997) similar to that reported for neurons of the nucleus accumbens (Chieng and Williams, 1998). When the disinhibi-

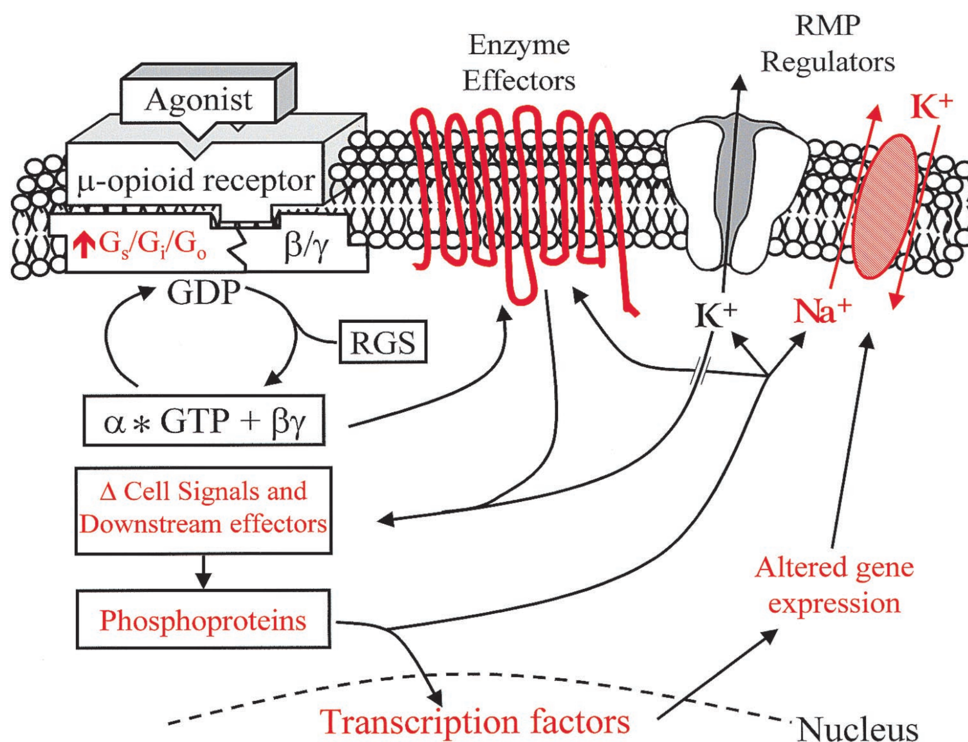

Fig. 2. Schematic illustration of the identified modifications in cell signaling pathways and proteins that have been proposed to occur following chronic exposure to μ-opioid receptor agonist. The cellular substances that have been identified to change following chronic treatment with opioid are highlighted in red. The impact of changes in these cellular proteins on the characteristics of the tolerance that develops accounts for homologous tolerance if related to a specific signaling pathway or heterologous tolerance if the modification leads to generalized changes in cell excitability. The possibility that a change in one cellular locus (e.g., adenylyl cyclase) could actually evoke changes in other cellular effectors (e.g., sodium pump) through modification of gene expression is clearly an area of potentially fruitful research.

tory effect of morphine was eliminated by $GABA_A$ receptor antagonism with bicuculline, neurons of the guinea pig nTS from animals treated chronically with morphine display nonspecific (i.e., heterologous) subsensitivity to muscimol, 2-chloroadenosine, clonidine, and morphine as well as supersensitivity to the excitatory effects of elevations of K^+ in the bathing solution. This suggests that the cellular basis for tolerance involves a general increase in excitability in neurons of the nTS as in those of the myenteric plexus. Similarly, neurons of the guinea pig LC display a reduced responsiveness to muscimol and μ-receptor activation following chronic treatment that is accompanied by a reduced capacity of the sodium pump similar to that of myenteric neurons (see Fleming, 1999).

Conclusions and Future Directions

The lack of a unifying hypothesis defining the cellular basis of tolerance to and/or dependence upon opioids can be largely attributed to the complexity of opioid action involving several different signaling pathways. The basis for this diversity can be found in the number of opioid receptors mediating the effects of the narcotics and the very nature of those receptors (i.e., GPCR superfamily). Multiple forms of tolerance to and dependence on opioids have been characterized in terms of both time to development (acute versus chronic) and specificity characteristics (homologous versus heterologous). These two characteristics and the differences that exist in cells and tissues exhibiting the phenomena suggest that multiple mechanisms must be considered at the very least and, furthermore, that the development of the various forms of tolerance may occur concurrently. The fact that multiple signaling pathways can be activated via a single agonist using a single receptor subtype through the G-protein transducers provides a mechanism to simultaneously involve many components in the process of cellular adaptation. 1) There is a rapid, short-lived homologous tolerance associated with very high agonist levels and clearly due to receptor desensitization/uncoupling. 2) There is a tolerance (possibly homologous) with a moderate half-life associated with alterations in second messenger and transduction proteins. 3) There is a long-lasting heterologous tolerance resulting from a generalized increase in cellular excitability, partial membrane depolarization, and reduction in Na^+, K^+ pump protein. The fact that these three mechanisms can overlap in a given neuronal population is especially clear in myenteric neurons. Whether these adaptations are independent of each other is uncertain. The first mechanism, uncoupling of the receptor from its transduction process, could either trigger or impede adaptations via the other two mechanisms. Mechanism 2 clearly can lead to transcriptional changes that could participate in the long-term regulation of other proteins involved in cell function. It is possible, therefore, that the alteration in AC-regulated proteins (i.e., mechanism 2) may induce the reduction in pump protein in mechanism 3 in some neurons, as schematically illustrated in Fig. 2. It is also possible that different mechanisms play a primary role in different neuronal populations.

The capacity of cells to preferentially activate different signaling pathways depending on the magnitude of receptor occupation provides an avenue to involve multiple pathways in adaptive processes. In addition to coinciding in time, however, the proposed cellular mechanisms that underlie the development of tolerance to opioids and other drugs must also account for both the acute effects of the agonist and the long-term changes in cellular responsiveness that are consistent with the observed specificity of the altered responsiveness. It is highly probable that multiple mechanisms are responsible for the development of tolerance and dependence to opioids and that these divergent signaling pathways are likely to be activated concurrently but to different degrees depending upon the level of receptor activation. Therefore, future efforts must begin to explore the impact of the level of receptor occupation on the signaling pathways that are activated and to develop some order in the processes that are identified.

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