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Minireview

Phosphorylation: A molecular switch in opioid tolerance

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Abstract

Protein phosphorylation is a key posttranslational modification mechanism controlling the conformation and activity of many proteins. Increasing evidence has implicated an essential role of phosphorylation by several major protein kinases in promoting and maintaining opioid tolerance. We review some of the most recent studies on protein kinase C (PKC), cyclic AMP dependent protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase G (PKG), and G protein receptor kinase (GRK). These kinases act as the molecular switches to modulate opioid tolerance. Pharmacological interventions at one or more of the protein kinases and phosphatases may provide valuable strategies to improve opioid analgesia by attenuating tolerance to these drugs.

Keywords: Phosphorylation; Protein kinase; Phosphatase; PKC; CaMKII; PKA; PKG; GRK; CREB; NO; cAMP; cGMP; Calmodulin; Calcium; NMDA

Contents

Introduction

Opioid analgesics are highly efficacious for acute pain; however, chronic treatment with opioids presents variable results. Drug tolerance invariably develops in almost all chronic pain patients who receive opioids. The development of drug tolerance will necessitate higher doses of the same drug or use of a more efficacious opioid drug, which leads to exacerbated adverse effects including respiratory depression, cognitive changes, and increased vulnerability to drug dependence. In some cases, even the highest tolerable dose of an opioid drug cannot achieve the desirable analgesic effect in patients. The mechanism underlying opioid tolerance,

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Table 1
Summary of tolerance models and protein kinase inhibitors used in the cited studies

Publication	Animal	Method to induce tolerance	Kinase inhibitor	Key findings
Aley and Levine, 1997a,b	Rats	Peripheral tolerance to DAMGO	H-7 chelerythrine	No effect
Bernstein and Welch, 1998a,b	Mice	Chronic (1 Mor plt×3 d+additional MS injections)	–	↓ PKA-mediated μOR phosphorylation
Fan et al. (1999)	Rats	Chronic (MS 10 mg/kg q12h×9 d)	KN-62, KN93, antisense to CaMKII	↓ Tolerance
Granados-Soto et al. (2000)	Rats	Chronic (MS 20 nmol/h i.t. infusion×5 d)	Chelerythrine GF109203X	↓ Tolerance ↓ Tolerance
Hua et al. (2002)	Rats	Chronic (MS 40 nmol/h i.t. infusion×5 d)	PKCα antisense	↓ Tolerance
Inoue and Ueda (2000)	Mice	Acute peripheral tolerance(MS 3 nmol, ipl, 4 h)	Calphostin C Go-6976 KT-5720	↓ Tolerance ↓ Tolerance No effect
Li and Roerig (1999)	Mice	Chronic (1 Mor plt×4 d)	–	↑ PKC activity ↓ Cytosolic PKCα, β, γ
Lou et al. (1999)	Rats	Chronic (MS 10 mg/kg q12h×9 d)	–	↓ CaMKII activity and CaMKIIα expression in hippocampus
Mao et al. (1995)	Rats	Chronic (MS 10 μg/d×8 d)	–	↑ PKCγ i.r.
Mayer et al. (1995)	Rats	Chronic (MS i.t. 10 μg/d×7 d)	GM1 ganglioside	↓ Tolerance
Narita et al. (1994a)	Rats	Chronic (MS i.c.v. infusion×3 d)	–	↑ Cytosolic PKC activity
Narita et al. (1994b)	Rats	Chronic (MS i.c.v. infusion×3 d)	H-7	↓ Tolerance
	Mice	Acute (DAMGO 10 ng, i.t.×3 h)	Calphostin C KT-5720	↓ Tolerance No effect
Shen et al. (2000)	Mice	Chronic (MS 1×25 mg/pellet+s.c. infusion, 3 d)	Antisense deoxy-oligonucleotides specific for alpha catalytic subunit of mouse PKA	↓ Tolerance
		Chronic (etorphine s.c. infusuin, 125 μg/kg/d×2 d)		↓ Tolerance
		Chronic (etorphine s.c. Infusuin, 250 μg/kg/d×2 d)		Partially ↓ tolerance
Smith et al. (2002)	Mice	Chronic (1 Mor plt×3 d)	Go-7874 sangivamycin	↓ Tolerance ↓ Tolerance
Smith et al. (2003)	Mice	Chronic ("45-fold morphine tolerance"=1 Mor plt×3 d+additional MS injections)	Gö6850, Go-7874 KT-5720, 4-cyano-3-methylisoquinoline Combination	Partially ↓ tolerance Partially ↓ tolerance Partially ↓ tolerance Fully ↓ tolerance
Tang et al., 2006a,b	Mice	Chronic (1 Mor plt×6 d) Acute (MS 100 mg/kg×4–6 h)	KN-93	↓ Tolerance ↓ Tolerance ↑ CaMKII expression and activity
Wang et al., 1994; Bilsky et al., 1996	Mice	Acute (MS, 100 mg/kg, 4–6 h)	H-7 H-8	↓ Tolerance No effect
Gene-deletion studies			Gene deleted	
Yukhananov and Kissin (2003)	PKCγ−/−mice	Chronic (1 Mor plt×6 d)	PKCγ	No effect
Zeitz et al. (2001)	PKCγ−/−mice	Chronic (1 Mor plt×4 d)	PKCγ	↓ Tolerance

MS: morphine sulfate; Mor plt: morphine pellet containing 75 mg morphine base/pellet, implanted s.c.; ipl: intraplantarly; i.r.: immunoreactivity; i.t.: intrathecally.

especially to analgesia, has been the subject of numerous studies. Phosphorylation of the G protein coupled receptors (GPCRs) by various kinases has been linked to receptor uncoupling and internalization, which affects cellular desensitization and resensitization (Lefkowitz, 1998; Gainetdinov et al., 2004). What was initially proposed as a desensitization mechanism for the adrenergic receptors has been found to also work for many other GPCRs as well (Lefkowitz et al., 1990; Gainetdinov et al., 2004). The initial evidence that receptor phosphorylation could affect cellular and antinociceptive tolerance to opioids came from studies applying non-selective protein kinase inhibitors (Wang et al., 1994). Subsequent studies, employing more selective chemical inhibitors or gene-specific down-regulation or deletion methods, have firmly implicated several major serine/threonine kinases and phosphatases in promoting and/or maintaining opioid tolerance. This short review will summarize some key findings in the past decade or so.

Protein kinase C (PKC)

PKC is a family of serine/threonine protein kinases with 11 isoforms (Nishizuka, 1995). Based on primary structure and activation co-factors, these kinases can be divided into three groups. The conventional PKC (cPKC) group includes PKCα, β_I, β_{II}, and γ, which are activated by calcium and diacylglycerol. In contrast, the novel PKCs (nPKC) including δ, ε, η, θ, and μ isoforms do not appear to require calcium for their activation. The two least characterized isoforms, ζ and ι (λ for the murine ortholog), belong to the class of atypical PKC (aPKC) and are activated by yet to be defined mechanisms. Activation of PKC results in the translocation of the kinase from

the cytosol to the plasma membrane, which has been used as an indicator for the activation of PKC (Nishizuka, 1995). Indeed, membrane-bound PKC, as revealed by the [^{3}H]PDBu autoradiography, is increased in opioid-tolerant animals (Mayer et al., 1995)[1]. Activation of PKC by chronic administration of opioids has been reported by other studies using enzymatic assays (Narita et al., 1994a; Li and Roerig, 1999; Granados-Soto et al., 2000) or by monitoring PKC translocation (Kramer and Simon, 1999b). The essential role of PKC in opioid tolerance can be directly demonstrated by utilizing PKC inhibitors to reverse and/or prevent opioid tolerance in various animal models (Narita et al., 1994b, 1995; Wang et al., 1994; Bilsky et al., 1996; Granados-Soto et al., 2000; Smith et al., 2002). The involvement of PKC in antinociceptive tolerance developed to peripherally administered opioids has also been studied. Morphine (3 nmol intraplantarly) induces acute (within 4 h) tolerance to peripheral analgesia in the bradykinin-nociception test in mice (Inoue and Ueda, 2000). The effect is blocked by the PKC inhibitors calphostin C and Go-6976. In the same setting, peripherally administered [D-Ala2,NMePhe4,Gly(ol)5] enkephalin (DAMGO) does not produce tolerance (Ueda et al., 2001). The lack of DAMGO peripheral tolerance correlates with its high potency in internalizing the mu opioid receptor, whereas morphine does not cause receptor internalization in these cells (Ueda et al., 2001). This differences on the receptor internalization exhibited by different opioids have been previously reported in other cells (e.g., Arden et al., 1995; Keith et al., 1998). Interestingly, significant DAMGO-induced acute tolerance was observed when DAMGO internalization mechanisms were impaired (by the antisense or dominant negative inhibition of dynamin) (Ueda et al., 2001). These data are in agreement with the concept that receptor internalization is a mechanism for receptor resensitization through dephosphorylation (Krueger et al., 1997; Zhang et al., 1998; Grecksch et al., 2006). Arguing against this internalization/resensitization hypothesis, DAMGO has been reported in other studies to induce tolerance when administered centrally (Vanderah et al., 2000) or peripherally (Aley and Levine, 1997a,b). In the studies by Aley and Levine, PKC was found not to be important in DAMGO-induced acute tolerance to its peripheral antinociceptive effect against prostaglandin E2-induced mechanical hyperalgesia in rats peripherally (Aley and Levine, 1997a,b). It should also be noted that morphine was recently found to be capable of inducing receptor internalization in certain cells (Haberstock-Debic et al., 2003, 2005).

The exact PKC isoform(s) promoting opioid tolerance is less clear. PKCγ immunoreactivity is increased in the spinal cord dorsal horn of morphine-tolerant rats (Mao et al., 1995; Granados-Soto et al., 2000; Narita et al., 2001). In mice lacking PKCγ, morphine produces significantly reduced antinociceptive tolerance and neuropathic pain (Malmberg et al., 1997; Narita et al., 2001; Zeitz et al., 2001). Desensitization to DAMGO-stimulated [^{35}S]GTPγS binding after repeated DAMGO administration is also absent in mice lacking PKCγ (Narita et al., 2001). However, PKCγ-deletion mice still produce some degree of antinociceptive tolerance to opioids (Zeitz et al., 2001). In fact, Yukhananov and Kissin reported that wildtype and knockout mice developed tolerance to the same extent (Yukhananov and Kissin, 2003). The discrepancy may be caused by the differences in genetic background of experimental animals (Yukhananov and Kissin, 2003). Yukhananov and Kissin used PKC-deletion mice with 129 PF2/J × C57Bl6 (B6; 129P-Pkcc^{tm1Stl}, Jackson Laboratory) genetic background, whereas the mice in Zeitz et al's study were on 129/OLA × C57BL6 background. In either study, it is not certain if control (wildtype) mice and the gene-deletion mice share the same background, based on the limited information provided by the vendor. In general, the best approach for this type of studies is to use littermates from heterozygous breeders. Additional debate on the role PKCγ in opioid tolerance comes from its distinctive expression patterns. Since PKCγ is not expressed in peripheral tissues, PKCγ is unlikely to be a mechanism for tolerance developed to the peripheral antinociception action of opioid drugs (e.g., Aley and Levine, 1997b). In spinal cord dorsal horn, only a small fraction (~5%) of neurons co-express PKCγ and the μ-opioid receptor (Polgar et al., 1999). It is also of importance to note that PKCγ is not present in primary afferent neurons (Malmberg et al., 1997), indicating that opioid receptors cannot directly signal to PKCγ in these sensory neurons.

PKCα is another isoform that has been found to be up-regulated in opioid tolerance (Granados-Soto et al., 2000) and in cells that have been treated with DAMGO (Kramer and Simon, 1999a). Antisense oligonucleotides targeting spinal PKCα are able to prevent morphine tolerance in rats (Hua et al., 2002). There are also data indicating that PKCβ can be regulated by opioids, although its role is less clear (Ventayol et al., 1997; Li and Roerig, 1999). Up-regulation of PKCα and PKCβ has also been observed in myenteric plexus neurons obtained from the guinea pigs that have been treated with morphine (5 × 75 mg morphine/pellet for 6 d) (Wang et al., 1996a). Tolerance following chronic opioid administration is prevented by infusing PKC inhibitors i.c.v. or spinally at the time opioids are administered (Narita et al., 1994a; Granados-Soto et al., 2000).

In summary, a number of studies from several different laboratories have independently identified a critical role for PKC in opioid tolerance. It is still a puzzle how persistent PKC activation is achieved in opioid tolerance as one would expect desensitization of the kinase after prolonged activation. One plausible mechanism may be through the interaction of PKC with the *N*-methyl-D-aspartate (NMDA) receptors. The NMDA receptors, members of ligand-gated ion channels, respond to the excitatory amino acid glutamate. Glutamate is the major excitatory neurotransmitter and mediates synaptic transmission that is critical for the normal functioning of the nervous system. For example, the NMDA receptors are essential for the generation of long-term potentiation and spatial learning and memory (Nakazawa et al., 2004). Abnormality of the NMDA receptors has been postulated to be associated with a number of

[1] Refer to Table 1 for more detailed information on the method of inducing opioid tolerance and, if applicable, specific protein kinase inhibitors used in each study.

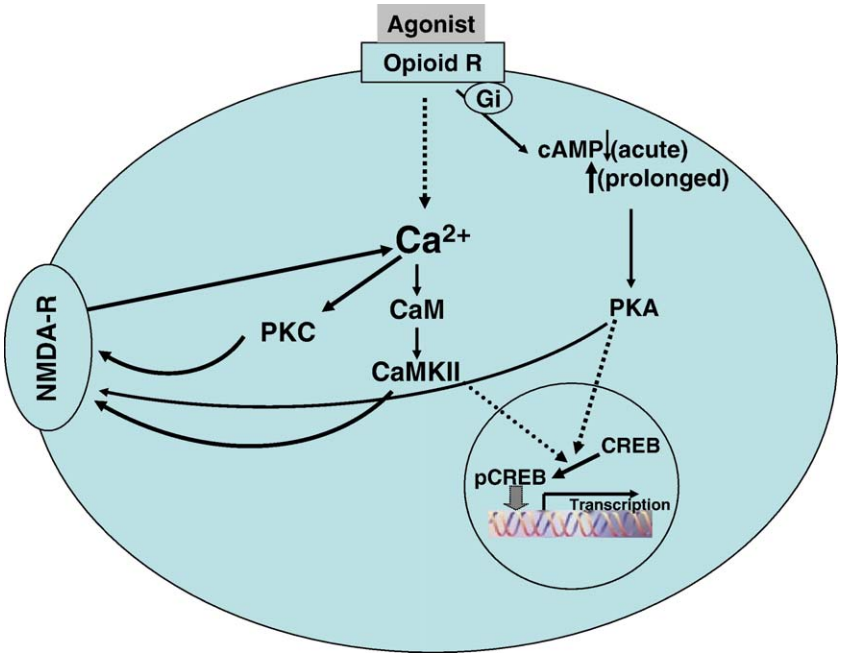

Fig. 1. Proposed actions of protein kinases in opioid tolerance. This is a simplified scheme showing the activation of PKA, PKC, and CaMKII in opioid tolerance. Adenylyl cyclase "supersensitization" and persistent activation of PKA have been well documented in opioid tolerance. Increased intracellular Ca^{2+} levels and activities of PKC and CaMKII have been reported; however, the mechanisms leading to and maintaining the persistent activation of PKC and CaMKII are not entirely clear. One potential mechanism by which PKC and CaMKII can sustain their prolonged activation is by activating the NMDA receptors via phosphorylation. PKC and CaMKII (as well as PKA) can phosphorylate the NMDA receptors (Leonard and Hell, 1997). Activation of the NMDA receptors causes influx of Ca^{2+}, which, in turn, may activate PKC and CaMKII. This interaction between the NMDA receptors and PKC and/or CaMKII in opioid tolerance is only a proposed mechanism, which has not been tested. For downstream signal transduction, these protein kinases phosphorylate numerous effectors including several transcription factors. CREB is shown as an example to illustrate how these kinases can phosphorylate phosphoproteins and regulate cellular events including proliferation, differentiation, and apoptosis. This overly simplified scheme does not suggest that all these mechanisms work at the same time in all cells, but rather intend to generalize a network of intracellular protein kinase signaling pathways that could potentially impact opioid tolerance.

pathophysiologic states, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral Sclerosis (Lipton, 2004). Phosphorylation of the NMDA receptors is a key means to regulate the function of the ion channels, which are the most permeable to Ca^{2+}. Phosphorylation of NMDA receptors by PKC has been shown to enhance NMDA receptor function (Kelso et al., 1992; Xiong et al., 1998). Therefore, activated PKC as a result of opioid activation can potentially increase the activity of the NMDA receptors. The activated NMDA receptors are permeable to Ca^{2+}, which, in turn, can activate more PKC. As a result, a positive feedforward loop between PKC and the NMDA receptors may exist in opioid tolerance (Fig. 1), although such a mechanism has yet to be tested. The NMDA receptor antagonists are highly effective in blocking opioid tolerance (Trujillo and Akil, 1991; Trujillo, 2000).

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)

CaMKII is a multifunctional, Ca^{2+}/calumodulin (CaM) activated kinase, whose α and β isoforms are richly expressed in the central nervous system. Both CaMKIIα and the NMDA receptor are essential for long-term potentiation in hippocampal neurons, and learning and memory (e.g., Mayford et al., 1996). It has been proposed that hippocampal CaMKII may act through learning and memory to affect opioid tolerance. Chronic hippocampal, but not striatal, application of CaMKII inhibitors was able to prevent opioid tolerance. In contrast, acute inhibition of hippocampal CaMKII failed to modulate morphine tolerance, suggesting the importance of learning/memory pathways (Fan et al., 1999; Lou et al., 1999). Recent studies indicate that CaMKII may also affect opioid tolerance independently of learning and memory (Tang et al., 2006b). Intrathecally or i.c.v. applied CaMKII inhibitors are able to acutely reverse already-established opioid tolerance (Wang et al., 2003; Tang et al., 2006a,b). Biochemical evidence supports the possibility of a direct interaction between CaMKII and the opioid receptors. The cloned human mu opioid receptor (μOR) contains consensus sites for phosphorylation by CaMKII (Mestek et al., 1995). Desensitization of the μOR is enhanced when a constitutively active form of CaMKII is over-expressed (Mestek et al., 1995; Koch et al., 1997), and absent when the receptor is replaced with a mutant (μOR S261A/S266A) lacking the consensus CaMKII site (Koch et al., 1997). Moreover, CaMKII and μOR are well co-localized in dorsal root ganglion and spinal neurons (Bruggemann et al., 2000). On the other hand, intracellular Ca^{2+}, CaM, and CaMKII can all be regulated by opioids. Cytosolic free Ca^{2+} is increased after the treatment with opioids (Fields and Sarne, 1997; Smart et al., 1997; Spencer et al., 1997; Quillan et al., 2002). Similarly, chronic treatments with opioids have been found to increase CaM activity (Nehmad et al., 1982) and mRNA levels (Niu et al.,

2000). Indeed, expression and activation of CaMKIIα are both increased in opioid tolerance (Lou et al., 1999; Wang et al., 2003; Liang et al., 2004; Tang et al., 2006b). The increased CaMKII activity is most likely due to increased Ca^{2+} levels and CaM activity upon the activation of opioid receptors. However, there may be alternative mechanisms. For example, it has been shown that μOR contains a CaM binding site. CaM can be released from its association with μOR upon the receptor activation (Wang et al., 1999; Sadee et al., 2005). The increase in free CaM may contribute to the activation of CaMKII.

Similar to PKC, the mechanisms sustaining the persistent activation of CaMKII has not been studied. One could also hypothesize a positive feedback loop between CaMKII and the NMDA receptors, which can account for the persistent activation of CaMKII (Fig. 1). In this pathway, activated CaMKII can phosphorylate and activate the NMDA receptors, leading to the influx of Ca^{2+} through the channels (Fukunaga et al., 1992; Kitamura et al., 1993). Increased cytosolic Ca^{2+} ions bind and change the conformation of CaM. Ca^{2+}-bound, activated CaM makes it possible for the autophosphorylation of CaMKII at position Thr286 and subsequent activation of the kinase (Strack and Colbran, 1998).

Besides the NMDA receptor, CaMKII affects a number of other downstream effectors including several transcription factors such as cAMP response element-binding protein (CREB) (Sheng et al., 1991), activating transcript factor 1 (ATF-1) (Shimomura et al., 1996), serum response factor (Misra et al., 1994), and CAAT-enhancer-binding protein β (C/EBP β) (Wegner et al., 1992). While most of these transcription factors have been reported to be regulated by opioids, phosphorylation and activation of CREB have been extensively studied and found to be important for opioid tolerance and dependence (Blendy and Maldonado, 1998; Nestler, 2001).

Protein kinase A (PKA)

The cAMP-dependent protein kinase or protein kinase A (PKA) is another major protein kinase that can be modulated by opioid drugs. In turn, PKA modulates a number of downstream effectors (Nestler, 2001, 2004). Inhibition of adenylyl cyclase is a key signaling pathway after the acute activation of opioid receptors by agonists. However, prolonged treatment with opioids produces an up-regulation of cAMP levels and increased PKA activity (Sharma et al., 1975; Wang et al., 1994). This phenomenon called "cAMP up-regulation" or "adenylyl cyclase superactivation" has long been considered as a cellular correlate for opioid tolerance (Sharma et al., 1975; Wang et al., 1994) and may involve a number of cellular changes (Avidor-Reiss et al., 1996; Rubenzik et al., 2001; Varga et al., 2003). The effects of the cAMP second messenger and PKA are mediated by a number of downstream effectors including the transcription factor CREB (cAMP response element-binding protein) (Montminy and Bilezikjian, 1987). CREB produces its effects via the regulation of other genes. This is initiated by the phosphorylation of CREB on a single serine residue, Ser133, by PKA. Upon phosphorylation, CREB dimers bind to specific "cAMP response element" (CRE) sites that are found on target genes. Since CRE is part of a transcriptional complex, binding of CREB initiates gene transcription. Opioid-mediated up-regulation of the cAMP pathway and activation of CREB have been demonstrated to occur in the locus coeruleus, an area important for physical dependence (Lane-Ladd et al., 1997). Similar activation of CREB has also been shown in the mesolimbic area including nucleus accumbens, ventral tegmental area, amygdala, and other regions (Nestler, 2004).

Targeting PKA by its inhibitors has produced mixed results on opioid tolerance in vivo. A number of studies fail to identify any effect of PKA inhibition on opioid tolerance (Narita et al., 1995; Bilsky et al., 1996; Inoue and Ueda, 2000) or cellular desensitization (Chakrabarti et al., 1998). PKA-mediated phosphorylation of μOR is found to be decreased after chronic morphine administration (Bernstein and Welch, 1998a; Chakrabarti et al., 1998). Others have reported that PKA inhibitors can partially (Smith et al., 2003) or completely (Bernstein and Welch, 1997) block antinociceptive tolerance to opioids. As early as in the mid-1970s, Way and colleagues reported that a single pretreatment with cAMP (i.v.), which is a PKA activator, was able to significantly enhance opioid tolerance and dependence (Ho et al., 1975). In addition, concurrent intrathecal and i.c.v. injections of antisense deoxyoligonucleotides specific for alpha catalytic subunit of mouse PKA prevent morphine or the low dose of etorphine (125 μg/kg/day for 2 days)-induced opioid tolerance (Shen et al., 2000). These discrepancies may be caused by different experimental models and inhibitory agents used, which is elegantly illustrated in the study by Smith et al. Depending on the level of opioid antinociceptive tolerance, PKC and PKA inhibitors exhibit full or partial effects (Smith et al., 2003). Both PKA and PKC inhibitors are required to reverse the "high level" of morphine tolerance (Smith et al., 2003). Similarly, Shen et al. find that antisense deoxyoligonucleotides targeting PKA only partially prevents the high dose of etorphine (250 μg/kg/day for 2 days)-induced opioid tolerance (Shen et al., 2000) Another study identifies PKA, but not PKC, as the mediator for maintaining cellular tolerance to DAMGO in morphine-treated neurosecretory cells isolated from the ovariectomized female guinea pigs (Wagner et al., 1998).

Cyclic GMP-dependent kinase (PKG)

PKG is a serine/threonine kinase that is widely expressed in the nervous systems and other tissues (Wang and Robinson, 1997). Although cGMP is an important second messenger in the CNS, little is known about its action in opioid tolerance. In one published study, the PKG inhibitor KT-5823 is found to prevent morphine tolerance if given intrathecally, but not intracerebroventricularly (Bernstein and Welch, 1997). More indirect evidence comes from the studies examining the major activation pathway of PKG by nitric oxide (NO), activation of NO-sensitive guanylyl cyclase, and increased intracellular cGMP levels (NO/cGMP/PKG pathway). The pathway is up-regulated at multiple points by opioids (Liang and Clark, 2004). Chronic treatment with opioids increases cGMP levels in the spinal cord

and several brain regions (Minneman and Iversen, 1976; Racagni et al., 1976; Burton et al., 1990; Bhargava and Cao, 1997). Chronic morphine also increases nitric oxide synthase (NOS) mRNA level and NOS-positive cells in the rat spinal cord (Machelska et al., 1997). Inhibitors of NOS have been shown to attenuate the development of antinociceptive tolerance to opioids (Bhargava, 1994; Majeed et al., 1994; Rauhala et al., 1994; Highfield and Grant, 1998). Moreover, neuronal NOS (nNOS) appears to be the isoform important for morphine tolerance (Heinzen et al., 2005; Santamarta et al., 2005). Morphine induces significantly less tolerance in nNOS-deficient mice, when compared with endothelial NOS (eNOS)-deficient mice or wildtype control mice (Heinzen and Pollack, 2004).

G protein receptor kinases (GRK)

The G protein receptor kinase (GRK) is a family of kinases that specifically phosphorylate the activated G protein coupled receptors (Pitcher et al., 1998). Cellular desensitization of the delta opioid receptor involves phosphorylation of the receptor by one or more G protein coupled receptor kinases (Pei et al., 1995). In μOR-transfected HEK293 cells, morphine activates, but does not internalize the receptor. Interestingly, over-expressing GRK2 in μOR-transfected HEK293 cells renders μOR capable of being internalized by morphine (Zhang et al., 1998). At the same time, morphine-mediated inhibition of adenylyl cyclase activity was attenuated in HEK293 cells transfected with μOR and GRK2. Indeed, opioid treatment leads to the up-regulation of various GRKs and β-arrestin 2 in vivo (Chakrabarti et al., 2001; Hurle, 2001). Cellular studies have shown that GRK phosphorylation of the receptor and subsequent β-arrestin binding are essential steps in the desensitization of many GPCRs (Lefkowitz, 1998). GRK phosphorylation increases the affinity of GPCRs to β-arrestin which is required in forming clathrin-coated pits and vesicles during receptor internalization. Perhaps due to a lack of suitable chemical inhibitors, to our knowledge, the effect of GRK inhibition on opioid tolerance has not been reported. It has been shown that β-arrestin 2-deletion mice do not develop tolerance to morphine (Bohn et al., 2000).

Protein phosphatases (PP)

If phosphorylation by protein kinases is a key in initiating opioid tolerance, the "off-switch" is provided by actions of protein phosphatases. However, this role of protein phosphatases has not been fully studied in opioid tolerance. In fact, the opposite effect has been reported thus far. One study finds that okadaic acid, an inhibitor of protein phosphatase PP1 and PP2A, enhances the morphine-antinociceptive effect in opioid tolerant mice, suggesting that phosphatase types 1 and/or 2A may actually contribute to the development of morphine tolerance. Inhibition of calcineurin (pp2B) does not produce the same effect in the study (Bernstein and Welch, 1998b). Since different protein kinases and phosphatases may have different substrate specificities, these data do not necessarily contradict with those from protein kinase studies. Additional evidence comes from the studies with immunosuppressants such as FK506 (tacrolimus) and cyclosporine A. These immunophilin ligands inhibit calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase (Mehr et al., 2003). Both cyclosporine A and FK506 have been reported to block the development and expression of opioid tolerance (Homayoun et al., 2003; Mehr et al., 2003). However, these agents also reduce NO production by inhibiting dephosphorylation of nNOS. Therefore, a major part of their effects may be due to the inhibition of NO/cGMP/PKG or other pathways. A more direct approach by activating phosphatases has not been experimentally tested so far.

Phosphorylation targets

Given increased activity of so many protein kinases by opioids, one probing question is, "what proteins are the phosphorylation targets by these kinases?" Obviously, a large number of receptors, ion channels, intracellular signaling molecules and transcription factors (e.g., CREB, ATF-1, C/EBPβ, NFκB) can serve as substrates (targets) for these protein

Table 2
Relative selectivity of protein kinase inhibitors at several major protein kinases

Kinase inhibitor	CaMKII	MLCK	PKA	PKC	PKG	Key references
Bisindolylmaleimide I (Gö6850 or GF109203X)	–	–	2.0	0.01	–	(Toullec et al., 1991; Davis et al., 1992a)
Calphostin C	–	>5.0	>50	0.05	>25	(Kobayashi et al., 1989; Tamaoki et al., 1990)
Chelerythrine	>100		170	0.66		(Herbert et al., 1990; Herbert et al., 1993)
4-cyano-3-methylisoquinoline		Weak	0.03	Weak	–	(Lu et al. (1996))
Go-6976	–	5.8	>100	0.008	6.2	(Martiny-Baron et al. (1993))
Go-7874	–	0.12	0.51	0.004	4.8	(Davis et al., 1989; Davis et al., 1992b)
H-7	–	97	3.0	6.0	5.8	(Hidaka et al., 1984; Kawamoto and Hidaka, 1984; Schachtele et al., 1988)
H-8	–	68	1.2	15	0.87	(Hidaka et al. (1984))
KN-62	0.9	>100	>100	>100	–	(Hidaka et al., 1990; Tokumitsu et al., 1990)
KN-93	0.37	–	–	–	–	(Sumi et al. (1991))
KT-5720	–	>2	0.056	>2	>2	(Kase et al. (1987))
KT-5823	–	0.018	1.2	0.723	0.158	(Kase et al. (1987))
Sangivamycin	–	–	–	10	–	(Loomis and Bell (1988))

Reported IC50 or Ki values (in μM) are listed. "–": not done. Full chemical names of these inhibitors are listed under Abbreviations.

kinases. Several recent publications have reviewed some of these substrates (e.g., Wang and Robinson, 1997; Dempsey et al., 2000; Nestler, 2004; Olive and Messing, 2004; Svenningsson et al., 2004; Yamauchi, 2005). These protein kinases can also phosphorylate themselves or each other to regulate the kinase activity. At the receptor level, the NMDA receptor is a potential phosphorylation target for PKC, CaMKII, PKA, and other protein kinases. The opioid receptor may be another substrate. μOR, the primary receptor mediating opioid analgesia (Sora et al., 1997), is phosphorylated in an agonist- (Wang et al., 1996b; Zhang et al., 1996) and calcium-dependent manner (Wang et al., 1996b). Cellular studies using the site-directed mutagenesis method clearly indicate that μOR phosphorylation at several sites is enhanced by opioids and is required for cellular desensitization (Pak et al., 1997; Burd et al., 1998; Wolf et al., 1999; Deng et al., 2000; Koch et al., 2000; Celver et al., 2001; El Kouhen et al., 2001; Wang et al., 2002). The μOR receptor phosphorylation has even been proposed to predict the state of receptor activity (Wang et al., 1994; Chavkin et al., 2001; Liu et al., 2001; Freye and Levy, 2005; Heinzen et al., 2005). Similarly, phosphorylation of the delta and kappa opioid receptors is increased after the treatment with opioid agonists (Trapaidze et al., 2000; Xiang et al., 2001; Heinzen et al., 2005; Navratilova et al., 2005).

Concluding remarks

Increasing evidence has implicated the importance of protein phosphorylation in opioid tolerance. However, we should keep in mind that many chemical protein kinase inhibitors used in these studies can inhibit more than one kinase or other pathways. (Table 2). In addition, most protein kinases exist in multiple isoforms; further studies are needed to identify exact isoforms that are critical in opioid tolerance. More efforts are needed to identify the exact cellular targets (at receptors/ion channel levels or intracellular effectors) that these protein kinase act on in opioid tolerance. It is possible that different kinases/mechanisms are involved in various tolerance models (e.g., acute vs chronic tolerance, cellular vs rodent models). Different opioid agonists may have differential effects on protein kinases in leading to tolerance. For example, different opioids have been shown to possess different efficacy in perpetuating receptor-mediated internalization (e.g., Arden et al., 1995; Keith et al., 1998; Haberstock-Debic et al., 2003; Haberstock-Debic et al., 2005), a process that is intimately linked to the action of one or more kinases. Therefore, results from such studies will need to be interpreted in the context of the specific model system and opioid drug. Some of the best cellular mechanisms are obtained from CHO or HEK cells transfected with cloned opioid receptors. Caution should be exercised when extrapolating the findings beyond the cellular system, as these cell lines do not always develop cellular tolerance to opioids (Piros et al., 1996; Wang and Sadee, 2000).

Once a kinase isoform is identified, the hurdle of achieving isoform-specific inhibition still exists. Presently, peptide-, antibody-, and gene-based inhibitors offer the most selective inhibition of a kinase. However, other than antisense deoxyoligonucleotides, few studies have applied these more selective tools. Further work is also needed to deliver peptide-, antibody-, and gene-based inhibitors in vivo, so that one day we may translate these findings into clinically useful treatments for opioid tolerance (Lu et al., 2006). Mouse gene-deletion ("knockout") studies have provided much needed selectivity in studying protein kinases, although such a method has its own drawbacks. Interpretation of results can be compromised by unmatched genetic backgrounds in mice with different genotypes or adaptive changes associated with gene manipulation (Banbury Conference on Genetic Background in Mice, 1997; Simpson et al., 1997). In particular, many knockout models are produced on 129 mouse strain; however, antinociceptive tolerance to opioids cannot be established in 129SvEv mice (Kolesnikov et al., 1998). Even with these obstacles, the reversible nature of phosphorylation makes it a very attractive therapeutic target. Therefore, studies in this direction will not only shed light onto the mechanisms underlying opioid tolerance, but may also lead to useful therapeutic agents that can sustain opioid analgesia by attenuating opioid tolerance.

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