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Protein Kinases and Pain

Mani Indiana Funez, Fabiane Hiratsuka Veiga de Souza,
José Eduardo Pandossio and Paulo Gustavo Barboni Dantas Nascimento
School of Ceilandia, Brasilia University, Brazil

1. Introduction

There is abundant evidence that protein kinases are involved in the physiopathology of acute and chronic pain. In the first section, we discuss the role of protein kinases in pain and the signalling pathways involved in both the acute and chronic states. The second section will present evidence supporting the contribution of protein kinase inhibition to pain control by different classes of drugs. Both well-known drugs and new molecules can control pain in the peripheral and central nervous systems. The third section highlights the progress in pharmaceutical development and protein kinase research for new pain control drugs in the first decade of the 21st century.

2. Role of protein kinases in acute and chronic pain

In this section, we will discuss the differential activation of protein kinases by pain mediators and the modulation of the acute and chronic pain processes by several kinases.

2.1 PKC

Protein Kinase C (PKC) is a family of phospholipid-dependent serine/threonine phosphotransferases; it can be divided into the following groups of isoforms: a) conventional or classical (α, βI, βII, γ), b) novel (δ, ε, η, θ), and c) atypical (ζ, λ (mouse)/ι (human)) isoforms (Nishizuka, 1992). Five subspecies of PKC, PKC-βI, PKC-βII, PKC-δ, PKC-ε, and PKC-ζ, are expressed in the dorsal root ganglion (DRG) of rats (Cesare et al. 1999). The PKC isoforms that are expressed in the DRG of mice include PKC-α, PKC-βI, PKC-βII, PKC-δ, PKC-ε, PKC-η, PKC-θ, PKC-ζ, and PKC-λ (Khasar et al., 1999a). Thus, there are some differences in the expression of DRG PKC isoforms between species.

Signal transduction through the PKC pathway has been strongly linked to pain. Inflammatory stimuli and mediators can activate PKC to induce pain. Nociceptive response caused by formalin injection into the mouse paw is characterised by two phases; the neurogenic response, which is due to direct nociceptor activation, and the inflammatory response, which is caused by inflammatory mediators (Hunskaar and Hole, 1987). In this model, PKC blockade by local treatment with chelerythrine inhibited the second phase of nociceptive response (Souza et al., 2002), which is driven largely by tissue inflammation, indicating a relationship between PKC activation and the inflammatory process. In the same

way, mechanical sensitisation induced by the inflammatory mediator bradykinin in rats is inhibited by a PKC inhibitor (Souza et al., 2002). *In vitro* experiments conducted in DRG neurons strongly suggest that bradykinin-induced heat sensitisation is dependent on PKC activation because it can be reversed by pharmacological inhibition with staurosporine or phosphatase inhibitors (Burguess et al., 1989; Cesare and McNaughton, 1996). *In vitro* experiments have shown noticeable PKC-activity in rat DRGs after 3 hours of prostaglandin E_2 (PGE_2) paw administration. This activity was accompanied by paw sensitisation to mechanical stimuli, as measured by behavioural experiments (Sachs et al., 2009).

Carrageenan injection in rat or mouse paws is another tool used to study inflammatory sensitisation. In the same way, pharmacological inhibition of PKCε reduces carrageenan-induced mechanical sensitisation in mice (Khasar et al., 1999a). Phosphorylation of PKCε in DRG neurons is increased after carrageenan-induced acute sensitisation (Zhou et al. 2003), and a PKCε agonist sensitises nociceptors to mechanical stimuli (Aley and Levine, 2003). Inflammatory sensitisation has a "sympathetic" component that involves the release of amines such as epinephrine and dopamine (Coderre et al. 1984; Nakamura and Ferreira 1987). Evidence suggests that mechanical sensitisation induced either by epinephrine in rats (Khasar et al. 1999b) or dopamine in mice (Villarreal et al., 2009b) is blocked by PKCε-selective inhibition. Cesare et al. (1999) found that bradykinin exposure induces PKCε translocation from the cytosol to a membrane-associated position in cultured DRG neurons, thus contributing to heat sensitisation.

The mechanical sensitisation induced by PGE_2 involves the peripheral activation of PKCε in rats and mice, as shown by specific pharmacological inhibition (Sachs et al., 2009; Villareal et al., 2009b). In PKCε-mutant mice, the nociceptive threshold is preserved, whereas the nociceptive response was significantly impaired, as evaluated in a model of visceral pain using peritoneal administration of acetic acid (Khasar et al. 1999a). Kassuya et al. (2007), found a noticeable increase in membrane-bound PKCα expression of mouse paw tissue after PGE_2 administration (Kassuya et al., 2007). Thus, the PGE_2–induced pain-related effects during inflammation may be mediated by PKCε and PKCα.

Multiple voltage-gated sodium channel (VGSC) isoforms are expressed in DRG neurons. For example, isoforms Na_v 1.8 and Na_v 1.9 are responsible for tetrodotoxin-resistant (TTX-resistant) currents due to Na^+ channel blocker insensitivity. These sodium currents can be modulated by PKC phosphorylation, which is induced by inflammatory mediators (Gold et al., 1998; Khasar et al., 1999a). Using whole-cell voltage-clamp recordings from DRG neurons, Gold et al. (1998) found that PKC inhibitors decreased the density of tetrodotoxin-resistant sodium current, whereas the PKC activator PMA produces changes that are opposite, suggesting that PKC modulates it. In addition, it was show a relationship between inflammatory mediators-induced changes in TTX-resistant sodium currents and PKC activity (Gold et al., 1998; Khasar et al., 1999a).

PKC peripheral activation contributes to central pain processing. During serotonin-induced rat paw sensitisation, another pain-sensitising mediator associated with inflammation, the response of animals to thermal stimulation and c-fos activation in the dorsal horn is attenuated by intraplantar application of the PKC inhibitor chelerythrine (Chen et al., 2006). During inflammation or in naïve animals, activation of glutamate receptors mGluRs in the spinal dorsal horn modulates acute nociception. These receptors are coupled to Gq/II protein phospholipase C (PLC)-phosphoinositide (PI) hydrolysis and PKC pre- and post-

synaptic activation (Neugebauer, 2002; Giles et al., 2007), suggesting that PKC modulates the synaptic transmission at the spinal level.

PKC activation is associated with chronic pain conditions. Mao et al. (1992) found an increase in membrane-bound PKC in the spinal cord of rats in a model of post-injury neuropathic pain. The role of PKC was confirmed using an intracellular inhibitor of PKC translocation/activation and analysing membrane-bound PKC translocation and pain behaviour. The data suggest a role for PKC in neuropathic pain states. Ahlgren and Levine (1994) found a reduction in streptozotocin-induced diabetic rat pain sensitisation after treatment with PKC inhibitors.

Using the partial sciatic nerve section model, Malmberg et al. (1997) verified that mice lacking PKCγ completely fail to develop neuropathic-associated sensitisation even though they respond normally to acute pain stimuli. In addition, PKCγ expression is restricted to a subset of dorsal horn neurons. Malmberg and co-workers suggest that targeting PKCγ is a promising tool for treating chronic pain. This isoform inhibition also attenuates opioid tolerance in the spinal cord (section 3).

The physiopathology of alcoholic neuropathy in rats seems to depend on PKCε activation and up-regulation in DRG neurons, as shown by selective pharmacological inhibition and western blot analysis performed after 70 days of ethanol administration (Dina et al., 2000). In addition, the role of PKCε in pain sensitisation is associated with neuropathy induced by the antineoplastic agent paclitaxel in rats (Dina et al., 2001).

The role of PKCε is well demonstrated during chronic inflammatory pain conditions. Aley et al. (2000) developed a model to study chronic inflammatory sensitisation that can be induced by a single episode of acute inflammation; after the induction, in a time-lapse of 5 days there is inflammatory-mediator prolonged-response. During this state, PKCε seems to be responsible for the maintenance of this "primed state" and the prolonged response to inflammatory mediators (Aley et al. 2000). Accordingly, the phosphorylation of PKCε in DRG neurons correlated with pain-associated prolonged inflammation after 3 days of the administration of Complete Freund's Adjuvant (CFA) to rat paws (Zhou et al., 2003).

Mechanical persistent inflammatory sensitisation can also be induced by intraplantar administration of inflammatory mediators like prostaglandins and sympathetic amines in rats and mice (Ferreira et al., 1990; Villarreal et al., 2009b). Studies suggest that PKC activity in the DRG is up-regulated by and is at least partially responsible for the persistent condition, as shown by analyses of PKC activity in rat DRGs (Villarreal et al. 2009a). Moreover, the local administration of a selective PKCε inhibitor abolished the persistent state induced by PGE_2 in rats and mice (Villarreal et al. 2009a; Villarreal et al., 2009b). Evaluation of the mechanisms downstream of PKCε activation found that $Na_v1.8$ mRNA levels in the DRG from rats was up-regulated and inhibition of PKCε activity reduced these levels (Villarreal et al., 2009a).

2.2 PKA

Cyclic adenosine-monophosphate (cAMP)-dependent protein kinase (PKA) is a serine/threonine phosphotransferase; in its inactive form, it is a tetrameric holoenzyme composed of two regulatory and two catalytic subunits (Taylor et al., 1990). When the

second messenger cAMP is generated the PKA-regulatory subunits bind cAMP, and the holoenzyme separates into the regulatory subunits and the catalytic subunits (Taylor et al., 1990). The catalytic subunits can phosphorylate their biological targets and regulate many cellular functions. There are different regulatory (RIα, RIβ, RIIα, RIIβ) and catalytic (Cα, Cβ) subunits; α subunits are expressed in non-neuronal and neuronal tissue, whereas β subunits are expressed predominantly in neuronal cells (Cadd and McKnight, 1989).

cAMP/PKA signalling is involved in nociceptor sensitisation by inflammatory mediators. Ferreira and Nakamura (1979) provided evidence that sensitisation of rat hind-paws by prostaglandins is dependent on cAMP generation. Since this original study, many subsequent studies have shown that cAMP generation is induced by a plethora of inflammatory stimuli. The mechanical nociceptor sensitisation that occurs during inflammation or induced by either inflammatory mediators (PGE_2, dopamine, serotonin) is blocked by treatment with PKA inhibitors in rats and mice (Taiwo and Levine 1991; Taiwo et al., 1992; Aley and Levine, 1999; Aley et al., 2000; Sachs et al., 2009; Villarreal et al., 2009b).

Adenylyl cyclase (AC)/cAMP/PKA activation may be necessary to induce and maintain mechanical nociceptor sensitisation (Aley and Levine, 1999). Moreover, PGE_2–induced inflammatory sensitisation increased PKA activity in mouse paws (Kassuya et al., 2007); and in rat DRGs (Sachs et al., 2009), which correlates with the behavioural data. Accordingly, the intraplantar administration of the catalytic subunit of PKA (PKACS) induces mechanical nociceptor sensitisation (Aley and Levine, 1999; Aley and Levine, 2003). Supporting the animal model data, *in vitro* studies using sensory neurons that were cultured and bathed in classic inflammatory mediators showed that prostaglandins can sensitise these cells to bradykinin and that this effect is dependent on PKA activation (Cui and Nicol, 1995; Smith et al., 2000). The role of PKA in formalin-induced nociceptive pain and inflammatory sensitisation was demonstrated in experiments in mice with a null mutation in the type I regulatory subunit (RIβ) of PKA. This mutation dampens the response during nociceptive pain and thermal stimulation (Malmberg et al., 1997).

Once activated, the PKA substrate in the nociceptive pathways can be voltage-gated sodium channels. In fact, *in vitro* studies have shown that TTX-resistant sodium current is modulated via PKA activation during inflammation (England et al., 1996; Gold et al., 1998). Additionally, during inflammation, PKA enhances the gating of transient receptor potential vanilloid channel-1 (TRPV-1) via direct phosphorylation (Lopshire and Nicol, 1998; Rathee et al., 2002). Therefore, PKA can directly phosphorylate ion channels, thus increasing the excitability of sensory neurons and contributing to some pain conditions. Studies using a model of persistent inflammatory sensitisation in rats and mice show that PKA could exert a role in the maintenance of the chronic state. The persistent sensitisation is abolished by injection of PKA inhibitors, and PKA expression and activity were up-regulated in DRG (Villarreal et al., 2009a, 2009b). The contribution of PKA to sensitisation maintenance seems to be due to the regulation of the $Na_v1.8$ sodium channel expression (Villarreal et al., 2009a).

In the neuropathic pain model of sciatic nerve ligature, PKARIβ-null animals present nociceptive responses that are similar to control animals (Malmberg et al., 1997). However, in a model of paclitaxel-induced pain neuropathy, pharmacological inhibition of PKA attenuates the response to thermal stimulation (Dina et al., 2001). Other subunits of PKA, different from PKARIβ, may be activated during neuropathy because PKA inhibitors do not present selectivity.

2.3 MAPKs

Mitogen-Activated Protein Kinases (MAPKs) are protein-serine/threonine kinases. There are many subfamily isoforms known, and are currently 14 mammalian members. They are important for pain regulation and control and are divided in extracellular-signal-regulated kinases (ERKs, 7 isoforms), stress-activated protein kinases or c-Jun N-terminal kinases (JNKs, 3 isoforms) and p38 mitogen-activated protein kinases (p38 MAPK, 4 isoforms). These enzymes are activated by direct phosphorylation of two sites in the kinase activation loop, at a tyrosine and a threonine residue; separated by a single, variable residue (Pearson et al. 2001).

Classically, upon receptor-dependent tyrosine kinase activation on the cellular surface, a cascade of biochemical reactions culminates in small GTPase (Ras) activation. This molecular event initiates a series of catalytic phosphorylation-based signalling, involving kinases such as the proto-oncogene serine/threonine-protein kinase (C-Raf), mitogen-activated protein kinase kinase 1 (MEK1), MEK2 and MAPKs. The dual phosphorylation of these proteins leads to conformational changes, allowing their respective catalytic domains to be accessible to their substrates, which are mainly transcription factors that regulate diverse genes, and others proteins that are regulated by phosphorylation. In addition, the MAPKs interact with inactivating phosphatases, which finely tunes their cellular activity. The same hierarchical cascade exists for JNK and p38 MAPK activation, consisting of three consecutive steps of phosphorylation and activation of different kinases (MAPKKK → MAPKK → MAPK).

Extracellular mitogens such as growth factors (cytokines and hormones) and phorbol esters (e.g., 12-O-tetradecanoylphorbol-13-acetate, TPA) activate ERK1 and ERK2, which regulates cell proliferation and promotes effects such as induction or inhibition of differentiation, stimulation of secretory responses in a variety of cell types such as neutrophils, modulating membrane activity, and generating active oxygen species (Blumberg, 1988). The stress-activated protein kinases, or JNKs, and p38 MAPK signalling pathways are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, osmotic shock and cellular redox state, and are involved in cell differentiation and apoptosis. There are 10 isoforms of the three JNKs due to alternative splicing of JNK-1, JNK-2, and JNK-3, and there are four p38 MAPK isoforms.

These MAPKs are involved in processing cellular pain. Dai et al. (2002) demonstrated that ERK is activated in DRG neurons by electrical, thermal and chemical stimuli using electrophysiological recordings and western blot analysis. The peripheral stimulation of ERK1/2 and p38 MAPKs is involved in the nociceptor sensitisation produced by epinephrine, nerve grow factor (NGF) and capsaicin (Aley & Levine 2003, Zhu & Oxford, 2007). Activation of nuclear factor-kappaB (NF-κB), a transcription factor linked to inflammation, and p38 MAPK leads to the formation of various pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Doyle et al. 2011). TNF-α may induce acute peripheral mechanical sensitisation by acting directly on its receptor TNFR1, which is localised in primary afferent neurons, resulting in the p38-dependent modulation of TTX-resistant Na^+ channel currents (Jin & Gereau 2006; Zhang et al. 2011).

In neurons, synaptic activity-induced increases in the intracellular Ca^{2+} concentration activate MAPKs. Ca^{2+}/calmodulin-activated protein kinase (CaMKII) is essential for

synaptic plasticity because it regulates transcriptional and translational modifications in gene expression and regulation. MAPKs are downstream effectors of multiple kinases, including CaMKII. Membrane depolarisation and calcium influx activate MAPK/ERK kinases. ERK and p38 MAPKs are up-regulated both in primary afferent nerves and the spinal cord in response to noxious stimulation, nerve injury and tissue injury. Inhibition of ERK or p38 MAPK phosphorylation or activity induces an antinociceptive effect in many of the animal pain models described throughout this section. Thus, in addition to the PKA and PKC signalling pathways, some cross-talk may exist with MAPK cascades upon inflammation or injury.

As an example, IL-6 exerts an important role in the development and maintenance of muscular sensitisation to nociception. The IL-6-mediated muscular pain response involves resident cell activation, polymorphonuclear cell infiltration, cytokine production, prostanoids and sympathomimetic amines release (Manjavachi et al. 2010). This response to IL-6 triggers the activation of intracellular pathways, especially MAPKs. Upon IL-6 stimulation, ERK, p38 MAPK and JNK phosphorylation is measurable by flux cytometry, and selective inhibitors of ERK and p38 MAPK partially reduced mechanical nociceptive behaviour (Manjavachi et al. 2010). Inflamed tissues release NGF that act upon nociceptors, activating the p38 MAPK cascade and leading to an increase of TRPV-1 translation and transport to nerve terminals, which contributes to the maintenance of nociceptive behaviour in animal models (Ji et al. 2002). Additionally, two separate p38 MAPK pharmacological inhibitors were effective at inhibiting the development of burn-induced sensitisation when administered as intrathecal pre-treatments (Sorkin et al. 2009).

A screen of MAPK activation in the dorsal horn in both phases of the formalin test demonstrated that p38 MAPK is activated in spinal microglia. Thus, a reduction in the level of spinal p38β, but not p38α, prevented the development of sensitisation following peripheral inflammation (Li et al., 2010). Any study of MAPK signalling must also consider the effect of nervous system cells other than neurons in the pain process.

The same kind of consideration is needed for chronic pain. Synaptic and nerve plasticity is a key element in pain chronification. Changes in structure and function as a result of input from the environment, lesions and pathologies may lead to neuropathic pain. These changes depend upon transcriptional and translational modifications in cell function that are mediated by MAPK signalling. Thus, MAPK modulation became a natural choice for research and the development of new drugs and pharmacological tools.

Pfizer Global Research and Development published a research paper in 2003 showing that the development of neuropathic pain is associated with an increase in the activity of the MAPK/ERK-kinase cascade within the spinal cord. They explored the chronic constriction injury model and the streptozocin-induced diabetic model to mimic neuropathic pain states. Global changes in gene expression and the effect of MAPK/ERK-kinase (MEK) inhibitor were analysed (Ciruela et al., 2003). These efforts lead to the selection of these kinases as targets of drug design for pain, with a focus on neuropathic pain.

The MAPK intracellular signalling cascades are also associated with synaptic long-term potentiation and memory and are associated with nociceptive behaviour in spinal cord injury (Crown et al., 2006). ERK 1/2 and p38 MAPK phosphorylation levels are up-

regulated in rat-spinal cords during mechanical sensitisation after spinal cord injury. Neurons are not the only cells involved in this process; microglial but not astrocytic p38α contributes to the maintenance of neuronal hyperexcitability in caudal areas after spinal cord injury (Gwak et al. 2009).

The IL-6/p38 MAPK/CX3C Receptor 1 signalling cascade is involved in neural–glial communication and plays an important role in triggering spinal glial activation and facilitating pain processing following peripheral nerve injury. Up-regulation of CX3CR1 expression by IL-6-p38 MAPK signalling enhances the responsiveness of microglia to chemokine CXCL1, or fractalkine, after nerve injury (Lee et al., 2010). TNF-α is important during the development of neuropathic pain by spinal nerve ligature (SNL) (Schäfers et al., 2003). The inhibition of spinal p38 MAPK activation prevents this event. However, the activation of ERK but not p38 MAPK is critically involved in the TNFα-induced increase in TRPV1 expression in cultured DRG neurons (Hensellek et al., 2007).

Injury to peripheral nerves may result in the formation of neuromas. Elevated levels of phosphorylated ERK1/2 can be identified in individual neuroma axons that also possess the voltage-gated sodium channel $Na_v1.7$. Painful human neuromas show accumulation of this sodium channel, and its function is modulated by ERK1/2 phosphorylation (Persson et al., 2011).

MAPK expression analysed in the spinal cord after SNL showed differential activation in injured and uninjured DRG neurons. Uninjured neurons had only p38 MAPK detectable induction. In contrast ERK, p38 MAPK and JNK were activated in several populations of injured DRG neurons (Obata et al., 2004). Differential activation of MAPK in lesioned and sound primary nerve afferents may be linked to the pathogenesis of neuropathic pain after partial nerve injury (Svensson et al., 2003).

2.4 Interplay between pathways

The specificity of activation for each signalling pathway may be determined by the stimuli (Juntilla et al., 2008), and the crosstalk between them could be induced during pathological states (Noselli, 2000). Pimienta and Pascual (2007) described MAPK intracellular signalling as "different signalling cascades crosstalk with each other in a way that their functional compensation makes possible the simultaneous integration of multiple inputs".

Considering only one inflammatory mediator, PGE_2, in three models performed in the same species (mice) with analyses of not the same tissue, differences between the signalling pathways involved can be detected:

a. Acute nociception induced by high-dose PGE_2 administration is dependent on ERK signalling mechanisms because its overexpression was detected in hind paw by western immunoblotting analyses (Kassuya et al., 2007). This effect was reversed by EP receptor antagonists (Kassuya et al., 2007).
b. In the same way, PGE_2 is a final mediator of nociceptor sensitisation that acts on the peripheral nerve endings through the prostanoid receptors, leading to sensitisation of sensory nerves. PGE_2-induced acute mechanical sensitisation, which is also associated with kinase activation, was completely prevented by PKA and PKCε, but not by ERK, pharmacological inhibition (Villarreal et al. 2009b). The persistent pain state induced by

chronic PGE_2 administration is completely abolished by PKA or PKCε inhibitors, but not by ERK inhibitors (Villarreal et al. 2009b).

Thus, we conclude that PKA, PKC and ERK are involved in the effects of PGE_2 (including nociceptor sensitisation and nociception). The inflammatory processes include several others mediators in addition to PGE_2. In the same work, Villarreal et al. (2009b) showed that dopamine-induced acute sensitisation involves PKA, PKC and ERK activation, whereas the dopamine-induced persistent sensitisation state is abolished by ERK inhibition and temporarily inhibited by PKA or PKCε inhibitors, suggesting that ERK plays the major role. So, what is the real meaning of these results?

The study of MAPK and other kinases must keep its momentum. The interplay between different signalling pathways is challenging to understand. The available experimental models allow individual probing of each mediator and the kinase transduction of its signalling. Biological systems and pathological states have multiple variables in a complex regulated environment that hinder our understanding of each molecule and their combined role. Nevertheless, the continuous efforts have already achieved interesting findings. In the next sections, additional mechanisms and protein kinases will be described in discussing the pharmacological mechanisms of different drug classes in pain control.

3. Role of protein kinases in pain control

Both acute and chronic pain are usually controlled by administration of pharmacological agents (analgesics and adjuvants) that attempt to tackle pain in both the central and peripheral divisions of the nociceptive pathway. Although a classical mechanism of action is well described for most drugs, additional mechanisms link their analgesic effects with some kinases-dependent pathways, mainly pathways related to PKA, PKC, MAPKs and cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG). In this section, the involvement of protein kinases in the mechanisms of action of drugs used for pain control, such as opioids, dipyrone, general and local anaesthetics and antidepressants will be analysed.

3.1 Opioids

Among opioids, morphine is widely used as a classical opioid analgesic for the clinical management of acute and chronic pain. Despite its wide use, tolerance to the analgesic actions of morphine is an important side effect of prolonged exposure. Individuals who are tolerant to the effects of morphine require larger doses to elicit the same amount of analgesia. Thus, antinociceptive tolerance and the high doses required to achieve effects have limited the use of morphine.

Many factors have been related to morphine tolerance, such as a change of the descending pain modulatory pathway, receptor desensitisation, down-regulation of opioid functional receptors, release of excitatory neurotransmission and other adaptive changes in cell signalling pathways. Interestingly, PKC, especially PKCγ, plays a major role in the changes associated with morphine tolerance. Song et al. (2010) demonstrated that an isoform-specific inhibitor could successfully down-regulate PKCγ in the spinal cord and reverse the development of morphine tolerance in rats. This result not only implicates this PKC isoform in the opioid tolerance mechanism but also has potential applications in pain management.

Beyond the involvement of PKC in opioid tolerance, PKC is involved in inflammatory and neuropathic pain. The capacity of opioids to alleviate inflammatory pain is negatively regulated by the glutamate-binding N-methyl-D-aspartate receptor (NMDAR). And increased activity of this receptor complicates the clinical use of opioids for treating neuropathic pain. Rodríguez-Muñoz et al. (2011) indicated that morphine disrupts the glutamate-binding NMDAR complex by PKC-mediated phosphorylation and potentiates the NMDAR-CaMKII pathway, which is implicated in morphine tolerance. Inhibition of PKC restored the antinociceptive effect of morphine on the μ-opioid receptor (MOR). Thus, the opposing activities of the MOR and NMDAR in pain control affect their relation within neurons of structures such as the periaqueductal grey (PAG), a region that is implicated in the opioid control of nociception. This finding could be exploited in developing bifunctional drugs that would act exclusively on NMDARs associated with MORs.

MORs are not the only opioid receptors that influence PKC. Berg et al. (2011), who were investigating the regulation of the κ-opioid receptor (KOR) in rat primary sensory neurons *in vitro* and in a rat model of thermal sensitisation, showed that the application of a KOR agonist (U50488) did not inhibit AC activity or release of calcitonin gene-related peptide (CGRP) *in vitro* and did not inhibit thermal sensitisation *in vivo*. It is important to note that AC activity, CGRP release, and thermal sensitisation process are related to PKC activation (see section 2). However, after a 15-min pretreatment with bradykinin, the agonist became capable of inhibiting AC activity, CGRP release, and thermal sensitisation. The *in vitro* effects of bradykinin on the KOR agonist were abolished by a PKC inhibitor; thus, Berg and co-workers suggest that PKC activation mediates BK-induced regulation of the KOR system. More studies are necessary to understand the mechanisms by which peripheral KOR agonist efficacy is regulated and the relationship of the KOR agonist effects with PKC activation.

In this regard, formalin-induced inflammatory nociception may inhibit morphine tolerance in mice. In this model, conventional PKC (cPKC) is up-regulated and treatment with an antisense oligonucleotide (AS-ODN) directed against cPKC abolished the development of morphine tolerance, suggesting that cPKC is involved in morphine tolerance development (Fujita-Hamabe et al., 2010). Additionally, formalin-induced inflammatory nociception inhibit morphine tolerance by a mechanism involving KOR activation, down-regulation of cPKC, and up-regulation of MOR activity (Fujita-Hamabe et al., 2010). The data suggest a key role to cPKC in opioid-induced tolerance and that nociception-activated mechanisms may modulate opioid-response, improving it. In addition, studying the effects of chronic ethanol–induced neuropathy in rats, Narita et al. (2007) showed that chronic ethanol exposure dysregulated MOR but not DOR and KOR, and was related to PKC up-regulation in the spinal cord, which may explain the reduced sensitivity to the morphine antinociceptive effect. Taken together, these findings suggest the PKC activation disrupts MOR function, which could be counteracted by the KOR system. How the DOR participates remains unclear.

Like PKC, PKA may also play a role in morphine antinociceptive tolerance. Previous studies have shown that chronic exposure to morphine results in intracellular adaptations within neurons that cause an increase in PKA activity. Unexpectedly, sustained morphine treatment produces paradoxical pain sensitisation (opioid-induced hyperalgesia) and causes an increase in spinal pain-related neurotransmitter concentrations, such as CGRP, in experimental animals. Studies have also shown that PKA plays a major role in the

regulation of presynaptic neurotransmitter (such as CGRP and substance P) synthesis and release. Tumati et al. (2011) previously showed that in cultured DRG neurons, sustained *in vitro* opioid agonist treatment up-regulates cAMP levels (AC superactivation) and augments CGRP release in a PKA-dependent manner. The authors also showed that selective knock-down of spinal PKA activity by intrathecal pretreatment of rats with a PKA-selective small interference RNA (siRNA) mixture significantly attenuates sustained morphine-mediated augmentation of spinal CGRP immunoreactivity, thermal and mechanical sensitisation and antinociceptive tolerance. These findings indicate that sustained morphine-mediated activation of spinal cAMP/PKA-dependent signalling may play an important role in opioid-induced pain sensitisation. More specifically, morphine acts acutely on MORs, which couple with G-proteins to inhibit AC and reduce PKA activity. However, during tolerance, MORs become uncoupled from G-proteins, AC inhibition is reduced, and PKA activity is increased. These findings also provide potential molecular targets for pharmacological intervention to prevent the development of such paradoxical pain sensitisation.

The majority of studies that have demonstrated an increase in PKA activity during opioid tolerance have been conducted in rats using brain regions associated with the reinforcing properties of opioids, such as the *locus coeruleus* and *nucleus accumbens*. Studying the expression of morphine antinociceptive tolerance at the behavioural level (tail-flick test) and the alterations in PKA activity at the cellular level in mouse brain (PAG, medulla, thalamus) and lumbar spinal cord, Dalton et al. (2005) support the hypothesis that an increase in PKA activity contributes to the tolerance to morphine-induced antinociception. However, the effect of chronic morphine treatment for 15 days on PKA activity was region-specific because increases in cytosolic PKA activity were observed in the lumbar spinal cord. In contrast, PKA activity/kinetics was not altered in the PAG, medulla or thalamus. These results demonstrate that spinal and supraspinal PKA activity are differentially altered during morphine tolerance. Thus, the neurons in mouse brain and lumbar spinal cord that make up the pain pathway from the brainstem to the spinal cord respond differently to chronic morphine treatment. To confirm these findings, future studies need to elucidate the differential responses to chronic morphine treatment using *in vivo* models of morphine antinociceptive tolerance concerning the PKA involvement.

Using a behavioural paw pressure test in rats, Yamdeu et al. (2011) demonstrated that up-regulation of NGF, through activation of the p38 MAPK pathway, lead to adaptive changes in sensory neuron opioid receptors that enhance susceptibility to local opioids. After intraplantar NGF treatment, this effect occurs in three consecutive steps: MOR expression is increased in DRG at 24 h, increased axonal MOR transport at 48 h, and increased MOR density at 96 h. Consequently, the dose-dependent peripheral antinociceptive effects of locally applied full opioid agonists such as fentanyl are potentiated, and the effects of partial opioid agonists such as buprenorphine are more efficacious, which is reversed by the intrathecal administration of p38 MAPK inhibitor SB203580. Thus, in rats, peripheral inflammation increases MOR expression in nociceptors by NGF activation of p38 MAPK. This mechanism may act as a counter-regulatory response to painful p38 MAPK–induced conditions, such as inflammatory pain, to facilitate exogenously or endogenously mediated opioid antinociception.

Recently, the roles of several MAPKs, including p38 MAPK and ERK, have been investigated in animal models of morphine tolerance and postoperative nociceptive

sensitisation. It is unknown, however, whether prior morphine-induced MAPK activation affects the resolution of postoperative nociceptive sensitisation. Horvath et al. (2010) investigated the effect of morphine-induced antinociceptive tolerance on the resolution of postoperative nociceptive sensitisation. They hypothesised that prior chronic morphine administration would inhibit or delay the resolution of postoperative nociceptive sensitisation via enhanced spinal glial proteins expression and MAPK signalling. Chronic morphine treatment attenuated the resolution of postoperative nociceptive sensitisation, as determined by thermal and mechanical behavioural tests, and enhanced microglial p38 MAPK and ERK phosphorylation. To better understand these results, prior chronic morphine exposure could prime microglia, causing exacerbated MAPK signalling pathway activation following subsequent paw incision injury. This would cause more robust microglial responses in rats with a history of morphine tolerance versus naïve rats, and this response is manifested by further neuronal sensitisation, behavioural hypersensitivity and inhibition of the resolution of the postoperative-associated nociceptive condition. The Horvath and co-workers study indicates that microglial MAPKs play a role in the mechanisms by which morphine attenuates the resolution of postoperative pain and suggests that patients who abuse opioids or are on chronic opioid therapy may be more susceptible to developing chronic pain syndromes following acute injury.

In conclusion, protein kinases (PKs) exert a crucial role in pain control responses mediated by opioids, mainly in tolerance-induced mechanisms. Thus, PKs could be the key to better understanding opioid pharmacodynamics.

3.2 General and local anaesthetics

3.2.1 General anaesthetics

Ketamine is an NMDAR antagonist that is available for clinical use as a general anaesthetic. Ketamine presents analgesic effect in acute and chronic pain models in both animals and humans (Mathisen et al. 1995, Rabben et al., 1999; Visser & Schug, 2006; Pascual et al., 2010).

The involvement of kinases in the analgesic effect of ketamine has been investigated. Using a model of neuropathic pain induced by SNL in rats, Mei et al. (2011) showed that SNL induced ipsilateral JNK phosphorylation up-regulation in astrocytes, but not microglia or neurons, within the spinal dorsal horn. Intrathecal ketamine relieved SNL-induced mechanical sensitisation and produced a dose-dependent effect on the suppression of SNL-induced spinal astrocytic JNK phosphorylation but had no effect on JNK protein expression, suggesting that the inhibition of spinal JNK activation may be involved in the analgesic effects of ketamine in this model.

The inhibition of MAPK phosphorylation by ketamine has also been related to a reduction in cytokine gene expressions in lipopolysaccharide (LPS)-activated macrophages (Wu et al., 2008). A therapeutic concentration of ketamine can decrease LPS-induced JNK phosphorylation, thus inhibiting TNF-α and IL-6 gene expression, which leads to the suppression of LPS-induced macrophage activation (Wu et al., 2008). In addition, ketamine reduced IL-1β biosynthesis in LPS-stimulated macrophages through the suppression of Ras, Raf, MEK1/2, and ERK1/2/IKK phosphorylation and the subsequent translocation and transactivation of the transcription factor NF-κB (Chen et al., 2009). The involvement of TNF-α, IL-6 and IL-1β in inflammatory nociceptive sensitisation is well known. Thus, the

inhibition of cytokine production by ketamine in different cells may be an additional mechanism that contributes toward its analgesic effect.

Beyond its own specific effects, ketamine also has analgesic effects when given in combination with opioids. As mentioned earlier, several studies have demonstrated that ERK1/2 is involved in nociception. However, activation of MOR by opioids leads to ERK1/2 phosphorylation (Fukuda et al. 1996; Gutstein et al. 1997; Gupta et al., 2011), and this can be potentiated by ketamine. Gupta et al. (2011) investigated whether the ability of ketamine to increase the duration of opioid-induced effects could be related to the modulation of opioid-induced signalling. The authors found that, in a cell culture model, ketamine increases the effectiveness of opioid-induced signalling by enhancing the level of opioid-induced ERK1/2 phosphorylation. Ketamine also delays the desensitisation and improves the resensitisation of ERK1/2 signalling. These effects were observed in heterologous cells expressing MOR, suggesting a non-NMDA receptor-mediated action of ketamine (Gupta et al., 2011). The authors concluded that the overall effect of ketamine appears to be keeping opioid-induced ERK1/2 signalling active for a longer time period, and this could account for the observed effects of ketamine on the duration of opioid-induced analgesia. However, these data were obtained from *in vitro* experiments, and the link with analgesia is not clearly understood. Data provided from *in vivo* studies could contribute to improve the understanding of opioid-induced analgesia and its potentiation by ketamine.

3.2.2 Local anaesthetics

Systemic or topical administration of lidocaine and other local anaesthetics reduce hypersensitivity states induced by both acute inflammation and peripheral nerve injury in animals and brings significant relief in some patients with neuropathic pain syndromes (Mao & Chen, 2000; Ma et al., 2003; Gu et al., 2008; Fleming & O'Connor, 2009; Suter et al., 2009; Buchanan& MacIvor, 2010; Suzuki et al., 2011).

The analgesic effect of lidocaine in neuropathic pain can be partially explained by its ability to attenuate MAPK activation. Intrathecal injection of lidocaine in rats with chronic constriction injury suppressed the phosphorylation of p38 MAPK in the activated microglia in the spinal cord (Gu et al., 2008). In ATP-activated cultured rat microglia, lidocaine inhibited p38 MAPK activation and attenuated the production of proinflammatory cytokines, including TNF-α, IL-1β and IL-6 (Su et al., 2010). Furthermore, lidocaine significantly inhibited LPS-induced Toll-like receptor 4, NF-κB, ERK and p38 MAPK activation, but not JNK activation in LPS-stimulated murine macrophages (Lee et al., 2008).

Spared nerve injury (SNI) induces mechanical sensitisation and p38 MAPK activation in spinal microglia. Bupivacaine microspheres induced a complete sensory and motor blockade and significantly inhibited p38 MAPK activation and microglial proliferation in the spinal cords of rats (Suter et al., 2009). Carrageenan-induced hind paw inflammation and sensitisation triggers phosphorylation of spinal p38 MAPK and enhances TNF and IL-1 production in the bilateral DRGs and spinal cord. Although bupivacaine inhibits oedema, hyperalgesia and the carrageenan-induced production of systemic cytokines (Beloeil et al., 2006a; Combettes et al., 2010), the inhibitory effects of bupivacaine on the expression of cytokines or phosphorylated p38 MAPK in spinal cord or DRGs have not been verified (Beloeil et al., 2006b).

ERK activation as a potential target for bupivacaine antinociception was also investigated. The activation of both ionotropic (AMPA, NMDA, TRPV1) and metabotropic (NK-1, bradykinin 2 receptor, mGluR) receptors results in ERK phosphorylation in superficial dorsal horn neurons in rats. Bupivacaine blocked ionotropic but not metabotropic, receptor–induced ERK activation by apparently blocking Ca^{2+} influx through the plasma membrane in the spinal cord (Yanagidate & Strichartz, 2006).

Taken together, the inhibition of MAPK activation by general and local anaesthetics seems to represent a common and important pathway to at least partially explain the mechanism of analgesic action exerted by these drugs through ion influx inhibition.

3.3 Antidepressants

Selected antidepressants suppress pain through diverse mechanisms and are now considered as an essential component of the therapeutic strategy for treatment of many types of persistent pain. Their main mechanism of action involves reinforcement of the descending inhibitory pathways by increasing the amount of norepinephrine and serotonin in the synaptic cleft at both the supraspinal and spinal levels. Based on this, tricyclic antidepressants (TCAs) are widely used for treating chronic pain, such as neuropathic and inflammatory pain. Intrathecal (i.t.) co-infusion of amitriptyline with morphine not only attenuates the development of morphine tolerance but also preserves its antinociceptive efficacy (Tai et al., 2006). Tai et al. (2007) showed that amitriptyline pretreatment reverses the spinal cord PKA and PKC upregulation and preserves morphine's antinociceptive effect in morphine-tolerant rats submitted to thermal behaviour test; this reversal may occur via preventing the up-regulation of PKA and PKC protein expression. It results in the trafficking of glutamate transporters from the cytosol to the plasma membrane of glial cells, thus reducing the excitatory amino acid (EAA) concentration in the cerebrospinal fluid (CSF) spinal cord by the morphine challenge. This study suggested that amitriptyline is a useful analgesic adjuvant in the treatment of patients who need long-term opioid administration for pain relief.

In addition to the traditionally used TCAs, such as amitriptyline, selective serotonin reuptake inhibitors (SSRIs) and mixed monoamine uptake inhibitors are also used as a first-line treatment for managing pain syndromes. As mentioned above, voltage-gated sodium channels (VGSCs) are subject to modulation by G protein-coupled receptor signalling cascades involving PKA- and PKC-mediated phosphorylation. Depending on the neuron type and its anatomical location, phosphorylation of the VGSCs by PKC may facilitate slow inactivation (Cantrell and Catterall, 2001). Activating the 5-HT2C subtype of serotonin receptors in prefrontal cortex neurons results in a negative shift in the voltage-dependence of fast inactivation accompanied with a reduction of the peak current due to a PKC-mediated phosphorylation process (Carr et al., 2002). Concurrent phosphorylation by PKA seems necessary for the maximal current reduction (Cantrell et al., 2002). These mechanisms can be activated by various neurotransmitters including serotonin (Cantrell and Catterall, 2001). Because SSRIs increase the extracellular concentration of serotonin it is logical that they would indirectly modulate sodium channels in the central nervous system. This action mediated by increased serotonin and, PKA and PKC activity, could account for the analgesic effect of SSRIs.

Thán et al. (2007) studied the pharmacological interaction between SSRIs and sodium channel blocking agents such as lamotrigine. They examined the interaction of VGSCs blockers and SSRIs at the level of spinal segmental neurotransmission in the rat hemisected spinal cord model. The reflex inhibitory action of VGSCs blocker was markedly enhanced when SSRI compounds were co-applied; and it was found serotonin receptors and PKC involvement in the modulation of sodium channel function (Thán et al., 2007).

In conclusion, it seems that antidepressants exert analgesic effects by a mechanism involving serotonin, PKA and PKC activation, and modulation of VGSCs. Understanding the PK dynamics in these processes would be key to improve pain management.

3.4 PKG signalling and pain control

As described in section 1, the activation of signalling pathways that are dependent on PKA, PKC and MAPKs is important for the sensitisation of nociceptors and pain processing. The PKG pathway, in turn, is related to the nitric oxide (NO)/cGMP/PKG/ATP-sensitive K^+ channel pathway, which plays an important role in peripheral antinociception (Rodrigues & Duarte, 2000; Sachs et al., 2004).

The relationship between the NO/cGMP pathway and peripheral antinociception was first demonstrated by Ferreira and co-workers (Durate et al., 1990; Ferreira et al., 1991). They showed that the antinociceptive effect of acetylcholine and morphine was blocked by a guanylyl cyclase inhibitor and an NO synthase inhibitor, and was potentiated by a specific cGMP phosphodiesterase inhibitor. Moreover, the antinociception achieved with these drugs was mimicked by NO donors such as sodium nitroprusside. The involvement of this pathway has also been demonstrated for other analgesics, such as dipyrone (Duarte et al., 1992), diclofenac (Tonussi et al., 1994), and some antinociceptive agents, such as *Crotalus durissus terrificus* snake-venom (Picolo et al., 2000), the potent κ-opioid receptor agonist bremazocine (Amarante & Duarte, 2002), xylazine (Romero & Duarte, 2009), the cannabinoid receptor agonist anandamida (Reis et al., 2009) and ketamine (Romero et al., 2011). In agreement with *in vivo* studies, data from electrophysiological experiments studying inflammatory sensitisation showed that capsaicin-induced elevations in intracellular Ca^{2+} levels of rat sensory neurons lead to an enhanced production of cGMP via the NO pathway. The elevated cGMP levels and the subsequent activation of PKG appear to inactivate the sensitisation, confirming the important regulatory role of this kinase in reversing the neuronal sensitisation (Lopshire & Nicol, 1997).

In addition to studies on the mechanism of antinociceptive action of analgesics, Duarte and co-workers showed that the ability of morphine and dipyrone to induce peripheral antinociception is dependent on the activation of ATP-sensitive K^+ channels (Rodrigues & Duarte, 2000). cGMP can directly or indirectly (via PKG stimulation) modulate the activity of ion channels. PKG is a protein kinase that is stimulated selectively but not exclusively by cGMP. Once stimulated, PKG inhibits phospholipase C activity, stimulates Ca^{2+}-ATPase activity, inhibits inositol 1,4,5-triphosphate, inhibits Ca^{2+} channels, and/or stimulates K^+ channels activity (Cury et al., 2011). Furthermore, Sachs et al. (2004) demonstrated that the antinociceptive effect of dipyrone on persistent inflammatory sensitisation is dependent on the PKG activation and its modulation of ATP-sensitive K^+ channels.

Taken together, these findings suggest the relevant role of PKG as an intermediate between cGMP generation and the opening of ATP-sensitive K^+ channels. The activation of this modulatory pathway may be an interesting target for new drug development.

4. Conclusions: A perspective of promising drug targets

In this section, protein kinases will be viewed as targets for pain control drug development. Several pre-clinical and clinical trials will be reviewed, focusing on the effectiveness and adverse effects of such drugs.

The genomic analysis of the eukaryotic protein kinase superfamily together with drug design approaches such as the bioisosteric replacement of pharmacophoric groups of lead compounds and 3D-quantitative structure-activity relationship analysis provide several new chemical entities to be tested and developed as drug candidates.

The continuous progress in protein structure determination and improved resolution allows the identification of pharmacological targets. The experimental results from genetically modified animals support new hypotheses and help to validate new concepts to better understand the pathological genesis and natural processes of our body.

Such progress in medicinal chemistry, biochemistry and pharmacology paradoxically leads to poor results in terms of new pharmaceutical entities and therapeutics. The pharmaceutical innovation decrease in recent decades is due to many aspects that are beyond the scope of this chapter. As targets of pharmaceutics, protein kinases play an important role in this history, providing several new therapeutic cancer targets. Drug discovery companies have targeted protein kinase inhibitors, which have led to billion dollar merges and a new branch of research and development that spread beyond the boundaries of cancer therapeutics (Garber, 2003).

At the beginning of the second decade of the 21st century, there are synthetic and medicinal chemistry service companies with strong backgrounds in kinase targets and kinase inhibitor drug discovery; these companies can develop new compounds on demand. There are sixteen pharmaceuticals actually licensed as protein kinase inhibitors, mainly to treat different cancers. The first drug, Trastuzumab, was licensed in 1998; this drug is a monoclonal antibody targeting membrane receptors that activates the MAPK pathway as well as the PI3 Kinase/AKT pathways. After this initial drug, many small molecules followed, targeting kinases as mechanism of action, mainly as ATP competitors.

The International Federation of Pharmaceutical Manufacturers & Associations has listed in its Clinical Trials Portal three entries for clinical trials focusing on pain and protein kinases. Two of these trials involve p38 MAPK inhibitors from a large pharmaceutical company and are testing for neuropathic pain following nerve trauma and from lumbosacral radiculopathy. The third trial involves tyrosine kinase (TrkA) receptor expression in children with retrosternal pain.

Experimental evidence suggests that p38 MAPK is activated in spinal microglia after nerve injury and contributes to neuropathic pain development and maintenance (Ji & Suter, 2007). p38 MAPK phosphorylates targets that transduce cellular signals to molecules and transcription factors that are involved in regulating the biosynthesis of inflammatory cytokines such as IL-1 and TNF-α. The inhibitor dilmapimod was associated with a

significant reduction in pain intensity in patients with neuropathic pain following nerve injury (Anand et al., 2011). The clinical efficacy of p38 MAPK inhibitors in acute pain was also demonstrated in an assay of acute postsurgical dental pain; these inhibitors increased the time to rescue medication and decreased pain intensity when compared with the placebo group (Tong et al. 2011).

Despite these clinical assays that are directly associated with pain, many other clinical and pre-clinical studies have some degree of relevance when pain management is the goal. The action of different protein kinases inhibitors in cancer, rheumatoid arthritis, postsurgical conditions, diabetes and so forth has significant impact on decreasing pain in subjects suffering from these pathologies.

In addition to these efforts, one long-sought goal is the development of inhibitors of PKC isoforms because this family of protein kinases is involved in the cellular signalling of nociception, anxiety and cognition (Van Kolen et al., 2008). Non-isoform-specific PKC inhibitors have proven to be too toxic for *in vivo* use. PKCε is the primary target for drug design. This isoform is activated during nerve sensitisation and phosphorylates ion channels in the peripheral nervous system such as TRPV-1, and N-type voltage-dependent calcium channels (VDCCs) in isolectin B4-positive nociceptors; in addition, it mediates interplay between other kinases that are important to nociceptor function, such as PKA and MAPK (Hucho et al., 2005). There are no specific ATP-binding competitors for PKCε. There are other compounds that target alternative domains, such as the pseudosubstrate sequence, which is responsible for keeping the kinase in an inactivate state. The lipid-binding, cellular localisation and actin-binding domains are also valid targets. The main goal is to develop isoform-specific inhibitors among the ten known isozymes and to provide tissue specificity because cardiac-specific PKCε inhibition blocks norepinephrine-mediated regulation of heart contraction (Johnson et al., 1996).

The pharmaceutical paradigm of "new targets for old drugs", where known medications are employed in new pathologies as an innovation strategy to keep new products flowing to the market also applies to protein kinases and pain control because many drugs utilised for chronic and neuropathic pain management, such as antidepressants and anaesthetics, depend on protein kinases for their mechanisms of action.

New lead drugs are also being proposed; these drugs utilise molecular hybridisation and bioisosteric replacement of pharmacophoric groups, where different bioactive molecular moieties of mechanistically diverse drugs are fused, giving birth to new chemical entities with dual activity profiles (Brando Lima et al., 2011) that incorporate protein kinase inhibition with another type of biological activity. The challenge of developing new molecular approaches to create drugs that manage pain is great, and as seen throughout this chapter, protein kinases are an important aspect of this problem.

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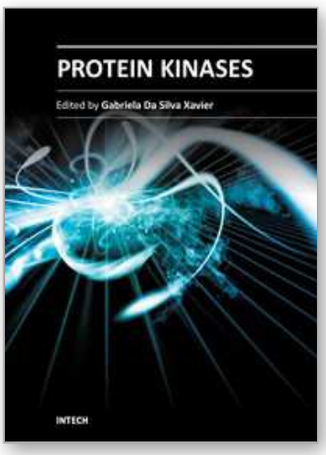
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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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