Signal transduction in neuropathic pain, with special emphasis on the analgesic role of opioids – Part II: Moving basic science towards a new pharmacotherapy*

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In the first part of this three-part article I explored the notion that pharmacological intervention, aimed at eliminating abnormal sensations such as hyperalgesia or paraesthesia arising as a direct result of nerve injury, activates adaptive responses that ensure adequacy of neurotransmission, regardless of whether such transmission ultimately evokes normal or abnormal sensations. Thus, by their nature, such adaptive responses will act to oppose and surmount any drug-induced intervention designed to diminish pain through attenuation of signal conduction. A corollary of this hypothesis is that even the most sophisticated novel pharmacological entities, when used to block the pain signal, represent substrates for evoking a repertoire of failsafe mechanisms that have evolved throughout a history of challenge and response. In Part II, I explore in greater depth how activation of these responses may explain why the treatment of neuropathic pains, particularly with opioids, can be so frustrating.

A new pharmacotherapy

Introduction

In Part II of this trilogy, I investigate the extent to which current basic science can be applied to the development of novel entities with the potential for entering preclinical evaluation. Following on from the treatment in Part I, I explore in greater depth the role of cholecystokinin (CCK) as an anti-opioid peptide. A re-evaluation of earlier preclinical studies confirms an important role for selective antagonists of the CCK\textsubscript{B} receptor subtype as co-analgesics with morphine in patients with intractable pain.\textsuperscript{1–3} In attempting to unravel mechanisms whereby CCK antagonizes opioid signalling, I adhere to the theme developed in Part I. That is, cross-talk between CCK and opioid signalling may in some way reflect a conflict between the need of an injured neuronal cell to enter programmed survival or apoptosis and that of the clinician to produce relief of pain. Within the context of CCK-mediated anti-opioid signalling, a role is proposed for the protein tyrosine kinase (PTK), PYK\textsubscript{2}, which was recently cloned from human brain. The possibility that phospholipase A\textsubscript{2} (PLA\textsubscript{2}) is an anti-opioid messenger of CCK is exciting because, as I discuss in...
the final part of this trilogy, this possibility may allow the development of new generic drugs in the management of neuropathic pain.

The recent discovery that opioid signalling converges with that of growth factors, such as epidermal growth factor and nerve growth factor is especially exciting. An understanding of how these two pathways converge upon modulation of phenotype expression through the activation of transcription of immediate early genes provides insights into mechanisms whereby opioids may exert effects upon adaptive plasticity responses in the mature nervous system. In Part III (to be published separately in 1999), I discuss the clinical significance of this signalling convergence, together with the possibilities for discovering new therapeutic options targeted at key mediators, such as phosphatidylinositol 3-kinase (PI-3K), and phosphatidylinositol 4,5-bisphosphate (PIP2).

Of more immediate interest to clinicians, in Part II I highlight important differences in signalling between commonly used opioid agonists, which include buprenorphine, morphine and fentanyl. It is becoming clear that, after activation of transducer proteins (i.e. G proteins), these opioids effect analgesia along quite different pathways. In this respect, buprenorphine is unique. In a new paradigm of neuropathic pain, transduction of the buprenorphine signal is unequivocally demonstrated to be entirely separate from that of morphine. The ability of buprenorphine to attenuate hyperalgesia and allodynia in this new paradigm is likely to have important clinical significance.

**CCK and opioids have been shown to exert opposite effects in several experimental paradigms and physiological functions. For instance, in rats, food intake increases after the administration of opioid agonists, but decreases after CCK. At the cellular level, CCK and opioids are known frequently to act in an opposite way, as shown for instance on ion transport in the intestine. Indeed, as summarized below, CCK frequently acts as an anti-opioid compound, especially in pain processing and control.**

**CCK as an anti-opioid peptide**
The results of numerous studies support this assertion. Thus, it has been shown that CCK antagonizes butorphanol-induced feeding, morphine’s effects on locomotor activity, and β-endorphin-induced catalepsy, and disrupts maternal behaviour. Similarly, CCK reduces the hypothermic and hypotensive effects of opioids, as well as the excitation of pyramidal neurones due to opioid receptor activation in the rat hippocampus. In addition, CCK blocks the increase in brain tryptophan hydroxylase activity that is elicited by morphine and prevents β-endorphin from inhibiting brain and liver DNA synthesis in rat pups.

**CCK as an anti-analgesic peptide**
Numerous reports have indicated that CCK antagonizes opioid antinociceptive effects. Indeed, administered by peripheral or central (intracerebroventricular (i.c.v.), intrathecal (i.t.)) routes, CCK can reduce morphine-, PL017- or ohmefentanyl- (two selective µ-agonists), and β-endorphin-induced analgesia, as well as the morphine-induced depression of the nociceptive flexion reflex in rats. In the mouse tail-flick test, CCK attenuates the analgesic effects of morphine. In addition, in the same species, CCK administered i.t. antagonizes the inhibition of tail flick by i.c.v. β-endorphin. Furthermore, the inhibitory effect of morphine and [d-Ala2-Me)Phe4-Glyol5] enkephalin (DAMGO) on C-fibre-evoked activity of rat spinal nociceptive neurones can be reduced by CCK.

Thus, from these data, it can be concluded that CCK has the pharmacological property of reducing the antinociceptive effects of endogenous opioids.

**Opioid analgesia and cholecystokinin**

**Introduction**
CCK was first identified as a hormone in the gastrointestinal system. It was subsequently shown to be present in the central nervous system (CNS) essentially in its sulphated octapeptide form (CCK-8). At least two types of CCK receptors (A and B) have been identified. Although CCKA ‘alimentary’ receptors are found primarily in the periphery, they are also present in some brain areas. CCKB ‘brain’ receptors have a widespread distribution in the CNS.
The CCK<sub>B</sub> receptor mediates the anti-opioid effects of CCK

Many data strongly suggest that the effects of exogenous CCK in fact mimic the action of the endogenous peptide. Thus, active immunization against CCK or the administration of an anti-CCK antiserum potentiates morphine and β-endorphin analgesia in rats.<sup>34–36</sup> Proglumide, a nonselective CCK receptor antagonist, and the selective CCK<sub>A</sub> antagonists, devazepide and lorglumide, enhance opioid analgesia in rodents and baboons.<sup>22,30,37–57</sup> Proglumide also potentiates the inhibitory effect of intrathecal morphine on the electrical activity of spinal nociceptive neurones.<sup>33</sup>

In addition to enhancing opioid antinociception in rodents and squirrel monkeys, the CCK<sub>B</sub> antagonist, L-365,260, produces, by itself, an analgesic effect in the latter species.<sup>34,57–61</sup> In the rat, another CCK<sub>B</sub> antagonist, CI-988, also enhances the analgesic effect of morphine on the flexor reflex and exerts a naloxone-reversible antinociceptive effect per se.<sup>26,62,63</sup> Moreover, it has been shown that treatment with antisense oligonucleotides targeting the cloned CCK<sub>B</sub> receptor produces a five-fold leftward displacement of the morphine analgesic dose–effect curve.<sup>64</sup>

An induction of the expression of the pro-CCK gene has been observed in rat dorsal root ganglia after peripheral axotomy, a model of neuropathic pain that induces autotetic behaviour.<sup>65</sup> Furthermore, this behaviour can be inhibited by combined treatment with morphine and the CCK<sub>B</sub> antagonist CI-988.<sup>65</sup> In axotomized rats, the depressive effect of CI-988 on the flexor reflex is more pronounced than in normal animals, and the (weak) inhibitory influence of morphine on this reflex is enhanced by CCK-receptor blockade by this antagonist.<sup>66</sup> These observations led the authors (Xu et al.) to propose that an increased release of CCK from terminals of (lesioned) primary afferent fibres could antagonize the actions of opioid analgesics either released endogenously or applied exogenously, resulting in the development of a neuropathic pain syndrome and in the ineffectiveness of opioids. Similarly, in another model of neuropathic pain due to photochemically-provoked spinal cord ischaemia, CI-988 (but not CAM 1481, a selective CCK<sub>A</sub> antagonist) relieved the lesion-induced allodynia-like symptoms.<sup>67</sup>

Taken together, these data suggest that the anti-opioid effects of CCK arise as a result of the stimulation by the peptide of CCK<sub>B</sub> rather than CCK<sub>A</sub> receptors. Indeed, the rank order of potency of the CCK antagonists (L-365,031, devazepide and L-365,260) in potentiating morphine analgesia in the rat correlates with their affinity for CCK<sub>B</sub> receptors.<sup>58</sup>

Exploring the mechanisms of CCK/opioid interactions

Neuronal injury is associated with an increased expression of the CCK<sub>B</sub> receptor

The importance of the pain state in revealing the possible anti-analgesic action of endogenous CCK is demonstrated by the observations of Stanfa and Dickenson.<sup>68</sup> These authors showed that CCK<sub>B</sub> receptor blockade by L-365,260 or PD 135158 potentiates the inhibitory effect of intrathecal morphine on the C-fibre-evoked responses of dorsal horn nociceptive neurones in normal rats<sup>68,69</sup> but not in animals with carrageenan-induced inflammation.<sup>68</sup> Conversely, CCK attenuates the effects of morphine only in rats with carrageenan inflammation, having no effect on the action of morphine in normal animals.<sup>68</sup> The effects of both CCK and its antagonists L-365,260 and PD 135138 are therefore dependent on the inflammatory state of the animal, with the agonist and the antagonists being active in opposite situations. This led to the proposal that, in normal rats, morphine may produce a maximal stimulation of CCK release, such that exogenous CCK is unable further to reduce the opioid analgesia, whereas, in rats with inflammation, there is a reduction in the spinal release of CCK, which could explain the enhanced potency of morphine in these animals.<sup>68,70</sup> In contrast, neuronal injury is associated with a very marked increase in the number of sensory neurones of all sizes that are expressing CCK<sub>B</sub> receptor messenger RNA (mRNA),<sup>71,72</sup> together with a dramatic increase in the number of sensory neurones in dorsal root ganglia that are synthesizing CCK.<sup>65,73</sup>

In the normal dorsal root ganglia in the rat,
mRNA for the CCK$_B$ receptor is present at very low levels. Thus, an injury-associated increase suggests an increased sensitivity to CCK for many primary sensory neurones of different modalities. It is important and of additional clinical relevance that several groups have reported that repeated administration of morphine also stimulates CCK biosynthesis in selective CNS regions, including the spinal cord, the brainstem and the hypothalamus.74–79 It is an interesting speculation that the anti-opioid effect of CCK may in some way be associated with a possible role of CCK in regeneration and repair. Medullary dorsal horn CCK concentrations in adult rats subjected to left infraorbital nerve section at birth were reported to be significantly raised in the left medulla in comparison with those in the control right medulla.80 On the basis of their observations, these workers (Jacquin et al.) concluded that neonatal deafferentation elevates CCK levels within the superficial medullary dorsal horn, reflecting reorganized intrinsic or higher-order inputs within a developing nervous system. In contrast, in acutely nerve-injured adult rats, chronic morphine-treated animals demonstrated a significant retardation of the regenerative process.81 Whether these effects of morphine in this study by Sinatra and Ford81 could be reversed by the administration of CCK-8, for example, is thought-provoking and may have clinical ramifications concerning the prolonged use of morphine in managing some neuropathic pains. On the basis of these very limited data, it is indeed a seductive possibility that the anti-opioid effect of CCK reflects a therapeutic dilemma: the enhancement of opioid-mediated analgesia by the concomitant use of CCK$_B$ receptor antagonists may coincidentally adversely affect processes of regeneration and remodelling subsequent to nerve injury.

**Cross-talk between CCK and opioid signalling**

There is a striking overlap in the anatomical distributions of CCK, opioid peptides and their respective receptors in the CNS.82,83 Both CCK and opioids are localized within principal nociceptive centres, including layers I and II of the spinal cord, the periaqueductal grey (PAG), and intralaminar nuclei of the thalamus.84–90 They also coexist in some neurones of the thalamus, PAG and allocortex.91,92 Thus, anatomical considerations allow an opportunity for cross-talk between opioidergic and CCK-ergic signalling. That is, messengers from one pathway are able to modulate signalling within the other.

A wide spectrum of biological effects induced by CCK peptides results from the activation of the two subtypes of CCK receptors, CCK$_A$ and CCK$_B$. Both subtypes are reported to stimulate phospholipase C (PLC) and phosphatidylinositol turnover through pertussis toxin (PTX)-insensitive G proteins that belong to the Gq/11 subfamily.85,93–105 The activation of Gq/11-coupled receptors results in activation of an inositol phospholipid-specific PLC, with subsequent hydrolysis of the inositol phosphates, PIP$_2$ (the preferred substrate), and phosphatidylinositol 4-phosphate, including phosphatidylinositol. The PLC-catalysed hydrolysis of PIP$_2$ generates two second messengers: inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ triggers the release of Ca$^{2+}$ from intracellular stores, which in turn facilitates the activation by DAG of protein kinase C (PKC) enzymes. It is important to note that there is a general consensus that, within the CNS, signal transduction at the CCK$_B$ receptor is brought about via Gq/11-mediated activation of PLC and the subsequent mobilization of intracellular Ca$^{2+}$ and the DAG-mediated activation of PKC isoforms.97,98,103,106–113

The results of numerous studies favour the idea that the dorsal horn of the spinal cord is an important site of interaction between CCK and opioids. At this level, the anti-opioid effect of CCK could be selective for $\mu$- and $\kappa$-, but not $\delta$-, opioid analgesia.24,114 At the biochemical messenger level, the mechanisms of this CCK/opioid interaction remain elusive; several have been proposed. These include: an allosteric interaction between opioid and CCK$_B$ receptors;16 a convergence of signalling by both receptors upon the same G protein, with opposite effects on potassium conductance;115 and opposing effects upon intracellular Ca$^{2+}$ levels achieved by the opioid action at a voltage-sensitive calcium channel (VSCC) and the CCK or CCK-8 action upon mobilization of intracellular Ca$^{2+}$.111 More recently, however, there has been
renewed interest in signalling interactions between activation of Gq/11 and Gi,116–119 PLC and Gi,120,121 and PLC and PI-3K (a putative messenger for Gi).122 In the next section, after a comprehensive review of these and other studies, I propose a model, at the messenger level, of the anti-opioid mechanism of the CCKB receptor. Additionally, I discuss the therapeutic implications of this new model.

**Convergence of signalling by opioids and CCK**

**Cross-talk between Gi and Gq/11**

In a variety of cell lines, activation of the Gq protein-coupled CCKB receptor results in PKC-dependent stimulated tyrosine phosphorylation of the mitogen-activated protein kinase (MAPK) cascade.101,123–135 Likewise, there is an emerging plethora of reports that demonstrate that three currently known mammalian MAPK signalling cascades (extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase/stress-activated protein kinase, and p38 kinase) can be activated by both acute and chronic opioid signalling transduced via the PTX-sensitive proteins Gi.136–153 In addition, at the messenger level, after activation of both Gq/11 (CCKB)- and Gi (opioid)-coupled receptors, the novel Ca2+-dependent PTK, PYK2 (for proline-rich tyrosine kinase), recently cloned from human brain,154 is an attractive candidate for the role of so-called ‘coincidence-detector’.155

Some key questions must now be asked. As we explore further the interaction between opioid and CCKB signalling, it becomes apparent that we need to investigate further the mechanism whereby Gβγ initiates the Shc–Grb2–Sos1 complex (see Part I, Figure 3). How does Gβγ effect tyrosine phosphorylation of the adapter protein, Shc? The scheme presented in Figure 3 of Part I, although valid, tells us nothing about Gq/11-mediated signalling.

**Gq/11**

In contrast to Gi-mediated activation of the MAPK cascade (Part I, Figure 3), activation by Gq/11-coupled receptors is mostly insensitive to inhibition by Gβγ sequestrants such as the carboxy-terminal fragment of the β-adrenergic receptor kinase and α-transducin,156–158 indicating that the signal is mediated predominantly by the α-subunit of Gq/11. Because the magnitude of MAPK activation by Gq- and Gi-coupled receptors is similar, it is unclear why a larger contribution by Gβγ subunits to the Gq-mediated signal is not detected. (One laboratory, however, has reported that a significant portion of the Gq-mediated muscarinic subtype 1 acetylcholine receptor signal in COS-7 cells is Gβγ-dependent.159)

The interesting observation that the magnitude of MAPK activation by Gq-coupled receptors (in this case an α-subunit signal) and Gi-coupled receptors (in this case a βγ-subunit signal) is similar, suggests a point of signalling convergence upstream of MAPK activation. Recent studies confirm that the ligand-activated CCKB receptor stimulates the formation of the Shc–Grb2–Sos1 complex upstream of p21ras and in a PKC-dependent manner.127,128,130,131,135 These new results also demonstrate that Shc proteins are tyrosine phosphorylated and associate with the Grb2–Sos1 constitutive complex in response to phorbol esters (direct activators of PKC), suggesting that activation of PKC is a potential signalling pathway leading to activation of the Shc–Grb2–Sos1 complex.130,135 The use of a potent and specific inhibitor of PKC, GF109203X, completely blocked the effect of a phorbol ester upon Shc tyrosine phosphorylation and its subsequent association with Grb2–Sos1.135

Taken together, these data allow the possibility that Gi (opioid)- and Gq (CCKB)-mediated pathways leading to MAPK activation may converge upstream of the formation of the Shc–Grb2–Sos1 complex, that is at the level of tyrosine phosphorylation of Shc. Convergence would be likely to be upon a PKC-dependent tyrosine kinase mechanism that results in the phosphorylation of Shc.

**In neurones, is PYK2 the enigmatic link between Gi and Gq/11?**

Recently, a novel Ca2+-dependent PTK, PYK2, was cloned from human brain.154 PYK2, also known as cell adhesion kinase-β160 or related adhesion focal tyrosine kinase,161 is a member of the focal adhesion kinase (FAK) family of non-receptor tyrosine kinases (non-RTKs). PYK2,
which is highly expressed within the CNS, is rapidly activated either by PKC or elevated levels of Ca\textsuperscript{2+} after stimulation of Gq-coupled receptors.\textsuperscript{154,162} It is important that tyrosine phosphorylation (i.e. activation) of PYK2 leads to binding of the SH2 domain of the PTK and of Src (also known as p\textsuperscript{60c-src} or c-Src) to tyrosine 402 of PYK2,\textsuperscript{163} and the activation of Src.\textsuperscript{163,164} The transient expression of a dominant interfering mutant of PYK2 or the PTK, Csk (a negative regulator of Src function), reduced Gi- or Gq/11-induced activation of MAPK; Gi- or Gq/11-induced MAPK activation was also inhibited by overexpression of dominant mutants of Grb2 and Sos.\textsuperscript{163,165} In addition, the catalytic activities of the Src family kinases are elevated by activation of the \(\alpha\)-subunit of Gq/11-coupled receptors.\textsuperscript{166,167} Together, these findings led to the proposal that PYK2 acts with Src to link Gi- and Gq/11-coupled receptors with Grb2 and Sos to activate the MAPK signalling pathway.\textsuperscript{162-164,168}

Additional support for this proposal was recently provided by the first demonstration that Gq-induced activation (i.e. phosphorylation of tyrosine residues Y402 and Y881) of PYK2 induced Src and Grb2 to bind to PYK2.\textsuperscript{162}

On the basis of these new findings, Gi- and Gq/11-mediated signalling may converge upon a Grb2-PYK2-Src complex (Figure 1). It is important to keep in mind that such findings do not constitute evidence for the existence of a synergistic interaction between inputs upon such a complex. However, Dickenson et al.,\textsuperscript{169} using Chinese hamster ovary (CHO-K1) cells transfected with the human brain adenosine A\textsubscript{1} receptor sequence, demonstrated that costimulation of the Gi/o-coupled adenosine A\textsubscript{1} receptor and the endogenous Gq/11-coupled P2Y\textsubscript{2} purinoceptor produced synergistic increases in MAPK activity. Consistent with known mechanisms for Gi/o-coupled receptor-mediated activation of MAPK, they demonstrated that the adenosine A\textsubscript{1} receptor stimulates MAPK activity in CHO cells by a pathway that is independent of PKC but involving tyrosine kinase, PI-3K and MAPK kinase (MAPKK (i.e. MEK)) activation (Figure 1). In attempting to elucidate the location at which the adenosine A\textsubscript{1} and P2Y\textsubscript{2} purinoceptor pathways converge to produce a synergistic increase in MAPK activity, these workers reviewed the earlier report of Eguchi et al.\textsuperscript{170} that, in rat vascular smooth muscle cells, the Gq-coupled angiotensin type II receptor stimulates MAPK activity via the release of Ca\textsuperscript{2+} from intracellular stores, with the resultant activation of a Ca\textsuperscript{2+}/calmodulin-dependent tyrosine kinase (?PYK2; see also Della Rocca et al.\textsuperscript{164}). Consistent with these results, Dickenson’s group demonstrated that the tyrosine kinase inhibitor, genisten, completely blocked the Gq/11-coupled P2Y\textsubscript{2} purinoceptor activation of MAPK. This finding, together with that of Eguchi et al.,\textsuperscript{170} that Gq/11-mediated activation of MAPK in smooth muscle cells was completely blocked by the calmodulin (CaM) inhibitor, calmidizolium, and genisten suggests a possible role for a Ca\textsuperscript{2+}/CaM-sensitive tyrosine kinase in mediating the convergence of Gq/11 and Gi/o signalling in the study by Dickenson et al.\textsuperscript{169}

Additional support for this conclusion may be derived from the work of Shimegi et al.\textsuperscript{171} using FRTL-5 thyroid cells. These workers observed that the Gi-mediated augmentation of Gq-stimulated PLA2 activity was always considerably higher than the effects of Gi-coupled receptor activity upon the Ca\textsuperscript{2+} response, indicating that a significantly greater interaction of the Gi- and Gq-mediated signals was occurring downstream of PLC. Although these results are also consistent with a synergistic interaction of MAPK and Ca\textsuperscript{2+} upon CaM-dependent PLA2 catalytic activity,\textsuperscript{172} they do not explain the synergistic increases in MAPK activity after costimulation of Gi/o-coupled receptors and endogenous Gq/11-coupled receptors.\textsuperscript{169} In summary, although it is well documented that PLC\textbeta isoforms can be activated by both the \(\alpha\)-subunit of Gq and \(\beta\gamma\)-subunits of Gi/o,\textsuperscript{171,173-176} and that there is the possibility for a synergistic interaction upon PLC of signals derived by activation of Gq/11 and Gi/o,\textsuperscript{164,177,178} I present emerging data for further convergence which is downstream of PLC and upstream of PLA2 (Figure 1).

Interesting recent evidence indicates that the association of the PTK, FAK (which is a close relative of PYK2), with Src enhances Src activity. Phosphopeptide mapping of Src isolated from complexes with FAK reveals the decreased phosphorylation of tyrosine 527 of Src, suggesting that the SH2-dependent association of Src with FAK
leads to the dephosphorylation (perhaps by an associated tyrosine phosphatase) of the regulatory site of Src, resulting in the activation of Src kinase activity (MD Schaller, JD Hildebrand and JT Parsons, unpublished observations reported in Parsons and Parsons179). It has been suggested that the formation of the FAK–Src complex may be a prerequisite for Src activation.179 This proposed role for FAK is entirely consistent with the observation that its close relative, PYK2, forms a complex with Src in response to stimulation by activated Gi163 (Figure 1).

**Summary**
In summary, it is known that PYK2 controls a broad array of processes in the CNS, including
short- and long-term neuronal plasticity. The activation of a Gq-coupled receptor leads to the rapid and robust tyrosine phosphorylation of PYK2, with a subsequent increase in catalytic activity of the kinase. Although PYK2 can be activated either by PKC or elevated levels of intracellular Ca\(^{2+}\), the elevation of intracellular Ca\(^{2+}\) concentrations appears to be crucial for PYK2 activation (Figure 1). Although the mechanism whereby Ca\(^{2+}\) regulates the activity of PYK2 is unknown, recent evidence suggests that calcium-bound calmodulin (Ca\(^{2+}\)-CaM) plays a central role in the activation of PYK2. It is interesting to note that, in the model of the regenerating rat sciatic nerve, substantial mobilization of CaM was suggested, resulting in intense localization of CaM immunoreactivity within the plasma membrane of unmyelinated axons. The recent report that local increases in intracellular Ca\(^{2+}\) levels are associated with rapid (seconds) translocation of CaM suggests that the CCK\(_B\)-generated signal is efficiently transduced to Src via the route Gq\(\alpha\rightarrow\)Ca\(^{2+}\)-CaM→PYK2→Src. CaM levels are also significantly increased throughout the CNS of rats that have received chronic morphine treatment, or have developed tolerance to morphine. This observation fuels the speculation about a role for Src-mediated ERK activation that may underscore both morphine tolerance and the diminished morphine responsiveness of neuropathic pain. Src-dependent tyrosine phosphorylation of the adapter protein, Shc, results in recruitment of the Grb2–Sos complex to the plasma membrane, where it catalyses p21ras guanine nucleotide exchange. The observed Gq-mediated association between PYK2 and Grb2 emphasizes the focal role of PYK2 in linking G protein-coupled receptors (GPCRs) with ERK activation (Figure 1). Disinhibition (by dephosphorylation) of Src is apparently conditional for the G\(\beta\gamma\)-mediated activation of ERK. This notion accords with the observation that Gi-coupled receptors (e.g. opioid) activate the ERK cascade by elevating the intracellular level of tyrosine kinase activity, most likely Src or an Src-family kinase (for further discussion see: Clapham and Neer; Sugden and Clerk; Yamaguchi et al.; Lopez-Ilasaca et al.). Finally, Wan and coworkers provide evidence for an alternative non-Src kinase link between Gi- and Gq/11-mediated signalling in vertebrates. They propose a scheme in which the Gq-coupled pathway requires the tyrosine kinases, Csk, Lyn and Syk, while the Gi-coupled pathway requires the tyrosine kinases Btk and Syk, to feed into the ERK cascade. Thus, in this scheme, both pathways converge upstream of Shc–Grb2–Sos upon the PTK, Syk. Such a mechanism is possibly not general and may have limited tissue distribution within non-neuronal cells.

Is PLA2 an anti-opioid CCK messenger?

PLA2 is required for regeneration of sensory axons

The early report by Yawo and Kuno provided some insights into the fundamental role of PLA2 in mediating the ‘resealing’ process after transsection of a nerve fibre. Using the cockroach giant axon as a model system, they demonstrated that sealing of a transected nerve was prevented by the addition of PLA2 inhibitors to the extracellular medium. Later, Nakamura presented findings suggesting the involvement of PLA2 activity in the axonal regeneration of brain adrenergic neurones. More recently, Edström et al., using an organ culture of adult frog dorsal root ganglia and their sciatic nerves, observed that, after a crush injury, axonal outgrowth was inhibited in a nontoxic way by low concentrations of different PLA2 inhibitors. In contrast, outgrowth was stimulated by a PLA2 activator. They concluded that, consistent with earlier reports, their results suggest that PLA2 activity plays an important role in nerve regeneration. Additionally, they provided unequivocal evidence for the localization of immunoreactivity for a low molecular weight form of PLA2 (sPLA2) within sciatic nerves. Similarly, Smalheiser and coworkers observed that PLA2 inhibitors delayed the initial outgrowth of NG108-15 cell neurites on laminin. After a series of investigations, these workers concluded that PLA2 mediates the ability of neurites to respond rapidly to external stimuli, possibly by regulating remodeling of the cytoskeleton.
PLA2 inhibits opioid receptor binding

It is interesting to note that it has been reported that PLA2 products of phospholipid hydrolysis, such as fatty acids and lysophosphatides, are potent inhibitors of high-affinity binding of opioids to neuronal membranes. Moreover, whereas such inhibition has been observed to be reversible after the binding of small amounts of PLA2 products to the receptor or to structures in close proximity to the receptor, the liberation of phospholipids from the receptor by PLA2 products results in irreversible inhibition. It has been proposed that these in vitro effects of PLA2 upon opioid receptor binding affinity are of physiological significance. More recent studies confirm that sPLA2 may play a role in the physiological and pathophysiological regulation of neurotransmitter receptor activity.

PLA2 and neuronal injury

A significant induction of PLA2 occurs after neuronal injury. Moreover, in the regenerating injured sensory nerve, it is likely that PLA2 activity is critically dependent upon both the external Ca\(^{2+}\) concentration and the rate of influx through VSCCs. Deviation in either direction from optimal values for these parameters results in marked effects upon regeneration and survival. Thus, it is possible that PLA2 functions both as a sensor and, because of its effects upon opioid receptor binding, as a modulator of VSCC current. In this case, this function represents an example of regeneration (i.e. survival) assuming dominance over the opioid-mediated attenuation of neurotransmission (by blocking the VSCC); this action of PLA2 is consistent with an ‘anti-opioid’ effect. It is also likely that the tyrosine kinase, PYK2, similarly functions as both a sensor and a modulator of VSCC current. In this case, the suppression of VSCC current would be as a result of tyrosine phosphorylation of the ion channel. It remains to be determined whether PYK2 and PLA2 act in series in order to effect fine control of VSCC (and possibly other ion channels) current.

In the intact PAG region of the midbrain, inhibition by opioids of γ-aminobutyric acid (GABA)-ergic synaptic transmission is dependent upon PLA2 activity. Additionally, increased PLA2 activity reduces the intrinsic sensitivity of adenosine triphosphate (ATP)-sensitive potassium channels to modulation by ATP, thereby increasing the probability for opening. In both cases, these effects of PLA2 have been interpreted as mechanisms of opioid-induced analgesia. Accordingly, the injury-associated induction of PLA2 activity, described earlier, could be argued to produce a leftward shift in the dose–response curve for opioid-induced analgesia. These apparent paradoxical effects of PLA2 upon the modulation of opioid receptor activity may depend on whether the neurone is intact or injured, or on the stage of regeneration.

Summary

In summary, in the injured neurone, PLA2 activity appears necessary for regeneration, and PLA2 products of phospholipid hydrolysis antagonize the high-affinity binding of opioids to neuronal membranes. PLA2-mediated modulation of the effects of opioid receptor activity at the N-type VSCC may represent fine tuning of VSCC current within optima which are critical for survival.

Functional coupling of opioid receptors to MAPK and PLA2

Fukuda et al., using CHO cells, provided the first report of a functional coupling of µ-, δ-, and κ-opioid receptor subtypes with activation of both MAPK and PLA2 (measured by an increase in the release of arachidonate). Previously, this coupling had not been described for native opioid receptors in neuronal cells or opioid receptors expressed from complementary DNAs (cDNAs). Their results are entirely consistent with the notion that full activation of cytosolic PLA2 requires both phosphorylation by MAPK and an increased cytosolic Ca\(^{2+}\) concentration. Accordingly, they observed that staurosporine, which almost completely abolishes MAPK activity induced by the activation of opioid receptors, strongly inhibits opioid receptor-mediated arachidonate release. Additionally, they showed that activation of the opioid receptor expressed in CHO cells leads to an increase in arachidonate release only in the presence of a calcium ionophore, confirming the requirement of an
elevation of intracellular Ca$^{2+}$ for PLA2 activation by opioid receptors. On the basis of these results, they speculate that PLA2 activity within neurones is upregulated only when opioid receptors are activated coincidently with a Gq/11-mediated increase in cytosolic levels of Ca$^{2+}$ via IP3, or with an increase in opening probability of VSCCs, for example as a direct result of depolarization-induced facilitation or disinhibition of Gβγ-induced blockade.

**PLA2: an anti-opioid messenger of the CCK$_B$ receptor?**

**Key points**

1) CCK-8 dose-dependently increases cytosolic (c)PLA2 activity in various cell lines stably expressing the Gq/11-coupled gastrin/CCK$_B$ receptor.222–230

2) Expression of µ-, δ- or κ-opioid receptors in numerous cell lines (see text for references) allows agonist-mediated stimulation (i.e. phosphorylation) of MAPK, including p42MAPK, p44MAPK and MEK (i.e. MAPKK). Whereas chronic opioid administration in vivo results in activation of MAPKs throughout the CNS,141–144,149,150 precipitate opioid withdrawal also results in brain region-specific activation of MAPK.145,231

3) The opioid receptor-mediated stimulation of MAPKs leads to an activation of cPLA2 (after phosphorylation by MAPKs) only when opioid receptors and coexisting Gq/11 Ca$^{2+}$-mobilizing (via IP3) receptors, or VSCCs are activated coincidently232, cPLA2 is thus a classic example of a ‘coincidence detector’.155

4) Consistent with the above observations, Gi/o-coupled (e.g. opioid) receptors activate cPLA2 only when a Gq-coupled receptor is also activated.171,233

5) Costimulation of a Gi/o-coupled receptor (e.g. opioid) and a Gq/11-coupled receptor (e.g. CCK) produces synergistic activation of MAPKs169 and cPLA2.234–236

6) After nerve injury there is a significant increase in CCK mRNA, CCK$_B$ receptor mRNA (see text for references), and induction of PLA2 (see text for references).

7) The PLA2 products of phospholipid hydrolysis, such as fatty acids and lysophosphatides, are potent inhibitors of high-affinity binding of opioids to neuronal membranes.204,205,236,237 It has been proposed that these in vitro effects of PLA2 are of physiological significance.204

8) Whereas chronic morphine exposure may retard nerve regeneration and induce permanent neurological deficits,81,238 activity of PLA2 appears critical for the induction and maintenance of nerve regeneration (see text for references).

9) The inhibitory effects of glucocorticosteroids,239 ketamine,240–242 phenytoin,243 and imipramine,244 upon PLA2 activity, together with potentiation of imipramine binding to synaptosomal membranes by PLA2245,246 may, in some part, contribute to the reported efficacy of these drugs in managing neuropathic pains.

**Transduction of the opioid analgesic signal**

**Do different opioid agonists activate different patterns of multiple G proteins?**

Whether different opioid agonists, on binding to the same receptor subtype (i.e. µ, δ or κ) can activate agonist-specific patterns of G proteins, and whether different opioid receptor subtypes couple selectively to different G proteins, are important clinical issues. As I hope to demonstrate within the scope of this report, answers to such difficult questions may ultimately provide the basis for a reappraisal and systemic evaluation of the role of individual opioids in the management of neuropathic pains.

Chakrabarti and coworkers247 investigated the identities of heterotrimeric G proteins that can interact with the µ-opioid receptor by using α-azidoanilido $[^{32}P]$Guanosine triphosphate (GTP)-labelling of α-subunits in the presence of opioid agonists in CHO-MORIVA3 cells (a CHO clone that stably expresses the µ-opioid receptor cDNA MOR-1). When membranes obtained from CHO-MORIVA3 cells were incubated with α-azidoanilido $[^{32}P]$GTP, selected µ-opioid ligands
induced an increase in labelling in four Gα subunits in this clone, three of which were identified as Gi3α, Gi2α and Go2α. The same pattern of simultaneous interaction of the μ-opioid receptor with multiple Gα-subunits was also observed in two other clones, one expressing about three times more and the other 10-fold fewer receptors as those expressed in the CHO-MORIVA3 cells. For three different μ-opioid ligands tested, there was a greater agonist-induced maximal increase of α-azidoanilido [32P]GTP incorporation into Gi2α and Go2α than for an unknown Gα (G?α) or Gi3α.

In an earlier study,248, the stimulation of cloned δ-opioid receptors from NG108-15 cells that were stably expressed in CHO cells also resulted in preferential labelling of Gi2α and Go2α, relative to Gi3α or G?α, whereas in a different study the same group observed that stimulated δ-opioid receptors in three neuroblastoma cell lines produced equivalent maximal labelling of all Gα subunits in all cell lines examined.249 However, examination of the coupling pattern of cloned κ-opioid receptors in CHO cells250 revealed that stimulation resulted in an equivalent maximum labelling of Gi3α, Gi2α and Go2α, while producing significantly less labelling of G?α. Any differences between κ-, δ- or μ-opioid agonist-induced increases of maximal labelling of Gα subunits could not be attributed to dissimilar receptor densities used in the various investigations because the studies examining both δ- and μ-opioid receptor/G protein interactions found that these patterns were independent of receptor density. Taken together, the results of these studies, in addition to those of other workers,251,252 although demonstrating receptor promiscuity in coupling and interactions with multiple G proteins, also suggest the possibility of some selectivity in the ability of opioid agonists when binding at different receptor subtypes maximally to load GTP on to individual Gα subunits.

After reviewing the results from several studies,247-250 Prather and coworkers250 concluded that the results obtained concerning differences in the efficacies of opioid agonists to induce selective labelling of Gα subunits may differ between laboratories and cell lines. Moreover, they proposed that making conclusions regarding opioid receptor/G protein selectivity may be further compli-

cated by their observations that the amount of agonist required to half-maximally activate Gα subunits does not always correlate with that needed to half-maximally stimulate effectors. For example, in one of their studies,250 although the ED_50 (mean effective dose) values of dynorphin-A (1-17) to produce labelling of Gα subunits were in agreement with the IC_50 (mean inhibitory concentration of 50%) values to inhibit forskolin-stimulated adenylyl cyclase activity, the respective values for U50-488H were not correlated. That is, all ED_50 values to induce photoaffinity labelling by U50-488H were significantly less than those required to half-maximally inhibit adenylyl cyclase activity. However, ED_50 values for labelling of Gα subunits were similar to their affinity (K_i) for the expressed κ-opioid receptor. These findings using U50-488H are consistent with the results of Traynor and Nahorski,253 in which the ability of μ-opioid agonists to activate G proteins was investigated using binding of the GTP analogue [35S]GTPγS to membranes from the human neuroblastoma SH-SY5Y cell line. For those opioids displaying similar values for half-maximal stimulation of [35S]GTPγS binding with those determined for K_i, these workers concluded that this reflected low intrinsic efficacy such that maximal loading of [35S]GTPγS would require full occupation of the receptor population. In contrast, a high ratio of K_i:EC_50 (i.e. K_i: mean effective concentration) would reflect a relatively greater receptor reserve.

In summary, the results of the above in vitro studies by Prather’s group247-250 and others251,252 indicate that many, if not all, opioid receptors, when stably transfected in cell lines, interact with multiple G proteins and that such coupling is generally not selective for any individual Gα subunits. Moreover, although opioid receptors in vitro can activate multiple effectors through different G protein α-subunits,254-259 the results generated by Prather’s group247-250 provide no indication of any consistent correlation between the amount of opioid agonist required to activate G proteins with that needed to stimulate effectors. Thus, although a particular opioid may exhibit typical partial agonist behaviour in achieving less than 100% maximal loading of [35S]GTPγS,253 this cannot be taken as an index of effector activity. In clinical practice with
buprenorphine, for example, which is typically described as a partial agonist, where analgesia is the measured response, full agonist behaviour is frequently observed.260

Standifer et al.,261 using differential blockade of opioid analgesia by antisense oligodeoxynucleotides (ODNs) directed against various G protein α-subunits, suggest that, in vivo, opioid receptors mediating analgesia seem to be coupled to fewer types of G protein α-subunits than do receptors studied in cell lines, with the exception of the δ-receptor. These workers emphasize that any loss of response in vivo after targeting of a specific Ga subunit by antisense ODN gives no indication that the particular Ga subunit was actually ‘generated’ at the opioid receptor. It could have been activated, for example, after receptor binding of some other ligand downstream of the opioid receptor. Consequently, although the results of Standifer et al.261 provide information on which Ga subunits mediate analgesia, they do not allow any conclusions to be made regarding the extent of specific opioid-mediated activation of multiple G proteins.

**Summary**

In summary, on the basis of the available data, attempting to differentiate opioid agonists according to patterns of activation of multiple G protein α-subunits would probably not provide any meaningful indication of differences in final outcomes. However, as reviewed later, the relative capacity of an opioid agonist to activate PTX-sensitive versus PTX-insensitive G proteins collectively, and not in terms of individual subunits, may have important significance for their clinical use in general, and for their role in managing some neuropathic pains, in particular.

**Different opioids target different effectors**

One of the most striking illustrations of the differential effects of opioid agonists upon a single effector is that provided by Baeyens’ group.262 These workers present the results of an in-depth study of the effects of an ATP-sensitive potassium (K_{ATP}) channel opener or blocker upon the antinociception induced by different opioid receptor agonists in a standard model of phasic pain, the mouse tail-flick test. Using a wide range of doses for each opioid (administered subcutaneously), together with a fixed dose of the K_{ATP} channel opener, cromakalim (administered i.c.v.), or a wide range of doses of cromakalim together with a fixed dose of each opioid, they observed marked differences between the opioids in the magnitude of the interaction with cromakalim. Buprenorphine-induced antinociception was markedly enhanced by cromakalim, whereas that of morphine and methadone was enhanced much less. It is surprising, however, that the antinociception induced by either fentanyl or levorphanol was not significantly modified (Figure 2). Similarly, buprenorphine-induced antinociception was particularly sensitive to the effects of the K_{ATP} channel blocker gliquidone (data not shown), whereas that of morphine and methadone required higher doses of gliquidone, and that of fentanyl and levorphanol was not significantly modified. The i.c.v. administration of the potassium channel blockers tetraethylammonium (10 µg per mouse) or 4-aminopyridine (25 ng per mouse), which are potent blockers of some voltage-sensitive, calcium-activated and sodium-activated potassium channels, did not significantly modify the antinociception induced by any of the opioid receptor agonists tested. From these results, Ocaña et al.262 conclude that opening of the K_{ATP} channel is involved in the antinociceptive effect of buprenorphine, morphine and methadone, but not in that of fentanyl or levorphanol.

In attempting to explain their findings, these workers argue that such differential effects cannot easily be attributed to differences in intrinsic efficacy but may possibly be explained by activation of different transducer pathways and effector mechanisms. That is, despite similar ligand binding properties to the µ-receptor, different conformational changes induced upon binding result in the activation of different G proteins. However, since this interpretation is based on the assumption that these agonists are mediating analgesia via the µ-opioid receptor subtype, the possibility cannot be excluded that such differential effects reflect actions at different opioid receptor subtypes. This alternative notion is supported by reports263–265 that the opening of the K_{ATP} channel plays an important role in the analgesia induced by highly selective agonists of δ-1
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([D-Pen², D-Pen⁵] enkephalin), but not δ-2 ([D-Ala²] deltorphin II), κ-(U-50488H, U-69593), or μ-selective (DAMGO, PL017) opioid receptor subtypes. On this basis then, it is interesting that receptor binding assays in several systems that include brain membranes from the rat, guinea pig, monkey and gerbil, consistently demonstrated that buprenorphine has equal, high affinity for μ- and κ- (most likely the κ-1 subtype) opioid receptors with approximately 10-fold lower affinity for the δ-opioid receptor. It is important that co-operative binding studies using the putative δ-1 receptor subtype-selective agonist [D-Pen², D-Pen⁵] enkephalin indicated that buprenorphine binds selectively to the δ-1 receptor subtype. In comparison, under the same study conditions morphine had affinity at the δ-1 receptor subtype, which was approximately 250 to 400-fold lower than that of buprenorphine. The binding affinity of fentanyl at the δ-1 receptor subtype was similar to or slightly less than that of morphine. However, whereas morphine demonstrated a binding affinity at the μ-opioid receptor, which was about one to two orders of magnitude greater than that at the δ-1 receptor subtype, fentanyl demonstrated considerable selectivity at the μ-opioid receptor. The binding affinity of fentanyl at the μ-opioid receptor was approximately three to four orders of magnitude greater than that at the δ-receptor subtype.

![Figure 2](image)

**Figure 2** Differential enhancement of opioid antinociception with the K<sub>ATP</sub> channel opener, cromakalim, in the tail-flick test in the mouse. Percentage antinociception was a mean value determined using 8–12 animals. Tail-flick latencies were measured at 10-minute intervals for up to two hours. For further details of methodology, see Ocaña et al. Within the dose range stated for each opioid (administered s.c.), the height of the left-hand column (■) represents the maximum observed effect of a single dose of cromakalim (32 µg i.c.v. per mouse) upon the antinociception induced by each opioid. At the dose of opioid for which this effect occurred (displayed above column), the magnitude of the interaction is expressed as the ratio of the percentage of antinociception of opioid plus cromakalim (32 µg) to that of opioid plus water (vehicle). Using a single dose of each opioid, the height of the right-hand column (□) represents the maximum observed effect using cromakalim administered i.c.v. within the range 4–64 µg. At the dose of cromakalim for which this effect occurred, the magnitude of the interaction is expressed as the ratio of the percentage of antinociception of opioid plus cromakalim to that of opioid plus cromakalim solvent (water plus 1% Tween 80). *p < 0.05; **p < 0.01 versus opioid + vehicle (i.e. minus cromakalim); NS: not significant. (Data adapted from Ocaña et al. 262 This figure is reproduced from McCormack and Chapleo, with the kind permission of Adis International Limited.)
Summary
Taken together, the above findings suggest that, at least for buprenorphine, morphine and fentanyl, the relative selectivity and binding affinity at the δ-1 receptor subtype may provide some explanation for the magnitude of the effect of the K<sub>ATP</sub> channel opener, cromakalim, upon the antinociception induced by each of these opioids<sup>262</sup> (Figure 2<sup>282</sup>). The important message from the study by Baeyens' group<sup>262</sup> is that opioid analgesia, in a standard model of phasic pain, can be mediated by actions upon different target effectors (Figure 2). It remains unclear whether such differential actions reflect binding of agonists to different opioid receptor subtypes or activation of different G proteins, or both. What is important is whether these differential effects can be exploited in a clinical setting. For example, what role does the K<sub>ATP</sub> channel play in signal transduction in neuropathic pain? In the management of neuropathic pains, could this ion channel become a target for the selective action of an opioid agonist such as buprenorphine, but not that of, say, fentanyl or levorphanol?

Opioid signalling via ADP-ribosylated G proteins

Intrathecal Bordetella pertussis toxin: a new paradigm of (centrally-originated) neuropathic pain
PTX was first introduced into research on receptor-coupled signalling under the name islet-activating protein in 1979, when Katade and Ui<sup>283</sup> published their experimental findings on a mechanism by which the toxin produced by Bordetella pertussis bacteria enhanced insulin-secretory responses of rat pancreatic islets. Since then, the toxin has proved to be the best probe for major G proteins that are involved in a variety of receptor-coupled intracellular signalings. A PTX substrate G protein was first purified from rat liver.<sup>284</sup> Shortly thereafter, bovine, porcine and rat brains proved to be the best sources for the purification of two PTX substrates, Gi (more precisely, Gi1, where i = inhibitory) and Go (where o = other, as a result of its distinct electrophoretic mobility). The α-subunits are the targets of PTX, with a cysteine unit four residues from the carboxy terminus being the site for the PTX-catalysed transfer of the adenosine diphosphate-ribosyl moiety of nicotinamide adenine dinucleotide. This covalent modification of the α-subunits of Gi/o proteins effectively uncouples the receptor system. Werling et al.<sup>285</sup> were one of the first groups to demonstrate that PTX treatment of brain membranes shifted μ-opioid receptors to the low-affinity state with concomitant loss of the guanine nucleotide regulatory effect on receptor binding. This was an early illustration of the classic effect of PTX-induced receptor uncoupling leading to loss of effector function, or receptor affinity alteration. In animal studies, with the notable exception of buprenorphine,<sup>286,287</sup> the administration of PTX by the i.c.v. or i.t. route results in attenuation of the antinociceptive action of opioids.<sup>288–290</sup> Thus, PTX is able to induce a tolerance-like state in animals that is behaviourally similar to opioid tolerance.<sup>291</sup>

Intrathecal PTX produces symptoms of neuropathic pain
Recently, Womer and colleagues,<sup>292</sup> conducted a series of elegant studies in which they investigated the effects of different doses of i.t. PTX in mice (0.01–0.5 μg per mouse) upon the latency to withdrawal of the tail in water at various temperatures (35–55°C). Preliminary studies by these workers demonstrated that mice treated with i.t. 0.5 μg PTX six days prior to tail-flick testing using a 55°C water bath, showed a hyperalgesic response. Baseline latencies to withdrawal were significantly shorter (<i>p</i> < 0.0001) for PTX-treated mice than for mice administered an i.t. injection of vehicle only, six days prior to tail-flick testing (Figure 3). To investigate further the relationship between the magnitude of the nociceptive stimulus (i.e. temperature of the water bath) and the hyperalgesic response, separate groups of mice were administered i.t. vehicle only, or 0.1 or 3.0 μg PTX, and, seven days later, tail-flick latencies were determined as a function of water bath temperature. Mice treated previously with PTX had significantly shorter tail-flick latencies at temperatures of 40°C and higher. An analysis of variance with repeated measures demonstrated a significant difference (<i>p</i> < 0.01) between the PTX-treated groups and the vehicle-treated group at water temperatures of 40, 45, 50 and 55°C. The time-course of onset and duration of
the effects of i.t. PTX administration were determined by injecting different groups of mice with either vehicle or PTX (0.01–0.3 µg) and measuring tail-flick latencies using a 45°C water bath at varying time points for up to 105 days (Figure 4). After the i.t. administration of vehicle, mean response latencies were always greater than 16 seconds. One day after PTX administration, tail-flick latencies were decreased (for clarity, only data for one dose of PTX are plotted in Figure 4).

During the course of tail-flick testing, Womer and colleagues observed that mice exhibited exaggerated tail-rattling after the tail was lightly touched, indicating the presence of mechanical allodynia. Using a non-noxious stimulus (gauze), stroking of the tail (for further details of method see Womer et al.292) elicited no positive responses out of 75 trials in the control mice. In contrast, in the PTX-treated mice there were 36 (48%) positive responses out of 75 trials. After further characterization of the time-course of the effects of a range of doses of PTX, Womer and coworkers292 determined that the persistent thermal hyperalgesia and allodynia produced by PTX are similar to those produced by neuropathic pain models involving nerve ligation or section.293–295

Summary
In summary, PTX administration in mice results in clearly demonstrable thermal allodynia (determined at 45°C which is not noxious in normal mice), thermal hyperalgesia (determined at 50°C and 55°C) and mechanical allodynia (stroking tail with gauze).

PTX may effect disinhibition of excitatory pathways
Although the mechanisms whereby PTX administration produces hyperalgesia and allodynia require further elucidation, Womer et al.292 speculate that the inactivation of Gi and Go by PTX leads to an inactivation of tonic and phasic inhibitory effector systems that are normally under the control of these G proteins. The inactivation of these regulatory systems would, they propose, lead to an apparent increase in the activity of excitatory pathways. The result would be a ‘wind-up’ of the central pathways of nociceptive processing, with exaggerated and aberrant responses to both innocuous and noxious stimulation. They conclude that the i.t. administration of PTX causes hyperalgesia and allodynia that appears similar to the symptoms reported by patients suffering from neuropathic pain, suggesting that deficiencies in inhibitory systems, compared with increases in excitatory systems, may play a role in the pathophysiology of at least some central or neuropathic pain states (see Womer et al.292 for further details of clinical relevance).

Buprenorphine: a unique opioid in a new paradigm of neuropathic pain
The elegant characterization by Womer’s group of the effects of PTX confirmed observations from earlier studies286,287 that morphine-induced antinociception is blocked in a dose-dependent manner by PTX, resulting in complete blockade after a 0.3 µg dose of PTX. Other workers have similarly demonstrated that i.t. PTX reduces the analgesic potency of etorphine and fentanyl,296 and that of the highly selective µ-agonist, PL017.291,297 Womer and coworkers292 add that their observations with morphine are consistent
with the fact that the analgesic signal transduced by morphine is mainly via PTX-sensitive inhibitory systems, and that, clinically, morphine is relatively less effective in treating central and neuropathic pain states than in treating acute pain. The results of other studies using antisense ODNs directed against Giα1, Giα2, Giα3, Goα, Gsα, Gqα and Gzα confirmed that, for morphine, morphine-6β-glucuronide, [D-Pen²-D-Pen⁵] enkephalin, DAMGO and U50,488H, whether administered i.c.v. or spinally, antinociception was transduced in all mice tested by both PTX-sensitive and PTX-insensitive pathways and, for none of these opioids, was antinociception eliminated by an antisense probe targeted at a PTX-insensitive G protein α-subunit.²⁶¹,²⁹⁸ Indeed, I am aware of only two studies, one using buprenorphine,²⁸⁶ and the other using naloxone benzoylhydrazone (NalBzoH),²⁶¹ that demonstrated unequivocally antinociception/analgesia that was entirely conditional upon agonist activation of a PTX-insensitive pathway(s).

In the rat tail-flick test, buprenorphine-mediated antinociception was not affected by the prior administration of PTX, whereas, in the same study, consistent with the recent results of Womer et al.,²⁹² morphine antinociception was eliminated within the range 0.1–30.0 mg morphine/kg administered subcutaneously (s.c.)²⁸⁶ (Figure 5). In phase 2a of the formalin test (i.e. the later phase of spontaneous behaviour that characterizes a prolonged tonic behavioural pain

Figure 4  Dose- and time-related effects of i.t. PTX on tail-flick latency in mice.

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**Figure 4** Dose- and time-related effects of i.t. PTX on tail-flick latency in mice.  
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*vehicle; — 0.3 µg PTX. (Adapted from Womer et al.²⁹² Note: for plots using 0.01–0.1 µg PTX, refer to this reference.)*
response) in the adult rat, buprenorphine antinociception was modified by PTX only at the highest doses tested (above 1 mg/kg s.c.), whereas, with morphine, antinociception was significantly attenuated below 3 mg/kg s.c. At high doses of morphine (10 and 20 mg/kg s.c.), the effects of PTX on morphine antinociception were progressively diminished.\textsuperscript{286} These novel data can be interpreted to imply that buprenorphine activates PTX-insensitive pathways in preference to PTX-sensitive pathways, the latter only becoming progressively available with increasing dose.\textsuperscript{259}

In the tail-flick test, analgesia (defined quantitatively as ‘doubling or greater of baseline flick latencies’) after the administration of NalBzoH (5 µg i.c.v.) was eliminated in all animals treated by a single i.c.v. injection of antisense ODN to Gz\textsubscript{a}.\textsuperscript{261} It is important that, like buprenorphine, the preferential activation of the PTX-insensitive Gz\textsubscript{a} subunit by NalBzoH may be attributable to an atypical receptor binding profile.\textsuperscript{300–304} However, it remains to be determined whether the receptor-binding affinity of NalBzoH to the supraspinal \(\kappa_3\) receptor, \textsuperscript{305,306} at which this compound mediates analgesia, is sensitive, for example, to regulation by guanine nucleotide/sodium (GW Pasternak, personal communication, 1996). In the tail-flick test, mice that are tolerant to buprenorphine demonstrate cross-tolerance to NalBzoH and, similarly, mice that are tolerant to NalBzoH demonstrate cross-tolerance to buprenorphine.\textsuperscript{307}

Finally, Avidor-Reiss \textit{et al.},\textsuperscript{308} using COS-7 cells transfected with adenylyl cyclase type V isoform and \(\mu\)-opioid receptor cDNAs, demonstrated that superactivation of either forskolin- or 12-0-tetradecanoylphorbol-13-acetate-stimulated cyclic adenosine monophosphate (cAMP) production after prolonged exposure to the \(\mu\)-agonists morphine or DAMGO is conditional upon activation of the PTX-sensitive Gi/o protein. Thus, the earlier observation by Thomas and Hoffman\textsuperscript{309} that chronic treatment of SK-N-SH human neuroblastoma cells with buprenorphine does not result in superactivation of adenylyl cyclase, whereas, in the same study, etorphine classically enhanced prostaglandin E\textsubscript{1}-stimulated cAMP synthesis, is entirely consistent with both the results of Avidor-Reiss and coworkers,\textsuperscript{308} reported above, and the observation of Wheeler-Aceto and Cowan\textsuperscript{286} that buprenorphine antinociception, in comparison with that of morphine, in both the tail-flick and formalin tests in the rat, is significantly, and at some dosages, exclusively, transduced by PTX-insensitive \(G\) protein-coupled pathways. Indeed, the thermodynamic equilibrium afforded by the atypical receptor binding profile of buprenorphine is consistent with activation of Gz, G\textsubscript{12} or G\textsubscript{13}, which are all PTX-insensitive, but not that of Gi/o,\textsuperscript{310} which are PTX-sensitive.

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**Figure 5** Morphine antinociception is uncoupled by pretreatment with PTX, whereas buprenorphine antinociception is relatively unaffected. (Adapted from Wheeler-Aceto and Cowan.\textsuperscript{286} Reproduced from McCormack \textit{et al.},\textsuperscript{311} with kind permission of Elsevier Science Ireland Ltd.)
Concluding remarks

The evidence presented in Part I strongly supports the view that, in the management of neuropathic pain, exogenous intervention activates counter-responses or ‘failsafe’ mechanisms. These mechanisms act to ensure signalling fidelity within the regenerating neurone, and, as a result, diminish the effectiveness of attempts at pain relief. Thus, clinicians may be faced with a therapeutic dilemma: the pharmacotherapy of pain in the dysfunctional neurone may possibly compromise regeneration, thereby perpetuating the pain state. No systematic studies have yet been undertaken in order to investigate such a possibility.

Table 1 Buprenorphine: a unique opioid and a lead candidate for future studies in neuropathic pain

<table>
<thead>
<tr>
<th>Morphine</th>
<th>Buprenorphine</th>
<th>Comments/significance</th>
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<tbody>
<tr>
<td>PTX-sensitive analgesia in both phasic and tonic pain models.286</td>
<td>PTX-insensitive analgesia.286</td>
<td>The intrathecal administration of PTX was recently characterized as a new paradigm of centrally-originating neuropathic pain.292 Unlike the effects of other opioids so far tested, buprenorphine analgesia is not blocked in this model.286 This distinction is likely to be clinically important.292</td>
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In the rat tail-flick test, morphine and methadone antinociception is only modestly enhanced or attenuated by K\textsubscript{ATP} channel openers or blockers, respectively.262,282 Fentanyl and levorphanol demonstrate no interactions with K\textsubscript{ATP} agents.262,282

In the formalin model of tonic pain, morphine, fentanyl and pethidine attenuate nociceptive behavioural responses with similar potency in both neonate and adult rats.313 (Ratio of neonate:adult = 3.7 (morphine), 1.8 (fentanyl), 2.1 (pethidine); p > 0.05). Buprenorphine is uniquely sensitive to the effects of K\textsubscript{ATP} channel openers and blockers.262,282

Buprenorphine is significantly more potent in the neonate formalin test.313 (Ratio of neonate:adult = 12.2; p < 0.05). Afferent processing in the early neonate rat is typified by several behavioural, anatomical and functional features, which, although not pathological, are characteristic of those observed in models of nerve injury using the adult rat.311 Notably, these features include a lack of segmental inhibition (‘disinhibition’) and afferent input in large myelinated fibres that make synaptic contacts within the superficial laminae of the dorsal horn, which in the adult are predominantly nociceptive.311 These new findings provide further evidence in support of a functionally-discrete transduction pathway for buprenorphine analgesia that is not accessible to other opioids.311
Table 1  continued

<table>
<thead>
<tr>
<th>Morphine</th>
<th>Buprenorphine</th>
<th>Comments/significance</th>
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<tr>
<td>In the cold ethanol (–20 °C) tail-flick test, dose–response curves for morphine and nalbuphine are superimposable. (^{314})</td>
<td>The dose–response curve of buprenorphine is displaced to the left (i.e. more potent) by three orders of magnitude relative to that of either morphine or nalbuphine. (^{314})</td>
<td>Cold hyperalgesia is relatively resistant to morphine in a model of chronic nerve injury, and, surprisingly, also in a model of inflammatory pain. (^{315}) Morphine is without effect on, or even enhances, the activity of many lamina (^{316}) spinal nociceptive neurones with projections to the parabrachial complex or thalamus (two brainstem regions receiving cold-specific afferents). Whereas in the rat tail-flick test at 55 °C, buprenorphine is more potent than morphine by a small factor (approx. (\times 10)), the difference of three orders of magnitude at –20 °C, cannot currently be explained and merits further investigation. The relevance of DNIC to the management of neuropathic pain is unknown. However, the powerful effect of buprenorphine upon DNIC may in some part explain the recent observation that the simultaneous administration of systemic buprenorphine with spinal morphine results in a supra-additive antinociceptive effect. (^{320}) (It should be noted that this synergy does not accord with classic dogma, which dictates that a ‘partial agonist’ in combination with a ‘full agonist’ will lead to a subadditive effect.)</td>
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<tr>
<td>Morphine blocks diffuse noxious inhibitory controls (DNIC) triggered by heterotopic noxious stimuli. (^{317-318}) (Note: blockade of DNIC has been elegantly argued as representing an analgesic mechanism of action of opioids. (^{317-319}))</td>
<td>In comparison with morphine, buprenorphine is a remarkably powerful blocker of DNIC, requiring a dose that is three orders of magnitude less than that of morphine. (^{319})</td>
<td></td>
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<tr>
<td>In common with most opioid agonists studied, receptor-binding affinity of morphine is regulated by guanine nucleotides (e.g. GTP) and sodium ions according to the classic subunit dissociation theory of G protein activation.</td>
<td>Buprenorphine demonstrates atypical receptor binding, which is generally not modulated according to classic theory. (^{275})</td>
<td>In some part, the enhanced residence of buprenorphine in the high-affinity binding state may explain buprenorphine’s capacity to activate PTX-insensitive pathways; activation (i.e. exchange of GDP (guanosine diphosphate) for GTP) of Gz, for example, is very slow and transient high-affinity states may result in only subthreshold stimulation. (Note: such effects would be in addition to conformational changes induced upon receptor binding. (^{321}))</td>
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In Part II, I have emphasized two recent discoveries. First is the importance of the Gβγ subunit in signalling in general, and in signalling convergence between opioids (and other agents that act through GPCRs) and growth factors (which act through RTKs), in particular. Secondly, I presented evidence in support of differences that exist between opioids in transduction of analgesia. Both of these discoveries may provide renewed optimism for the refined use of opioids in managing some neuropathic pains. That such differences between opioids may be exploited in order to achieve more effective pain relief is exemplified by buprenorphine, which transduces analgesia via PTX-insensitive pathways (Figure 5), and clearly demonstrates several important differences between other opioids such as morphine and fentanyl, for example (Table 1).

How can signalling along PTX-insensitive pathways produce divergence such that buprenorphine, for example, activates the K_{ATP} channel, whereas fentanyl and levorphanol have no effect (Figure 2)? In some part, the answer may be provided by looking at the work of Fields and Casey. Using purified Gα subunits (i.e. the α-subunit of the PTX-insensitive Gz) obtained from baculovirus infection of Sf9 cells, these workers observed that, once phosphorylated, Gαz has a greatly decreased ability to associate with Gβγ. Most importantly, these workers concluded that rapid phosphorylation of Gαz•GDP by activated PKC would not allow further recycling of Gαz. Support for this view was provided by Kozasa and Gilman, who examined nine Gα subunits and observed that only the PTX-insensitive G proteins served as substrates for phosphorylation by various isoforms of PKC. Thus, they confirmed the earlier observations by Fields and Casey that phosphorylated α-subunits of PTX-insensitive G proteins have reduced affinity for Gβγ in comparison with the unmodified Gα subunits. Subsequently, it has been proposed by two independent groups that this prevention of subunit re-association could potentiate Gβγ-mediated signalling by creating a pool of free Gβγ that could continue to modulate effector activity.

Support for a functional role of the selective phosphorylation by PKC of PTX-insensitive Gα subunits follows the observation by Hinton et al. that immunolocalization of PKC within the CNS corresponds closely with that of Gαz. Given the colocalization of opioid and CCK receptors throughout the CNS, then, after neuronal injury, the CCK_{B}-mediated activation of PKC (via DAG) in mediating the anti-opioid effects of CCK (via PYK2) may, paradoxically, also enhance analgesic signalling mediated along PTX-insensitive pathways.

With the discovery that PLA2 may be a messenger of the CCK_{B} receptor, there arise new targets for attenuating the effects of CCK upon opioid analgesia. It is interesting to note that the inhibitory effects of glucocorticosteroids, ketamine, phenytoin and imipramine on PLA2 activity, together with the potentiation of imipramine binding to synaptosomal membranes by PLA2 may, in some part, contribute to the reported efficacy of these drugs in managing neuropathic pains (refer to key points previously listed under ‘PLA2: an anti-opioid messenger of the CCK_{B} receptor?’).

In Part II, in attempting a more in-depth analysis of signalling and of signalling convergence within the injured neuronal cell, I have set the scene for Part III. In this final part, in addition to evaluating the options for developing more effective therapies for the management of neuropathic pains, I also look again at existing therapies, in particular the opioids.

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