British Journal of Pharmacology (1999) 128, 220–226
http://www.stockton-press.co.uk/bjp

Involvement of phospholipid signal transduction pathways in morphine tolerance in mice

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1 Opioid tolerance involves an alteration in the activity of intracellular kinases such as cyclic AMP-dependent protein kinase (PKA). Drugs that inhibit PKA reverse morphine antinociceptive tolerance. The hypothesis was tested that phospholipid pathways are also altered in morphine tolerance. Inhibitors of the phosphatidylinositol and phosphatidylcholine pathways were injected i.c.v. in an attempt to acutely reverse morphine antinociceptive tolerance.

2 Seventy-two hours after implantation of placebo or 75 mg morphine pellets, mice injected i.c.v. with inhibitor drug were challenged with morphine s.c. for generation of dose-response curves in the tail-flick test. Placebo pellet-implanted mice received doses of inhibitor drug having no effect on morphine's potency, in order to test for tolerance reversal in morphine pellet-implanted mice. Injection of the phosphatidylinositol-specific phospholipase C inhibitor ET-18-OCH_3 significantly reversed tolerance, indicating a potential role for inositol 1,4,5-trisphosphate (IP_3) and protein kinase C (PKC) in tolerance. Alternatively, phosphatidylcholine-specific phospholipase C increases the production of diacylglycerol and activation of PKC, without concomitant production of IP_3. D609, an inhibitor of phosphatidylserine-specific phospholipase C, also reversed tolerance. Heparin is an IP_3 receptor antagonist. Injection of low molecular weight heparin also reversed tolerance. PKC was also examined with three structurally dissimilar inhibitors. Bisindolylmaleimide I, Go-7874, and sangivamycin significantly reversed tolerance.

3 Chronic opioid exposure leads to changes in phospholipid metabolism that have a direct role in maintaining a state of tolerance. Evidence is accumulating that opioid tolerance disrupts the homeostatic balance of several important signal transduction pathways.

Keywords: Morphine tolerance; phospholipid metabolism; phosphatidylinositol cascade; protein kinase C; analgesia, inositol 1,4,5-trisphosphate

Abbreviations: DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PtdCholine, phosphatidylcholine; PtdCholine-PLC, phosphatidylcholine-specific phospholipase C; PtdIns, phosphatidylinositol; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns-PLC, phosphatidylinositol-specific phospholipase C

Introduction

The significance of phosphorylation events in acute and chronic opioid exposure has been investigated using primarily *in vitro* methodological approaches. The conclusion of most studies is that signal transduction events are changed in response to the chronic presence of opioid. Behavioural measures have been increasingly used to implicate specific signal transduction events in mediating opioid tolerance and dependence. Recently, Bernstein & Welch (1997) reported that an inhibitor of PKA (KT5720) injected i.c.v. reversed antinociceptive tolerance in morphine pellet-implanted mice. Phospholipid signal transduction systems have also been implicated in opioid tolerance, with most studies focusing on protein kinase C (PKC). For example, PKC inhibitors such as chelerythrine chloride, H7 and calphostin C were able to prevent or reverse acute antinociceptive tolerance to *mu*- and *delta*-opioid agonists (Fundytus & Coderre, 1996; Bilsky *et al*., 1996; Narita *et al*., 1995; 1996). Furthermore, tolerance has been associated with changes in the activity and levels of PKC throughout the neuraxis (Narita *et al*., 1994; Mao *et al*., 1995; Ventayol *et al*., 1997). The purpose of this study was to examine the involvement of different components of the phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCholine) pathways in morphine tolerance. The hypothesis was tested that central administration of inhibitors of multiple components of phospholipid pathways would lead to an acute reversal of morphine tolerance. Our data indicate that both the PtdIns and PtdCholine pathways contribute to morphine tolerance.

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Methods

Methods of handling mice

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN, U.S.A.) weighing 25–30 g were housed six to a cage in animal care quarters maintained at $22\pm2^{\circ}C$ on a 12-h light–dark cycle. Food and water were available *ad libitum*. The mice were brought to a test room ($22\pm2^{\circ}C$, 12 h light–dark cycle), marked for identification and allowed 24 h to recover from transport and handling. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical College of Virginia of Virginia Commonwealth University.

Surgical implantation of pellets

Mice were anaesthetized with ether before depilation of the hair around the base of the neck. Adequate anaesthesia was noted by the absence of the righting-reflex, and lack of response to toe-pinch, according to IACUC guidelines. The skin was cleansed with 10% providone iodine (General Medical Corp., Prichard, WV, U.S.A.) and rinsed with alcohol before making a 1 cm transverse incision at the base of the neck. The underlying subcutaneous space toward the dorsal flanks was opened using a sterile glass rod. Maintenance of a stringent aseptic surgical field minimized any potential contamination of the pellet, incision and subcutaneous space. A placebo pellet or a 75 mg morphine pellet was inserted in the space before closing the site with Vetbond Tissue Adhesive (3M Animal Care Products, St. Paul, MN, U.S.A.), and again applying providone iodine to the surface. The animals were allowed to recover in their home cages, where they remained throughout the experiment.

Intracerebroventricular injections

Intraventricular injections were performed as described by Pedigo *et al.* (1975). Mice were anaesthetized with ether and a transverse incision was made in the scalp. A free-hand 5 μl injection of the drug or the vehicle was made into the lateral ventricle. The extensive experience of this laboratory has made it possible to inject drugs with a greater than 95% accuracy. Immediately upon testing the animals were euthanized to minimize any type of distress, according to IACUC guidelines.

The tail-flick test

The tail-flick test used to assess for antinociception in mice was developed by D'Amour & Smith (1941) and modified by Dewey *et al.* (1970). Before injections the base-line (control) latency for each mouse was determined. Only mice with a control reaction time from 2–4 s were used. The test latency after drug treatment was assessed at the appropriate time, and a 10 s maximum cut-off time was used to prevent damage to the tail. Antinociception was quantified according to the method of Harris & Pierson (1964) as the percentage of maximum possible effect (% MPE) which was calculated as: % MPE = [(test – control) (10 – control)$^{-1}$] × 100. Per cent MPE was calculated for each mouse using at least six mice per dose.

Experimental design

Drugs that inhibit the PtdIns and PtdCholine pathways were tested for their ability to reverse morphine tolerance.

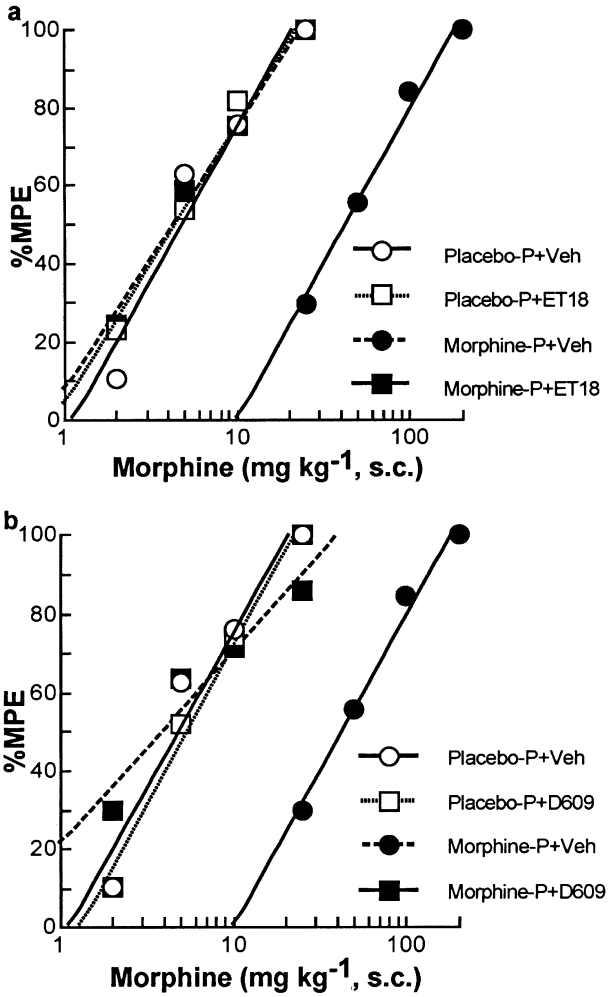

Figure 1 (a) Inhibition of PtdIns(4,5)P_2-specific PLC reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with ET-18-OCH_3 (9.5 nmol, i.c.v.). Immediately after i.c.v. injection the mice received morphine s.c., and were then tested 30 min later. (b) Inhibition of PtdCholine-specific PLC reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with D609 (94 nmol, i.c.v.). Immediately after i.c.v. injection the mice received morphine s.c., and were then tested 30 min later.

Table 1 Role of phospholipase C in morphine tolerance in mice

Group	*Treatment i.c.v.*	*ED_{50} mg kg^{-1}* (95% c.l.)	*Potency ratio* (95% c.l)	
Placebo-P	Veh	4.9 (3.8–6.5)	–	–
Morphine-P	Veh	42.7 (36.3–50.2)*	vs Pbo + Veh	8.6 (6.4–11.6)*
Placebo-P	ET-18-OCH_3	4.4 (3.3–5.8)	vs Pbo + Veh	0.9 (0.6–1.4)
Morphine-P	ET-18-OCH_3	4.3 (3.3–5.7)	vs Pbo + ET	1.0 (0.7–1.5)
			vs MP + Veh	9.2 (6.7–12.6)‡
Placebo-P	D609	5.4 (3.9–7.5)	vs Pbo + Veh	0.9 (0.7–1.7)
Morphine-P	D609	3.9 (2.2–6.9)	vs Pbo + D609	1.1 (0.7–1.7)
			vs MP + Veh	8.4 (4.9–13.9)‡

Seventy-two hours after aurgical implantation of placebo or 75 mg morphine pellets, mice received morphine s.c. and were tested 30 min later in the tail-flick test. Immediately before morphine, the mice were injected i.c.v. with ET-18-OCH_3 or D609 in distilled water. *Significantly different from Placebo-P/Veh group. ‡Significantly different from Morphine-P/Veh group.

Baseline tail-flick latencies were obtained before administration of morphine s.c. and vehicle or test drug i.c.v. Test latencies were measured 30 min after morphine administration, and injection of i.c.v. drugs. Since the overall hypothesis was that inhibition of the PtdIns and/or PtdCholine pathways would reverse tolerance, dose and time-course experiments were first conducted in tolerant animals. Using a dose of morphine s.c. calculated to elicit 20% MPE, increasing doses of inhibitor drug were injected i.c.v. in different groups of tolerant mice. Doses of inhibitor drug were adjusted to maximally reverse tolerance (i.e., an increase from 20% MPE to 80–100% MPE). Following this, the time the inhibitor drug was injected was varied in several groups to estimate the peak effect. Based on these parameters, placebo pellet-implanted mice received morphine calculated to elicit 20% MPE and the inhibitor drug. If the drug enhanced antinociception in placebo pellet-implanted mice, the dose was reduced until it had no effect, and was then re-tested in morphine pellet-implanted mice. Thus, inhibitor drug doses were adjusted to reverse morphine tolerance without affecting placebo pellet-implanted mice. For the sake of brevity, data on modulating agent dose and time-course studies are not shown. Morphine dose-response curves were generated for calculation of ED_{50} values and 95% confidence limits. These values were calculated using least squares linear regression analysis followed by calculation of 95% confidence limits by Bliss (1967). Tests for parallelism according to Tallarida & Murray (1987) were conducted before calculation of potency ratio values and 95% confidence limits by the method of Colquhoun (1971). A potency ratio value greater than one, with the lower 95% confidence limit greater than one, was considered a significant difference in potency.

Drugs and chemicals

The 75 mg morphine pellets were obtained from the National Institute on Drug Abuse (Bethesda, MD, U.S.A.). Morphine sulphate was dissolved in pyrogen-free isotonic saline (Baxter Healthcare Corp., Deerfield, IL, U.S.A.). Et-18-OCH_3 (1-O-Octadecyl-2-O-methyl-*rac*-glycero-3-phosphorylcholine), D609 (Tricyclodecan-9-yl-xanthogenate, potassium), bisindolylmaleimide I, HCl (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) and Go 7874 hydrochloride from Calbiochem (San Diego, CA, U.S.A.) were all dissolved in distilled water. Sangivamycin (7-deaza-7-carbamoyladenosine) (Calbiochem, San Diego, CA, U.S.A.) was solubilized in 10% DMSO in distilled water. Low molecular weight heparin (6000 Da) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in distilled water.

Results

Role of phospholipase C (PLC) in morphine tolerance

The tolerance observed was consistent with the first published characterization of morphine pellets in mice (Patrick *et al*., 1975). The animals in this study were also injected i.c.v. with vehicle or PLC inhibitors. Doses of PLC inhibitors were adjusted to have no effect in placebo pellet-implanted mice, so their effects in tolerant mice could be assessed. As seen in Figure 1a, the phosphatidylinositol-specific phospholipase C (PtdIns-PLC) inhibitor ET-18-OCH_3 (9.5 nmol, i.c.v.) significantly reversed morphine tolerance (Table 1). In addition, the phosphatidylcholine-specific phospholipase C (PtdCholine-PLC) inhibitor D609 (94 nmol, i.c.v.) also significantly reversed tolerance (Figure 1b; Table 1). Control experiments revealed no antinociceptive effects of ET-18-OCH_3 and D609 in s.c. saline injected placebo and morphine pellet-implanted mice (i.e. $<15\%$ MPE).

Role of IP_3-sensitive Ca^{2+} pools in morphine tolerance

PtdIns-PLC converts phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) into DAG and IP_3. The hypothesis was tested that Ca^{2+} released from IP_3-sensitive Ca^{2+} pools contributes to the expression of morphine tolerance. Low molecular

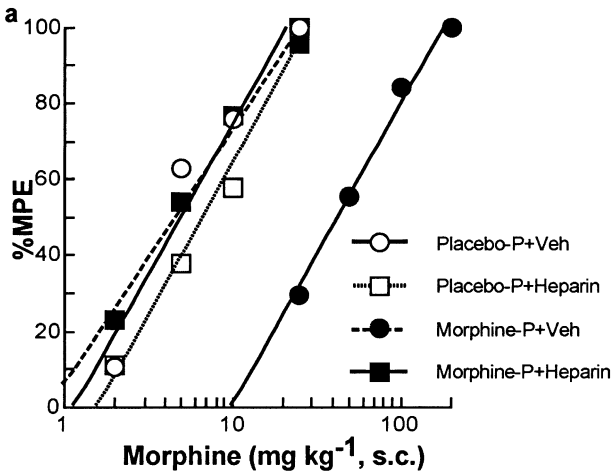

Figure 2 Antagonism of IP_3 receptors with low-molecular-weight heparin reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with low-molecular-weight heparin (6000 Da) (10 μg, i.c.v.). Immediately after i.c.v. injection the mice received morphine s.c., and were then tested 30 min later.

Table 2 Role of IP_3-sensitive CA^{2+} pools in morphine tolerance in mice

Group	*Treatment i.c.v*	*ED_{50} mg kg^{-1}* (95% c.l.)	*Potency ratio* (95% c.l.)	
Placebo-P	Veh	4.9 (3.8–6.5)	–	–
Morphine-P	Veh	42.7 (36.3–50.2)*	vs Pbo + Veh	8.6 (6.4–11.6)*
Placebo-P	Heparin	6.7 (4.8–9.4)	vs Pbo + Veh	0.7 (0.5–1.1)
Morphine-P	Heparin	4.6 (3.4–6.3)	vs Pbo + Hep	0.7 (0.4–1.2)
			vs MP + Veh	8.6 (6.0–12.0)‡

Seventy-two hours after surgical implantation of placebo or 75 mg morphine pellets, mice recieved morphine s.c. and were tested 30 min later in the tail-flick test. Immediately before morphine, the mice were injected i.c.v. with low molecular weight heparin in distilled water. *Significantly different from Placebo-P/Veh group. ‡Significantly different from Morphine-P/Veh group.

weight heparin (6000 Da) is a membrane permeable selective antagonist of IP_3 receptors. Heparin (10 μg, i.c.v.) had no effect in placebo pellet-implanted mice but significantly reversed tolerance in morphine pelleted mice (Figure 2; Table 2). Control experiments revealed no antinociceptive effects of hepain in s.c. saline injected placebo and morphine pellet-implanted mice (i.e. $<10\%$ MPE).

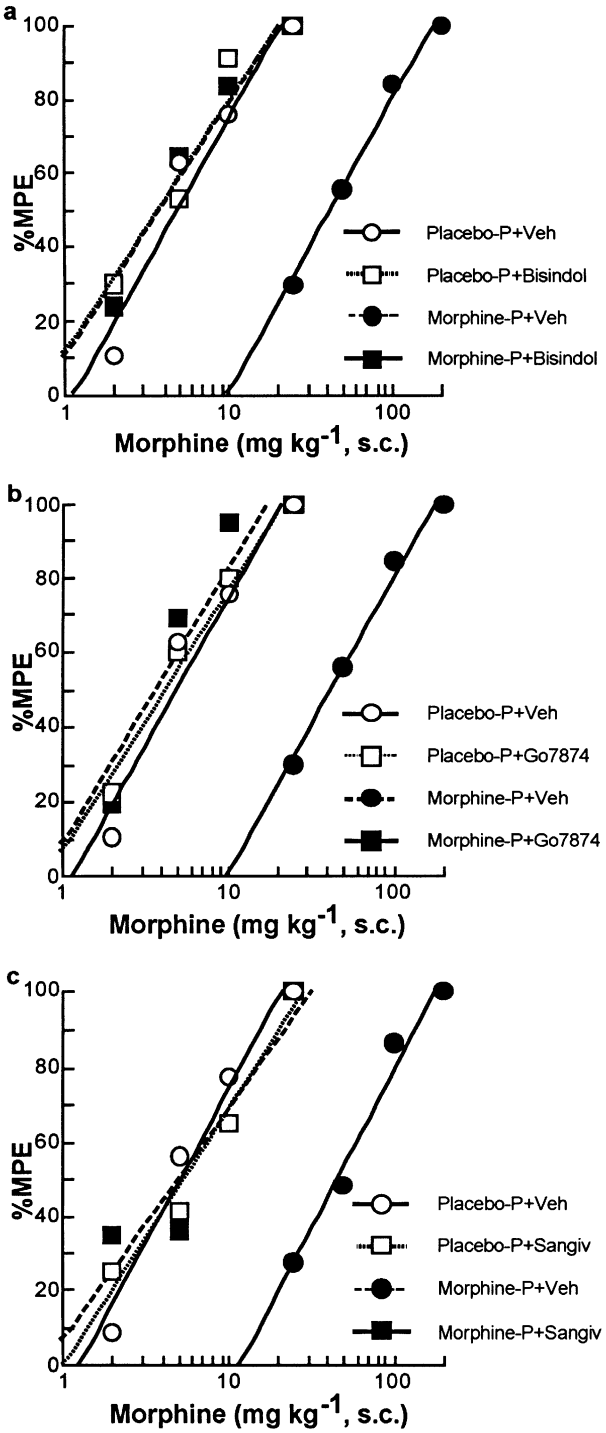

Figure 3 The protein kinase C inhibitors. (a) Bisindolylmaleimide I, (b) Go7874 and (c) sangivamycin reverse morphine tolerance. Baseline tail-flick latencies were obtained before injection mice with bisindoylmalemide I (11.1 nmol, i.c.v.), Go7874 (1.0 nmol, i.c.v.) or sangivamycin (8.1 nmol, i.c.v.). Immediately after i.c.v. injection the mice received morphine s.c., and were then tested 30 min later.

Role of PKC in morphine tolerance

The production of DAG by PLC leads to the translocation and activation of PKC in the membrane. Three structurally dissimilar PKC inhibitors were tested for their ability to reverse morphine tolerance. I.c.v. injection of bisindolylmaleimide I (11.1 nmol, i.c.v.), Go7874 (1.0 nmol, i.c.v.) and sangivamycin (8.1 nmol, i.c.v.) each significantly reversed tolerance in morphine pellet-implanted mice (Figure 3a, b, c, Tables 3 and 4).

Discussion

Role of PLC in morphine tolerance

To our knowledge, this is the first report demonstrating the reversal of morphine toleerance by selective inhibitors of PLC. ET-18-OCH_3 is a highly selective ether lipid analogue inhibitor of PtdIns-PLC, which converts PtdIns (4,5)P_2 into equimolar concentrations of DAG and IP_3. ET-18-OCH_3 does not antagonize PtdCholine-PLC or phospholipase D (for a review see Powis & Phil, 1994). It is thought that ET-18-OCH_3 disturbs phospholipid metabolism by accumulating in the membrane (Helfman *et al*., 1983). Our results indicate that both products of hydrolysis, IP_3 and DAG, contribute to the expression of tolerance (Figure 1a, Table 1). It is noteworthy that antagonists of metabotropic glutamate receptors $mGluR_1$ and $mGluR_2$, positively linked to activation of the PtdIns system, attenuated naloxone precipitated withdrawal in rats (Fundytus *et al*., 1997). Thus, both IP_3 and DAG may be important in the maintenance of opioid tolerance and physical dependence. The results with heparin, as discussed later, indicates a role for IP_3-sensitive Ca^{2+} pools in morphine tolerance. In addition, DAG production leads to the translocation and activation of PKC, which appears from this study to contribute to tolerance.

The possibility that PKC activation contributes to morphine tolerance was supported by the reversal of tolerance with D609, a PtdCholine-PLC inhibitor (Figure 1b, Table 1). The hydrolysis of PtdCholine by PtdCholine-PLC provides another source of DAG, without concomitant production of IP_3. Stimulation of this pathway leads to the translocation and activation of PKC (Billah & Anthes, 1990; Dennis *et al*., 1991). Therefore, the results with both ET-18OCH3 and D609 indicate that phospholipid metabolism may be more active in neurons crucial to the expression of opioid tolerance. As a consequence, it is predicted that both PKC activity and the levels of IP_3 would also be increased, as discussed later.

It remains to be revealed in which brain regions the PtdIns and PtdCholine systems may be affected, since all agents were administered i.c.v. The periaqueductal gray (PAG), lining the aqueduct between the third and fourth ventricle, is readily accessible to drugs injected into the lateral ventricles of the brain. This region, besides possessing *mu*-opioid receptors and receiving β-endorphin-containing terminals from cell bodies in the arcuate nucleus, plays an important role in modulating the activity of bulbospinal monoaminergic antinociceptive systems (Aston-Jones *et al*., 1991; Reichling *et al*., 1988). These descending pathways have been shown to inhibit the transmission of nociception at the level of the spinal dorsal horn. *Mu*-opioid agonists injected directly into the PAG elicits not only dose-dependent antinociception, but significantly reduces the levels of intra-neuronal Ca^{2+} (Zhang *et al*., 1992). Alternatively, phorbol ester injection i.c.v. antagonizes opioid antinociception (Narita *et al*., 1997; Ohsawa & Kamei, 1997).

Table 3 Role of protein kinase C in morphine tolerance in mice

Group	Treatment i.c.v	ED_{50} mg kg^{-1} (95% c.l.)	Potency ratio (95% c.l.)	
Placebo-P	Veh	4.9 (3.8–6.5)	–	–
Morphine-P	Veh	42.7 (36.3–50.2)*	vs Pbo + Veh	8.6 (6.4–11.6)*
Placebo-P	Bisindolylmaleimide	3.8 (2.9–4.9)	vs Pbo + Veh	0.8 (0.6–1.2)
Morphine-P	Bisindolylmaleimide	3.9 (2.8–5.5)	vs Pbo + Bis	1.0 (0.7–1.5)
			vs MP + Veh	10.1 (7.1–14.3)‡
Placebo-P	Go 7874	4.2 (3.0–6.0)	vs Pbo + Veh	0.9 (0.6–1.4)
Morphine-P	Go 7874	3.7 (2.7–4.9)	vs Pbo + Go	0.8 (0.5–1.2)
			vs MP + Veh	11.2 (8.5–14.8)‡

Seventy-two hours after surgical implantation of placebo or 75 mg morphine pellets, mice received morphine s.c. and were tested 30 min later in the tail-flick test. Immediately before morphine, the mice were injected i.c.v. with bisindolylmaleimide I of Go 7874 in distilled water. *Significantly different from Placebo-P/Veh group. ‡Significantly different from Morphine-P/Veh group.

Table 4 Further characterization of protein kinase C in morphine tolerance in mice

Group	Treatment i.c.v	ED_{50} mg kg^{-1} (95% c.l.)	Potency ratio (95% c.l.)	
Placebo-P	Veh	5.3 (3.7–7.4)	–	–
Morphine-P	Veh	46.3 (29.3–54.5)*	vs Pbo + Veh	8.7 (5.9–12.7)*
Placebo-P	Sangivamycin	5.6 (3.6–8.5)	vs Pbo + Veh	0.9 (0.5–1.6)
Morphine-P	Sangivamycin	5.1 (3.6–7.3)	vs Pbo + Sang	1.0 (0.6–1.7)
			vs MP + Veh	8.1 (5.8–11.2)‡

Seventy-two hours after surgical implantation of placebo or 75 mg morphine pellets, mice received morphine s.c. and were tested 30 min later in the tail-flick test. Immediately before morphine, the mice were injected i.c.v with sangivamycin in 10% DMSO distilled water. *Significantly different from Placebo-P/Veh group. ‡Significantly different from Morphine-P/Veh group.

Since the PAG is readily accessible to inhibitors of the phospholipid pathway, it is tempting to speculate that this region may be affected in morphine tolerance.

The issue of whether a stress component from free-hand i.c.v. injection could be modulated by these inhibitors should be addressed. It cannot be completely ruled out that i.c.v. injections are somewhat stressful. Recent evidence indicates that i.c.v. injection of saline in awake mice may be mildly stressful, since the slight increase in hot-plate latency threshold was blocked by nociceptin/Orphanin FQ, an endogenous ligand of opioid receptor like 1 receptors (Suaudeau *et al*., 1998). However, handling and injection stress was minimized by anaesthetizing the animals, as required by IACUC procedures. Furthermore, the direct influence of these phospholipid pathway inhibitors was examined in non-tolerant animals. First, the inhibitors did not alter the threshold for nociception in placebo pellet-implanted animals injected with vehicle s.c. Second, the potency of morphine s.c. was nearly identical in placebo pellet-implanted mice injected with vehicle or inhibitor i.c.v.

Role of IP_3 in tolerance

It is notable that low molecular weight heparin injected i.c.v. significantly reversed morphine tolerance. Yet interpreting these results is difficult without supportive *in vitro* evidence. Heparin is a potent and selective IP_3 receptor antagonist that prevents Ca^{2+} release from intracellular Ca^{2+} pools (Jonas *et al*., 1997). Yet heparin must be injected into cells or perfused onto permeabilized cells because of its high molecular weight (i.e., 12,000–30,000 Da) and lack of membrane permeability. Some evidence indicates that the low molecular weight heparin (i.e., 6000 Da) used in this study is membrane permeable. Perfusion of low molecular weight heparin over a non-permeabilized cerebellar slice preparation attenuated glutamate-stimulated increases in free intracellular Ca^{2+} (Jonas *et al*., 1997). Obviously, other studies measuring intracellular Ca^{2+} release are needed to confirm its membrane permeable properties. However, it is interesting to speculate that heparin may have reversed tolerance by antagonizing IP_3 receptors. In similar fashion, we have shown that ryanodine also reversed morphine tolerance (Smith *et al*., 1999), presumably by blocking Ca^{2+} release from Ca^{2+}/caffeine-sensitive intracellular pools (Friel & Tsien, 1992; Smith & Stevens, 1995).

Alternatively, heparin may act through other cellular mechanisms. Heparin is a potent inhibitor of G protein-coupled receptor kinases (GRKs) (Kunapuli *et al*., 1994). GRKs regulate the responsiveness of *mu*- and *delta*-opioid receptors through agonist-specific receptor phosphorylation, desensitization and internalization (Kunapuli *et al*., 1994; Morikawa *et al*., 1998; Zhang *et al*., 1998). For example, heparin significantly reduced the magnitude and rate of *delta*-opioid receptor desensitization in cultured SK-N-BE and NG108-15 cells (Hasbi *et al*., 1998; Morikawa *et al*., 1998). Thus, it is equally plausible that heparin reversed tolerance by transiently inhibiting GRK. Finally, if low molecular weight heparin did not permeate the cells, extracellular sites should be considered. Heparin can act on cell-surface heparin sulphate proteoglycan sites which were shown to be crucial in enabling the expression of long-term potentiation in the hippocampus (Lauri *et al*., 1999). The function of proteoglycan sites on other neuron types remains to be investigated.

Role of PKC in morphine tolerance

Our data is consistent with the hypothesis that selective inhibitors of PKC reverse morphine tolerance. Others have prevented or reversed acute tolerance to *mu*- or *delta*-opioid

agonists with PKC inhibitors such as chelerythrine chloride, H7 and calphostin C (Fundytus & Coderre, 1996; Bilsky *et al*., 1996; Narita *et al*., 1995; 1996). Both chelerythrine chloride and calphostin C are selective inhibitors of PKC. Our data with bisindolylmalemide I, Go 7874 and sangivamycin further supports the role for PKC in tolerance. Furthermore, both acute tolerance and tolerance resulting from chronic opioid administration appear to be mediated, in part, by changes in PKC levels or activity. Others have shown that Ca^{2+} sensitive PKC enzyme activity is increased in the pons/medulla, but not the midbrain, of morphine tolerant rats (Narita *et al*., 1994). Alternatively, measurements of immunoreactive PKC have yielded mixed results. Chronic morphine, heroin or methadone administration significantly reduce PKC-*alpha*/*beta* immunoreactivity in rat cerebral cortex, brainstem and hypothalamus (Ventayol *et al*., 1997). In opposite fashion, PKC-*gamma* immunoreactivity is significantly increased in spinal cord laminae I and II of morphine tolerant rats (Mao *et al*., 1995). At the very least, these studies, combined with the behavioural data, indicate that PKC plays a major role in expression of opioid tolerance. The recently developed PKC-*gamma* knock-out mouse may provide new insights into the role of one PKC isoform in tolerance (Malmberg *et al*., 1997).

This work was supported by National Institute on Drug Abuse grants DA01647 and DA07027.

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(Received February 3, 1999
Revised May 24, 1999
Accepted June 16, 1999)