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PKC and PKA inhibitors reverse tolerance to morphine-induced hypothermia and supraspinal analgesia in mice

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Abstract

Morphine antinociceptive tolerance in the tail-flick test is completely reversed by inhibitors of protein kinase C (PKC) or cAMP-dependent protein kinase (PKA). The effects of these inhibitors on tolerance to supraspinally mediated antinociception, such as the hot-plate test was unknown, as well as their effects in tests of mechanical nociception. The PKC inhibitors bisinolylmaleimide I ((2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) and Gö-7874 {2[1[(3-Dimethylaminopropyl)-5-methozyindol-3-yl]-3-(1H-indol-3-yl) hydrochloride} completely reversed the tolerance to morphine in both the hot-plate and tail-pinch tests. Similarly, the PKA inhibitor KT-5720 (8*R*, 9*S*, 11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[cde]trinden-1-one also reversed tolerance in both tests. The role of PKC and PKA in mediating tolerance to morphine-induced hypothermia was also investigated. Bisinolylmaleimide I, Gö-7874 and KT-5720 only partly reversed the 32-fold level of tolerance induced by the morphine pellets. However, co-administration of bisinolylmaleimide I with KT-5720 or Gö-7874 with KT-5720 completely reversed the tolerance. This demonstrates that tolerance in a non-behavioral system involves the actions of PKC and PKA.

Keywords: Morphine tolerance; Phosphatidylinositol cascade; Adenylyl cyclase cascade; Analgesia; Hypothermia; (Mouse)

1. Introduction

An increasing number of studies indicate that second messenger pathways activating cAMP-protein kinase (PKA) and Ca^{+}-dependent/independent protein kinase (PKC) play a role in the expression of opioid antinociceptive tolerance. Bernstein and Welch (1997) reported that intracerebroventricular (i.c.v.) injection of mice with the PKA inhibitor KT-5720 (8*R*, 9*S*, 11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[cde]trinden-1-one, but not the protein kinase G inhibitor KT-5823, reversed the tolerance to morphine-induced antinociception in the tail-flick test. In addition, i.c.v. injections of the anti-sense oligodeoxynucleuotide to PKA mRNA blocked the antinociceptive tolerance to morphine in mice (Shen et al., 2000). Phospholipid signal transduction systems have also been implicated in opioid tolerance. We demonstrated that inhibitors of phosphatidylcholine- and phosphatidylinositol-specific phospholipase C reversed morphine tolerance in mice tested in the tail-flick assay (Smith et al., 1999b). Furthermore, PKC plays an important role in the expression of opioid antinociceptive tolerance in both mice and rats. In mice tested in the tail-flick assay, the PKC inhibitors chelerythrine chloride (intrathecal, i.t.), calphostin C (i.t.), and H7 ([1-(5-Isoquinolinesulfonyl)-2-methylpiperazine, 2HCl]) (i.c.v.) were able to prevent or reverse the acute antinociceptive tolerance that developed to administration of a single dose of *mu*- or *delta*-opioid receptor agonist (Bilsky et al., 1996; Narita et al., 1995, 1996). In addition, structurally dissimilar PKC inhibitors were demonstrated to reverse antinociceptive tolerance in mice 3 days after morphine pellet implantation (Smith et al., 1999b, 2002, 2003). In rats, concomitant infusion of H7 i.c.v. prevented the development of morphine and buprenorphine tolerance, as measured in the tail-flick test (Narita et al., 1994). Chronic co-infusion of i.t. morphine with either bisindolylmaleimide I or chelerythrine prevented the development of morphine tolerance, as measured in the paw-

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withdrawal test to noxious heat (Granados-Soto et al., 2000). Furthermore, PKC inhibitors were able to reverse tolerance after 5 days of morphine infusion. Recent studies demonstrated that i.t. infusion of anti-sense oligodeoxynucleuotide to PKC*alpha* mRNA blocked the tolerance to i.t. infused morphine in rats (Hua et al., 2002).

Surprisingly, nearly all of these studies utilized the radiant heat tail-flick model of nociception to assess for the ability of protein kinase inhibitors to prevent or reverse opioid antinociceptive tolerance. The tail-flick reflex is mediated by spinal neurons, and is influenced by descending bulbospinal antinociceptive systems. We broadened our research by utilizing pain models that activate other nociceptive systems in order to determine the effectiveness of the PKC and PKA inhibitors to reverse opioid tolerance. The hot-plate test is accepted as a measure of supraspinally integrated antinociception, indicated by a coordinated licking or biting response to the fore- or hindpaws in response to noxious heat stimuli. The tail-pinch assay was chosen to measure mechanical nociception since it is more selective for activating high threshold A*delta* mechanoreceptors, as opposed to heat tests that activate lower threshold A*delta* and C-polymodal nociceptors (for a review see Le Bars et al., 2001). Our results demonstrate that the PKC inhibitors, bisinolylmaleimide I and Gö-7874, as well as the PKA inhibitor, KT-5720 completely reversed morphine tolerance in both nociceptive tests. In addition, we tested the effectiveness of these kinase inhibitors to reverse the tolerance to morphine-induced hypothermia. Administration of the PKC or PKA inhibitors alone only partly reversed the hypothermic tolerance, whereas co-administration of both classes of inhibitors completely reversed this tolerance.

2. Methods

2.1. Methods of handling mice

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25–30 g were housed 6 to a cage in animal care quarters maintained at 22 ± 2 °C on a 12-h light–dark cycle. Food and water were available ad libitum. The mice were brought to a test room (22 ± 2 °C, 12-h light–dark cycle), marked for identification and allowed 24 h to recover from transport and handling. The Institutional Animal Care and Use Committee (IACUC) at the Virginia Commonwealth University School of Medicine approved all procedures. IACUC procedures comply with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals August 7, 2002 amended guide.

2.2. Surgical implantation of pellets

Mice were anesthetized with 8% vaporized diethyl ether before shaving the hair around the base of the neck. Adequate anesthesia 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, Prichard, WV) and rinsed with alcohol before making a 1-cm horizontal 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 75 mg morphine pellet was inserted in the space before closing the site with Clay Adams Brand, MikRon® AutoClip® 9 mm Wound Clips (Becton Dickinson, Sparks, MD) and again applying iodine to the surface. The animals were allowed to recover in their home cages where they remained throughout the experiment.

2.3. Intracerebroventricular injections

I.c.v. injections were performed as described by Pedigo et al. (1975). Mice were anesthetized 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 greater than 95% accuracy. Immediately upon testing, the animals were euthanized to minimize any type of distress, according to IACUC guidelines.

2.4. Hot-plate test

The hot-plate test was performed as described by O'Callaghan and Holtzman (1975). The mice were first placed on a Syscom Model 35D hot plate set at 56 °C to obtain baseline response latencies before drug administration. The mice were observed for licking either their fore- or hind limb in response to the heat. The latencies ranged between 5- and 6-s. The mice were tested again at the appropriate time after being administered test drugs i.c.v. and morphine s.c. A 30-s cut-off was employed in order to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson (1964) as the percentage of maximum possible effect (% MPE) which was calculated as: %MPE=[(test latency − control latency) (30 − control latency)$^{-1}$] × 100. Percent MPE was calculated for each mouse using at least six mice per dose.

2.5. Tail-pinch test

The tail-pinch test was performed as described by Takagi et al. (1966) with the use of a 5-cm artery clamp. Baseline latencies were obtained. A positive response to application of the clamp to the distal 1/2 of the tail was recorded as an attempt to turn and bite the clamp. Baseline latencies occurred within 2- to 3-s. The mice were tested again at the appropriate time after being administered test drugs i.c.v. and morphine s.c. A 12-s cut-off was used to

indicate a negative response to noxious pressure. Antinociception was quantified according to the method of Harris and Pierson (1964) as the percentage of maximum possible effect (% MPE), which was calculated as: %MPE=[(test latency − control latency)(12 − control latency)$^{-1}$] × 100. Percent MPE was calculated for each mouse using at least six mice per dose.

2.6. Hypothermia measurements

Hypothermia was measured through use of a rectal temperature probe. Baseline temperatures were recorded before test drug administration, measuring approximately 37 °C for each mouse. The body temperature was recorded again after administration of each test drug i.c.v. and morphine s.c., ranging in temperatures from 30 to 36 °C. These temperatures were used to calculate the change in body temperature, ΔT_b = test temperature − baseline temperature, as a measure of hypothermia.

2.7. Reversal of morphine tolerance

Drugs that inhibit PKC were injected i.c.v. to test for their ability to affect morphine tolerance. In separate groups of mice, baseline hot-plate and tail-pinch latencies were obtained before i.c.v. injection of vehicle or test drug and morphine s.c. Test latencies were measured 30 min after morphine and test drug administration, based on a previous publication (Smith et al., 1999b). Doses used to reverse tolerance were based on those reported by Smith et al. (1999b, 2002) and Bernstein and Welch (1997). 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 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.

2.8. Drugs and chemicals

The 75 mg morphine pellets were obtained from the National Institute on Drug Abuse, Bethesda, MD. Morphine sulfate (Mallinckrodt, St. Louis, MO) was dissolved in pyrogen-free isotonic saline (Baxter Healthcare, Deerfield, IL). Bisindolylmaleimide I HCl (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) and Gö-7874 hydrochloride {2[1[(3-Dimethylaminopropyl)-5-methozyindol-3-yl]-3-(1H-indol-3-yl) hydrochloride} from Calbiochem, San Diego, CA were dissolved in distilled water. The corresponding vehicle-injected mice were injected with distilled water. KT-5720 (8*R*, 9*S*, 11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7*b*,11*a*-triazadibenzo[*a*,*g*]-]cycloocta[cde]trinden-1-one (Calbiochem) was dissolved in 10% dimethyl sulfoxide (DMSO), 20% emulphor, 70% distilled water. We have previously published on the use of this vehicle for i.c.v. injections (Smith et al., 1999a,b, 2002, 2003). The corresponding vehicle-injected mice were injected with 10% DMSO, 20% emulphor, 70% distilled water.

3. Results

3.1. Influence of PKC and PKA inhibitors in the hot-plate test

Morphine administered s.c. elicited dose-dependent antinociception in the hot-plate test in both placebo and morphine pellet-implanted mice. Furthermore, the potency of morphine was decreased by at least 6.5-fold in the morphine pellet-implanted mice compared to the placebo-pelleted mice. We tested doses of two PKC inhibitors previously demonstrated to reverse morphine tolerance in the tail-flick test (Smith et al., 1999b, 2002). As seen in Fig. 1A, Gö-7874 (4 nmol) injected i.c.v. completely reversed tolerance in the morphine pellet-implanted mice, while having no effect in placebo-pelleted mice. Gö-7874 elicited no other behaviors in either the placebo- or morphine-pelleted mice. Like the earlier studies, the PKC inhibitor bisindolylmaleimide I (11.1 nmol) also completely reversed morphine tolerance in the hot-plate test (Fig. 1B). The PKA inhibitor KT-5720 was also tested for its ability to reverse morphine tolerance. The 2.5 nmol dose that reversed morphine tolerance in the tail-flick test (Bernstein and Welch, 1997), also reversed tolerance in the hot-plate test (Fig. 2A). Table 1 demonstrates that the ED_{50} and potency-ratio values of the morphine-pelleted mice treated with PKC or PKA inhibitors did not differ significantly from similarly treated placebo-pelleted non-tolerant mice. Thus, morphine tolerance was acutely reversed by treatment with these inhibitors.

3.2. Influence of PKC and PKA inhibitors in the tail-pinch test

Morphine elicited dose-dependent antinociception in the tail-pinch test, and morphine's potency was significantly decreased by at least 6.5-fold in the morphine pellet-implanted groups. Like the hot-plate data, KT-5720 (Fig. 2B), Gö-7874 (Fig. 3A) and bisindolylmaleimide I (Fig. 3B) injected i.c.v completely reversed morphine tolerance in the tail-pinch assay. Table 2 demonstrates that the ED_{50} and potency-ratio values of the morphine-pelleted mice treated with PKC or PKA inhibitors did not differ significantly from similarly treated placebo-pelleted non-tolerant mice.

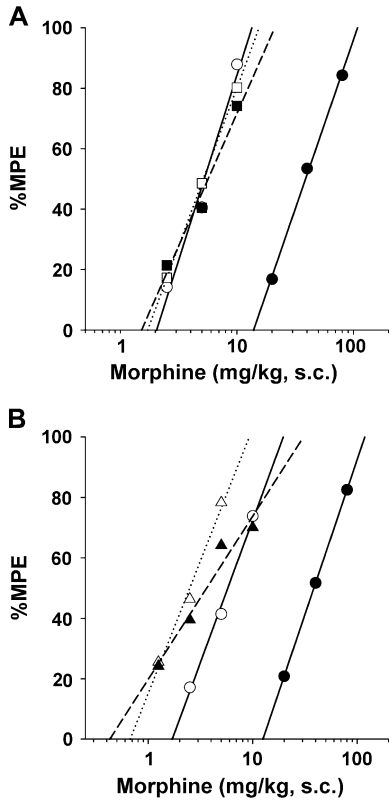

Fig. 1. (A) Reversal of morphine tolerance in the hot-plate test with Gö-7874. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or Gö-7874 was injected i.c.v. immediately followed by s.c. morphine. Hot-plate latencies were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/Gö-7874 (4.0 nmol) (□, dotted line); morphine-P/veh (●, solid line); morphine-P/Gö-7874 (■, dashed line). Each data point represents six mice. (B) Reversal of morphine tolerance in the hot-plate test with bisindolylmaleimide I. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or bisindolylmaleimide I was injected i.c.v. immediately followed by s.c. morphine. Hot-plate latencies were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/bisindolylmaleimide I (11.1 nmol) (△, dotted line); morphine-P/veh (●, solid line); morphine-P/bisindolylmaleimide I (▲, dashed line). Each data point represents six mice.

3.3. Tolerance reversal to the hypothermic effects of morphine

Morphine elicited dose-dependent hypothermia in mice in both the placebo and morphine pellet-implanted mice. Previous studies from our laboratory and those of our colleagues indicate that peak hypothermia occurs 30 min after morphine administration (Lichtman et al., 1993; Wiley and Martin, 2003). Furthermore, morphine's potency was decreased by 32-fold in the morphine pellet-implanted mice compared to the placebo-pelleted mice. However, unlike the hot-plate and tail-pinch tests, 4 nmol of Gö-7874 only partly reversed the tolerance to morphine-induced hypothermia (Fig. 4A). Increasing the Gö-7874 dose to 12 nmol elicited no further reversal in tolerance. Tolerance to the hypothermic effect of morphine was only partly reversed by bisindolylmaleimide I (Fig. 4B). In similar fashion, 2.5 nmol of KT-5720 only partly reversed the tolerance to morphine-induced hypothermia (Fig. 5). KT-5720 elicits toxicities at higher doses, so further testing was not conducted (Bernstein and Welch, 1997). PKC and PKA inhibitors were co-administered in other experiments in an attempt to completely reverse the tolerance to the hypothermic effects of morphine. Co-administration of Gö-7874 with KT-5720 (Fig. 6A) or bisindolylmaleimide I with KT-7520 (Fig. 6B) completely reversed the tolerance to morphine-induced hypothermia. Table 3 demonstrates that the co-administration of the PKC and PKA inhibitors completely reversed the tolerance to the hypothermic effects of morphine based on ED_{50} and potency-ratio values. These data indicate that when a high level of morphine tolerance is achieved, a threshold is crossed whereby administration of PKC or PKA

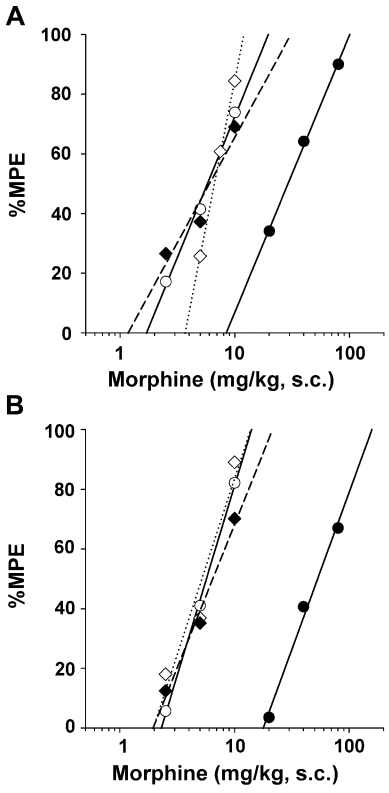

Fig. 2. (A) Reversal of morphine tolerance in the hot-plate test with KT-5720. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or KT-5720 was injected i.c.v. immediately followed by s.c. morphine. Hot-plate latencies were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/KT-5720 (2.5 nmol) (◇, dotted line); morphine-P/veh (●, solid line); morphine-P/KT-5720 (◆, dashed line). Each data point represents six mice. (B) Reversal of morphine tolerance in the tail-pinch test with KT-5720. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or KT-5720 was injected i.c.v. immediately followed by s.c. morphine. Tail-pinch latencies were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/KT-5720 (2.5 nmol) (◇, dotted line); morphine-P/veh (●, solid line); morphine-P/KT-5720 (◆, dashed line). Each data point represents six mice.

Table 1
Complete reversal of morphine tolerance by PKC inhibitors and the PKA inhibitor KT-5720 in the hot-plate test

Group	Treatment (i.c.v.)	ED_{50} (mg/kg (95% C.L.))	Potency ratio (95% C.L.)
Placebo-P	Gö-7874 (4 nmol)	6.7 (5.8 to 7.7)	–
Morphine-P	Gö-7874 (4 nmol)	5.7 (4.1 to 7.8)	vs. Pbo Gö-7874 0.6 (0.5 to 1.2)
Placebo-P	Bisindol. (11.1 nmol)	2.5 (1.5 to 4.1)	–
Morphine-P	Bisindol. (11.1 nmol)	5.7 (4.1 to 7.8)	vs. Pbo Bisindol. 1.5 (0.3 to 5.0)
Placebo-P	KT-5720 (2.5 nmol)	6.6 (5.7 to 7.7)	–
Morphine-P	KT-5720 (2.5 nmol)	6.0 (3.5 to 10.4)	vs. Pbo KT-5720 1.1 (0.5 to 1.9)

Placebo pellets or 75 mg morphine pellets were implanted, and 72-h later vehicle, Gö-7874, bisindolylmaleimide I or KT-5720 was injected i.c.v. immediately followed by s.c. morphine. Hot-plate latencies were obtained 30 min later. The effect of vehicle i.c.v. in placebo- and morphine-pelleted mice is illustrated in the figures.

inhibitors alone is unable to completely reverse morphine tolerance.

4. Discussion

4.1. Reversal of tolerance in the hot-plate test

The hypothesis was tested that PKC and PKA play a role in the expression of morphine tolerance to supraspinally integrated antinociception, as measured in the hot-plate test. The PKC inhibitors bisindolylmaleimide I and Gö-7874, and the PKA inhibitor KT-5720, completely reversed morphine tolerance. Lesion studies indicate that an intact central nervous system is required for animals to respond appropriately to the hot-plate test. One study compared the hot-plate, formalin test scores and tail-flick latencies in rats after lesions of the medial frontal and anterior cingulate cortices (Pastoriza et al., 1996). Hot-plate latencies were increased by 82%, whereas formalin test scores and tail-flick latencies were unaffected, indicating that these cortical regions were responsible for supraspinally mediated responses to noxious heat. Other evidence supports the important role of these cortical regions in mediating antinociception. Microinjection of the *mu*-opioid receptor agonist Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) into the anterior cingulate cortex resulted in dose-dependent antinociception in the hot-plate test (Lee et al., 1999). PKC_{alpha} and PKC_{gamma} have been identified specifically in neurons in the anterior cingulate cortex using both in situ hybridization and immunocytochemical approaches (Ito et al., 1990; Thomas and Everitt, 2001). Furthermore, cAMP binding to the regulatory subunit of PKA has been used to detect the activity of PKA in the cingulate cortex (Tanaka et al., 1999). Thus, the PKC and PKA inhibitors injected i.c.v. could have penetrated into these cortical regions, or acted on neurons projecting from these regions, to reverse morphine tolerance in the hot-plate test.

In contrast, the tail-flick test is regarded as a spinally mediated reflex originating in spinal cord segments S3 to Co3 (Grossman et al., 1982). The tail-flick reflex remains intact after transecting the spinal cord, and spinalized mice respond to morphine acting on spinal *mu*-opioid receptors, and other analgesic agents (Advokat, 2002; Siuciak and Advokat, 1989). However, the tail-flick reflex can also be influenced by descending antinociceptive pathways that terminate in laminae I and II of the spinal dorsal horn. For example, the dorsal and ventral periaqueductal gray projects into the rostral ventromedial medulla. The rostral ventro-

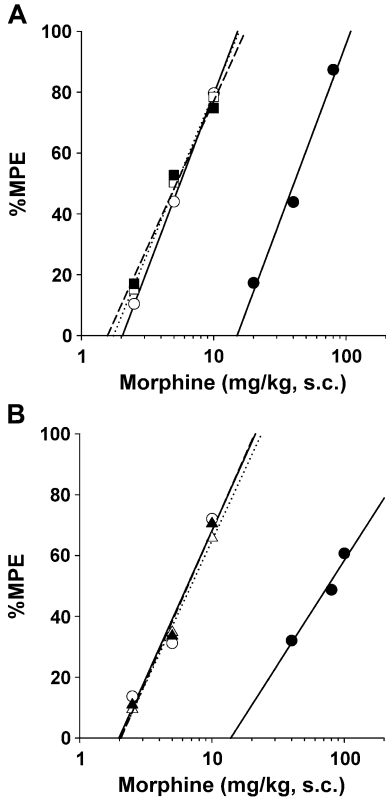

Fig. 3. (A) Reversal of morphine tolerance in the tail-pinch test with Gö-7874. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or Gö-7874 was injected i.c.v. immediately followed by s.c. morphine. Tail-pinch latencies were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/Gö-7874 (4.0 nmol) (□, dotted line); morphine-P/veh (●, solid line); morphine-P/Gö-7874 (■, dashed line). Each data point represents six mice. (B) Reversal of morphine tolerance in the tail-pinch test with bisindolylmaleimide I. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or bisindolylmaleimide I was injected i.c.v. immediately followed by s.c. morphine. Tail-pinch latencies were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/bisindolylmaleimide I (11.1 nmol) (△, dotted line); morphine-P/veh (●, solid line); morphine-P/bisindolylmaleimide I (▲, dashed line). Each data point represents six mice.

Table 2
Complete reversal of morphine tolerance by PKC inhibitors and the PKA inhibitor KT-5720 in the tail pinch test

Group	Treatment (i.c.v.)	ED_{50} (mg/kg (95% C.L.))	Potency ratio (95% C.L.)
Placebo-P	Gö-7874 (4 nmol)	6.4 (4.8 to 6.9)	–
Morphine-P	Gö-7874 (4 nmol)	6.3 (4.7 to 7.0)	vs. Pbo Gö-7874 0.8 (0.6 to 1.3)
Placebo-P	Bisindol. (11.1 nmol)	7.0 (4.8 to 10.1)	–
Morphine-P	Bisindol. (11.1 nmol)	6.5 (4.6 to 9.2)	vs. Pbo Bisindol. 0.9 (0.5 to 1.7)
Placebo-P	KT-5720 (2.5 nmol)	5.2 (4.0 to 6.7)	–
Morphine-P	KT-5720 (2.5 nmol)	6.5 (4.5 to 9.4)	vs. Pbo KT-5720 1.2 (0.8 to 2.0)

Placebo pellets or 75 mg morphine pellets were implanted, and 72 h later vehicle, Gö-7874, bisindolylmaleimide I or KT-5720 was injected i.c.v. immediately followed by s.c. morphine. Tail-pinch latencies were obtained 30 min later. The effect of vehicle i.c.v. in placebo- and morphine-pelleted mice is illustrated in the figures.

medial medulla, and in particular the nucleus raphe magnus, sends inhibitory projections into the dorsal spinal horn.

4.2. Reversal of tolerance in the tail-pinch test

In the tail-pinch test, the two PKC inhibitors and KT-5720 completely reversed tolerance to morphine-induced antinociception. The tail-pinch assay was chosen to measure mechanical nociception since it is more selective for activating high threshold A*delta* mechanoreceptors, as opposed to heat tests that activate lower threshold A*delta* and C-polymodal nociceptors (for a review see Le Bars et al., 2001). Previously, we demonstrated that bisindolylmaleimide I, Gö-7874 and KT-5720 completely reversed a moderate level of morphine tolerance in the radiant heat tail-flick test (Bernstein and Welch, 1997; Smith et al., 1999b, 2002). Thus, the nerves transmitting mechanical and heat nociception throughout the neuraxis that become tolerant to morphine, appear to be equally sensitive to protein kinase inhibitors.

4.3. Influence of intracerebroventricular vs. intrathecal protein kinase inhibitor administration

In most published studies, PKC and PKA inhibitors are administered either i.c.v. or i.t. to prevent the development of tolerance, or to reverse tolerance already established by the chronic administration of opioids. Diffusion from the i.c.v. location into supraspinal sites might alter the activity of bulbospinal pathways that ultimately influence the tail-flick reflex. For example, H7 completely reversed morphine tolerance when injected i.c.v., or was able to prevent the development of tolerance when it was concurrently infused i.c.v. with morphine for 3 days (Narita et al., 1994; Bilsky et al., 1996). We demonstrated that a single i.c.v. injection of various PKC or PKA inhibitors reversed morphine tolerance for 30 min or for as long as 24 h (Smith et al., 1999b, 2002).

Alternatively, diffusion of protein kinase inhibitors following i.t. administration could affect the activity of PKC or PKA in spinal neurons chronically exposed to opioids administered by i.t. or parenteral routes. For example, i.t. administration of calphostin C prevented the development of acute antinociceptive tolerance to the i.t. administered *delta* receptor agonist [D-Ala^2]deltorphin II and the *mu*-opioid receptor agonist DAMGO in mice (Narita et al., 1995, 1996). In rats infused spinally with morphine for 5 days, direct spinal co-infusion of chelerythrine or bisindo-

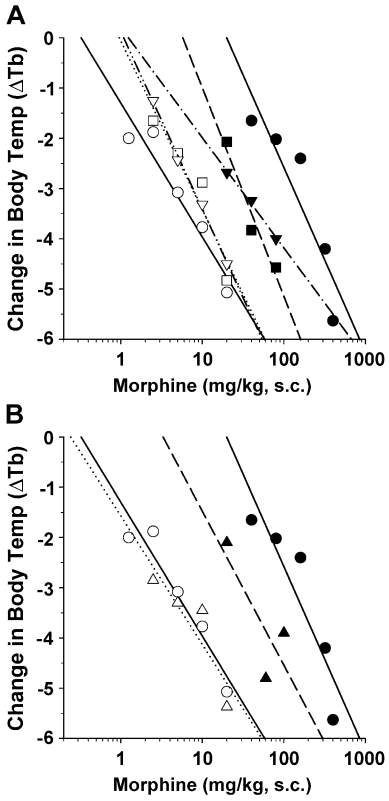

Fig. 4. (A) Partial reversal of tolerance to the hypothermic effects of morphine with Gö-7874. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or Gö-7874 was injected i.c.v. immediately followed by s.c. morphine. Rectal temperatures were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/Gö-7874 (4.0 nmol) (□, dotted line); placebo-P/Gö-7874 (12.0 nmol) (▽, dash-dot-dot-dash line); morphine-P/veh (●, solid line); morphine-P/Gö-7874 (4 nmol) (■, dashed line); morphine-P/Gö-7874 (12.0 nmol) (▼, dash-dot-dash line). Each data point represents six mice. (B) Partial reversal of tolerance to morphine-induced hypothermia with bisindolylmaleimide I. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or bisindolylmaleimide I was injected i.c.v. immediately followed by s.c. morphine. Rectal temperatures were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/bisindolylmaleimide I (11.1 nmol) (△, dotted line); morphine-P/veh (●, solid line); morphine-P/bisindolylmaleimide I (▲, dashed line). Each data point represents six mice.

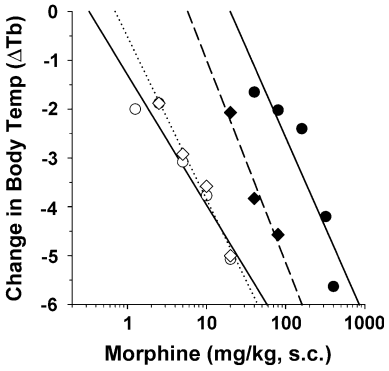

Fig. 5. Partial reversal of tolerance to morphine-induced hypothermia with KT-5720. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or KT-5720 was injected i.c.v. immediately followed by s.c. morphine. Rectal temperatures were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/KT-5720 (2.5 nmol) (◇, dotted line); morphine-P/veh (●, solid line); morphine-P/KT-5720 (◆, dashed line). Each data point represents six mice.

lylmaleimide I prevented the development of morphine tolerance, while acute bolus administration of PKC inhibitors reversed antinociceptive tolerance (Granados-Soto et al., 2000). In addition, i.t. infusion of antisense oligodeoxynucleoide to PKC_{alpha} prevented the development of morphine tolerance (Hua et al., 2002). Overall, PKC and PKA may contribute to the expression of opioid tolerance by affecting the activity of bulbospinal antinociceptive pathways, or by affecting the activity of spinal neurons that are tolerant following exposure to *mu*- or *delta*-opioid receptor agonists.

4.4. Tolerance to morphine-induced hypothermia

Placebo and morphine pellet-implanted mice injected with morphine s.c. developed dose-dependent hypothermia. In addition, mice implanted with 75 mg morphine pellets exhibited a 32-fold tolerance to morphine-induced hypothermia. This degree of tolerance was much greater than the approximately 6.5-fold antinociceptive tolerance in the hot-plate and tail-pinch tests. Hypothermic tolerance was similarly demonstrated when morphine was chronically injected by repeated administration (Bhargava, 1981; Rosow et al., 1982; Zarrindast et al., 2001). Identification of the opioid receptors through which morphine elicits hypothermia is complex, and may depend on the dose. For example, morphine-induced hypothermia in mice is mediated through composite actions on *mu*, *delta* and *kappa* opioid receptors, based on the ability of selective opioid receptor antagonists to block hypothermia (Baker and Meert, 2002). In rats, antisense oligodeoxynucleotide to *kappa* opioid receptors blocked the hypothermic effects of high (i.e., 30 mg/kg) doses of morphine (Chen et al., 1996). Thus, morphine-induced hypothermia may be mediated through multiple opioid receptor subtypes, especially at higher doses.

4.5. Reversal of tolerance to morphine-induced hypothermia

It is notable that the PKC inhibitors and KT-5720 did not block morphine-induced hypothermia. Instead, these drugs only partly reversed the tolerance to morphine-induced hypothermia at doses that completely reversed antinociceptive tolerance. Although the 32-fold level of hypothermic tolerance may have contributed to the difficulty in shifting the curves to the left, increasing the dose of Gö-7874 to 12 nmol did not further reverse the tolerance. It is notable that the 32-fold level of hypothermic tolerance required the co-administration of PKC and

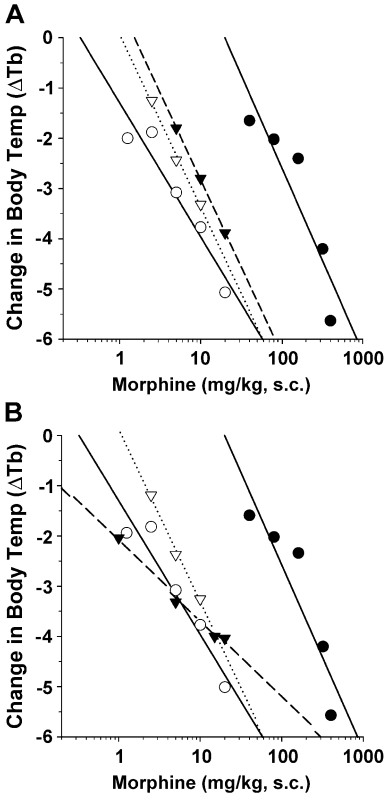

Fig. 6. (A) Complete reversal of tolerance to the hypothermic effects of morphine by co-administering Gö-7874 with KT-5720. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or Gö-7874 + KT-5720 were co-administered i.c.v. as a single injection, immediately followed by s.c. morphine. Rectal temperatures were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/Gö-7874 (12 nmol) + KT-5720 (2.5 nmol) (▽, dotted line); morphine-P/veh (●, solid line); morphine-P/Gö-7874 + KT-5720 (▼, dashed line). Each data point represents six mice. (B) Complete reversal of tolerance to the hypothermic effects of morphine by co-administering bisindolylmaleimide I with KT-5720. Mice were surgically implanted with placebo pellets, or 75 mg morphine pellets. Seventy-two hours later, vehicle or bisindolylmaleimide I + KT-5720 were co-administered i.c.v. as a single injection, immediately followed by s.c. morphine. Rectal temperatures were obtained 30 min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/bisindolylmaleimide I (11.1 nmol) + KT-5720 (2.5 nmol) (▽, dotted line); morphine-P/veh (●, solid line); morphine-P/bisindolylmaleimide I + KT-5720 (▼, dashed line). Each data point represents six mice.

Table 3
Complete reversal of tolerance to morphine-induced hypothermia by the co-administration of PKC inhibitors with the PKA inhibitor KT-5720

Group	Treatment (i.c.v.)	ED_{50} (mg/kg (95% C.L.))	Potency ratio (95% C.L.)
Placebo-P	Gö-7874 (12.0 nmol) + KT-5720 (2.5 nmol)	7.8 (5.5 to 11.1)	–
Morphine-P	Gö-7874 + KT-5720	11.2 (5.6 to 22.3)	vs. Pbo Gö-7874 1.4 (0.6 to 2.5)
Placebo-P	Bisindol. (11.1 nmol) + KT-5720 (2.5 nmol)	6.2 (4.0 to 9.6)	–
Morphine-P	Bisindol. + KT-5720	3.5 (1.2 to 10.7)	vs. Pbo Bisindol. 0.9 (0.5 to 1.7)

Placebo pellets or 75 mg morphine pellets were implanted, and 72 h later Gö-7874 + KT-5720 bisindolylmaleimide I + KT-5720 was injected i.c.v. immediately followed by s.c. morphine. Rectal temperatures were obtained 30 min later. The effect of vehicle, Gö-7874, bisindolylmaleimide and KT-5720 alone i.c.v. in placebo- and morphine-pelleted mice is illustrated in the figures.

PKA inhibitors to completely reverse the tolerance. We demonstrated a similar requirement for PKC and PKA inhibitor co-administration to reverse a 45-fold level of morphine antinociceptive tolerance in the tail-flick test (Smith et al., 2003). With a 5- to 8-fold level of antinociceptive tolerance in the tail-flick test, the PKC and PKA inhibitors administered alone completely reversed this level of tolerance (Smith et al., 1999b, 2002; Bernstein and Welch, 1997). Even in this study, the PKC and PKA inhibitors administered alone completely reversed the approximately 6.5-fold level of tolerance in the hot-plate and tail-pinch tests. Thus, when a certain level of tolerance is exceeded, a threshold appears to be crossed whereby these inhibitors injected alone fail to completely reverse tolerance to morphine-induced hypothermia and antinociception.

The i.c.v. administered PKC and PKA inhibitors may have diffused to thermoregulatory sites critical to integrating both central and peripheral thermal sensory information. The preoptic anterior hypothalamus possesses *mu*, *delta* and *kappa* opioid receptors (Mansour et al., 1987), and is the primary site regulating temperature set-point. Iontophoretic application of morphine increased the firing activity of warm-sensitive neurons in the preoptic anterior hypothalamus (POAH), which can mediate hypothermia by activating heat dissipation responses (Baldino et al., 1980). Furthermore, *kappa* receptor agonist stimulation within the POAH was demonstrated to elicit hypothermia (Xin et al., 1997). Thus, the POAH could be the site at which hypothermic tolerance occurs, and where the PKC and PKA inhibitors act to reverse tolerance. However, other brain sites could also mediate morphine-induced hypothermia, and be involved with the expression of morphine tolerance. The nucleus accumbens and preoptic area also possess opioid receptors that alter body temperature when injected with opioids. The pharmacological actions of β-endorphin closely resemble those of morphine. Microinjection of β-endorphin into the preoptic area or nucleus accumbens resulted in hypothermia (Tseng et al., 1980). Administration of PKC and PKA inhibitors i.c.v. could diffuse to these sites, or affect neurons projecting from these sites, to reverse tolerance to morphine-induced hypothermia.

In summary, since the majority of published studies have used the tail-flick assay to test for protein kinase inhibitors to prevent or reverse opioid tolerance, it was important to determine whether the inhibitors would reverse tolerance in other nociceptive tests. Clearly, during chronic morphine administration multiple nociceptive pathways are similarly affected by increases in PKC and PKA activity. Furthermore, the issue of whether PKC and PKA mediate tolerance to the physiological effects of morphine needs to be addressed. Chronic morphine administration appears to increase the activity of PKC and PKA to affect thermoregulation. Physiological centers that control respiratory and cardiovascular systems may be affected as well.

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