

Prevention of fentanyl-induced delayed pronociceptive effects in mice lacking the protein kinase C γ gene

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Abstract

It has recently been reported in several nociceptive models of rats that the antinociceptive effect of fentanyl, an opioid analgesic widely used in the management of per-operative pain, was followed by paradoxical delayed hyperalgesia dependent on *N*-methyl-D-aspartate (NMDA) mechanisms. Events upstream of the NMDA receptor, especially the activation of the protein kinase C γ (PKC γ), have been involved in the persistence of pain states associated with central sensitisation. In order to evaluate the contribution of the PKC γ in early and delayed fentanyl nociceptive responses, we studied these effects in knock-out mice deficient in such a protein. We found that fentanyl antinociception was followed by the spontaneous appearance of prolonged hyperalgesia in the paw pressure and formalin tests, and allodynia in the Von Frey paradigm. In PKC γ deficient mice, an enhancement of the early fentanyl antinociceptive effects was observed, as well as a complete prevention of the fentanyl delayed hyperalgesic/allodynic effects. Finally, naloxone administration in mice that had recovered their pre-fentanyl nociceptive threshold, precipitated hyperalgesia/allodynia in wild-type but not in mutant mice. This study identifies the PKC γ as a key element that links opioid receptor activation with the recruitment of opposite systems to opioid analgesia involved in a physiological compensatory pain enhancement. © 2003 Elsevier Ltd. All rights reserved.

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1. Introduction

Although morphine and its derivatives are the most effective painkillers in humans, there is now a growing number of animal and clinical studies reporting the appearance of abnormal and prolonged pain states after acute or chronic opioid treatment (Mao, 2002; Simonnet and Rivat, 2003). Opioid-induced pain hypersensitivity currently develops alongside analgesic tolerance, and both phenomena seem to share common cellular mechanisms including the activation of the *N*-methyl-D-aspartate (NMDA) glutamatergic receptors (Mao et al., 1994, 1995a; Simonnet and Rivat, 2003). Indeed, behavioural studies conducted on non-suffering rats suggested that the activation by opioid compounds of NMDA pronociceptive processes may be involved in acute tolerance

after a first opioid exposure (Larcher et al., 1998; Célérier et al., 1999, 2000; Laulin et al., 2002) and in the development of the analgesic tolerance classically observed following chronic opioid treatment (Laulin et al., 1999; Célérier et al., 2001; Mao et al., 1995a). Similarly, acute opioid exposure may produce a paradoxical enhancement of the hyperalgesia induced by carrageenan-elicited tissue damage and therefore facilitating the establishment of pain sensitisation in a NMDA reversible manner (Rivat et al., 2002).

Protein phosphorylation through various protein kinases is a major mechanism for the regulation of the NMDA receptor function. The Ca²⁺-sensitive protein kinases C, especially the gamma isoform (PKC γ) (Tanaka and Nishizuka, 1994) catalyse the NMDA receptor phosphorylation leading to enhancement of the NMDA mediated glutamate responses and to long-term potentiation of synaptic transmission (Chen and Huang, 1992; Mao et al., 1995b; Coderre and Katz, 1997; Suen et al., 1998). Previous studies suggest that PKC γ partici-

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pates in the increased pain sensitivity that occurs after injury, and in the sensitisation produced on dorsal horn neurons by neuropathy and inflammation (Malmberg et al., 1997; Martin et al., 1999, 2001). Interestingly, μ -opioid receptor stimulation triggers the activation of NMDA receptors by increasing intracellular PKC activity. Therefore, protein kinases, and particularly PKC γ , may represent a key element which links the opioid receptor activation and the recruitment of the glutamatergic/NMDA systems implicated in the promotion of pain.

The aim of this study was to characterise the influence of PKC γ in the early opioid antinociceptive effects and in the delayed pronociceptive effects of opioids. For this purpose, we used the μ -opioid agonist fentanyl, an analgesic widely utilised in the management of per-operative pain and previously reported to induce paradoxical NMDA-dependent delayed hyperalgesia and long-term neurobiological changes in rats (Célérier et al., 2000; Rivat et al., 2002). No selective antagonists are still available for the many isoforms of PKC, and the knock-out mice with a deletion of the gene encoding the PKC γ (Abeliovich et al., 1993) represent a unique tool to achieve the purpose of this study. It is important to underline that PKC γ is not expressed until after birth, and the possible compensatory responses due to its loss during development, are therefore significantly reduced (Basbaum, 1999). Firstly, we examined whether the PKC γ gene deletion has any influence on the early antinociceptive effects of fentanyl by using the tail-immersion test. Secondly, the influence of the PKC γ gene deletion on fentanyl-induced delayed allodynic/hyperalgesic effects was evaluated in the paw pressure, Von Frey and formalin tests. At the end of the experiments, an injection of naloxone was performed in order to assess whether fentanyl treatment may induce long-term changes in opioid system activity.

2. Methods

2.1. Animals

The B6;129P-Pkcc γ^{tm1St1} mutant and B6;129PF1/J wild-type mice were provided by Jackson (Maine, USA) and bred in the laboratory. The genotype of these mice was verified using polymerase chain reaction of tail-tip DNA. The animals were maintained at a controlled temperature (21 ± 1 °C) and humidity ($55 \pm 10\%$), with access to food and water ad libitum. Lighting was maintained at 12-h cycles (on at 8 a.m. and off at 8 p.m.). All the experiments were performed during the light phase of the dark/light cycle. All animal procedures met the guidelines of the National Institute of Health detailed in the 'Guide for the Care and use of Laboratory Animals', the European Communities Council Directive

86/609/EEC regulating animal research and were approved by the local ethical committee.

After arrival in the laboratory, mice were left to become accustomed to the colony room for 5 days. To avoid stress from the experimental conditions, which might modify the nociceptive threshold by producing stress-induced analgesia (Amir and Amit, 1978), the experiments were performed by the same experimenter in quiet conditions in a test room close to the colony room. For a minimum of one week before the beginning of the experiments, the animals became used to being handled by the experimenter for subcutaneous injections and familiarized to the experimental room environment. For tail-immersion, tail-pressure and Von Frey tests, the animals were also presented to the experimental conditions of the test (nociceptive apparatus and special handling) without any nociceptive stimulation.

2.2. Drugs

Fentanyl and naloxone were purchased from Sigma-Aldrich Chemie (Steinheim, Germany) and the formaldehyde solution from Scharlau Chemie S.A. (Barcelona, Spain). All compounds were dissolved in saline (0.9%). Fentanyl and naloxone were administered by subcutaneous (s.c.) route.

2.3. Nociceptive test

We investigated the influence of PKC γ deletion on the acute (min) and long-lasting (days) pharmacological effects of fentanyl on nociception. The nociceptive threshold of mice was evaluated by using experimental models associated with thermal (tail-immersion test), mechanical (tail-pressure and Von Frey tests) and chemical irritatory/inflammatory (formalin test) stimuli.

2.3.1. Tail-immersion test

Mice were gently placed in a restrainer cylinder. The nociceptive threshold was assessed as previously described (Janssen et al., 1963), by measuring the time to withdraw the tail immersed in a thermostated water bath (50 ± 0.1 °C, Clifton-Scientific Instruments, England), with a cut off latency of 10 s to prevent tissue damage.

2.3.2. Paw-pressure test

A constant increased pressure was applied to the tail of the animal until the withdrawal. The analgesymeter LE 7306 (Letica Scientific Instruments, Barcelona, Spain, tip diameter of the stylus: 1 mm) was used. The pressure was consecutively applied on the distal, median and proximal part of the tail. The nociceptive threshold (expressed in gr) was considered as the average of these three experimental values. A 600 g cut-off value was set to prevent tissue damage.

2.3.3. Von Frey test

A set of calibrated Von Frey monofilaments was used to deliver mechanical stimulation. The bending force of the filaments was in a range from 1 to 32 mN. The mice were placed in individual plastic boxes on a raised metal grid (6 × 6 mm apertures) under a plastic chamber (18 × 13 × 15 cm) and allowed to habituate for 1 h. The testing filament was probed against the central edge of the hind paw. The filaments were applied in an ascending series, perpendicularly to the plantar surface of the paw. Each filament was tested 10 times at an interval of a few seconds. If paw withdrawal caused by stimulation was observed, it was registered as a response. An increase in the number of responses to sub-threshold Von Frey filaments suggests the development of allodynia, a nocifensive behaviour to a normally non-noxious stimulus.

2.3.4. Formalin test

The formalin test was performed as previously described by Li et al. (2001a). In brief, animals were placed on a glass surface inside a cylindrical clear plastic enclosure (19 cm in diameter × 30 cm in height) for nociceptive testing. After 20 min of habituation to the experimental context, mice were given a s.c. injection of 25 µl of 0.1% formalin into the dorsal surface of one forepaw by using a 0.1 ml Hamilton microsyringe (Bonaduz, Switzerland) with a 30 ga needle. Mice were then placed back in the plastic enclosure and the time (s) that the animal spent licking the injected forepaw over the first 5 min (phase I) and from 10–40 min post injection (phase II) was recorded.

2.4. Experimental protocols

Since we previously observed that the early antinociceptive effect of opioids was followed by a delayed hyperalgesic effect for several days, we examined the involvement of PKC γ in both the short- (1–4 h) and long-term (1–6 days) changes in nociceptive threshold induced by an acute fentanyl administration. Fentanyl administration was performed using a procedure designed to mimic its use in human surgery: the mice received four consecutive s.c. injections (15 min interval between each injection) of various doses of fentanyl (20, 60 and 100 µg/kg). In all the experiments, the basal nociceptive threshold measurements were performed during the days preceding the scheduled experimental day to be sure of the basal value stability.

We first evaluated the effects of the PKC γ gene deletion on the basal nociceptive stimulus threshold in non-treated mice by using the tail-immersion, the paw-pressure and the Von Frey tests. Subsequently, the effects of this mutation on fentanyl-induced early antinociceptive effects were evaluated. The nociceptive threshold was estimated by using the tail-immersion test, 30

min before and 60, 120, 150, 180, 210 and 250 min after the first fentanyl injection. As preliminary experiments conducted on wild-type mice showed that fentanyl-delayed hyperalgesia was not observed in the tail-immersion test, we evaluated the influence of the PKC γ gene deletion on the fentanyl-delayed nociceptive effects by using the tail-pressure, Von-Frey and formalin tests. The nociceptive threshold was evaluated daily for 6 days after fentanyl treatment by using tail-pressure and Von Frey tests. Formalin-induced licking behaviour was evaluated on day 2, i.e. 48 h after fentanyl treatment. At the end of the experiments, when the animals had recovered their pre-fentanyl nociceptive threshold value, a naloxone test was performed. This was made to assess the magnitude of naloxone-precipitated hyperalgesia in saline and fentanyl-treated mice. The nociceptive threshold was evaluated 5 min after the opioid receptor antagonist injection (1 mg/kg, s.c.) by using the tail-pressure and Von Frey tests.

2.5. Statistical analysis

Data from the tail-immersion test were analysed by using a three-way analysis of variance (ANOVA). The factors of variation were treatment (between subject), genotype (between subject) and time (within subject). The data were then analysed for each day by using a two-way ANOVA (genotype and treatment as between factors), followed by one-way ANOVA for individual differences when significant interaction between these two factors was revealed.

Data from the paw pressure test were assessed using a one-way ANOVA with the time as a factor of variation (within subject), followed by a Dunnett's test when the main global effects were revealed.

For the Von Frey test, the results were expressed as a percentage of the maximal value of the basal stimulus-response curve obtained before fentanyl treatment. The data were analysed by three-way ANOVA with the treatment and the genotype as between factors, and the intensity of stimulation as within factor. When significant interactions between factors were observed, a two-way ANOVA (treatment and genotype as between factors) was performed for each intensity of stimulus. A subsequent one-way ANOVA was performed when appropriate.

Data from the formalin paradigm were analysed by using a two-way ANOVA (treatment and genotype as between factors) followed by a one-way ANOVA for individual differences when significant interaction between these two factors was revealed.

Statistical significance criterion was $P < 0.05$ in all the cases.

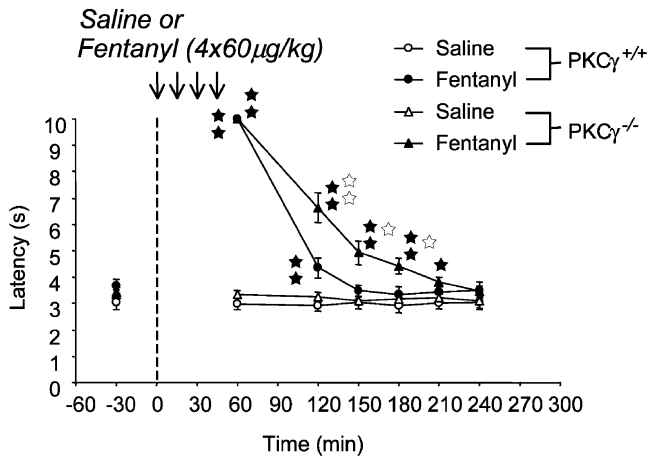


Fig. 1. Early antinociceptive effects of fentanyl in wild-type ($PKC\gamma^{+/+}$) and mutant ($PKC\gamma^{-/-}$) mice as measured by the tail-immersion test. Fentanyl was injected four times ($60 \mu\text{g}/\text{kg}$ per injection, s.c.) at 15 min intervals (arrows). Number of mice per group: 9–13. Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA, treatment effect) and * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA, genotype effect).

3. Results

3.1. The fentanyl antinociceptive effectiveness is increased in mutant mice

The influence of the $PKC\gamma$ deletion on the early antinociceptive effects of fentanyl was evaluated by using the tail-immersion test (Fig. 1). The tail-immersion test was chosen in this experiment since it enables multiple nociceptive measurements to be determined in a short period of time by using an elevated cut-off. This minimizes the duration of the ceiling effect after the administration of this potent and effective opioid drug. Before any pharmacological administration, no differences in the basal nociceptive threshold were found between the different wild-type and mutant groups. In wild-type mice, fentanyl administration ($4 \times 60 \mu\text{g}/\text{kg}$) induced a significant increase in the nociceptive threshold during 120 min (see Table 1 for three-way ANOVA) (one-way

Table 1
 $PKC\gamma$ influence on fentanyl antinociceptive effects in the tail-immersion test (three-way ANOVA)^a

	<i>F</i>	<i>P</i>
Day (D)	(6,240) = 156.9	<0.001
Treatment (T)	(1,40) = 87.62	<0.001
Genotype (G)	(1,40) = 5.99	<0.05
D×T	(6,240) = 153.1	<0.001
D×G	(6,240) = 6.05	<0.05
T×G	(1,40) = 4.39	<0.05
D×T×G	(6,240) = 6.57	<0.001

^a Factors of variation were day (within subject), treatment (between subject) and genotype (between subject).

ANOVA for treatment: $F_{1,21} = 752.4$, $P < 0.001$ at 60 min; $F_{1,21} = 13.52$, $P < 0.01$ at 120 min). In mutant mice, a longer fentanyl antinociceptive effect was observed. Indeed, fentanyl induced a significant increase in the nociceptive threshold during 210 min in mutant mice (one-way ANOVA for treatment: $F_{1,21} = 1757.7$, $P < 0.001$ at 60 min; $F_{1,21} = 63.00$, $P < 0.001$ at 120 min; $F_{1,21} = 15.88$, $P < 0.01$ at 150 min; $F_{1,21} = 10.43$, $P < 0.01$ at 180 min; $F_{1,21} = 7.12$, $P < 0.05$ at 210 min). Values obtained in wild-type and mutant mice differed from 120 to 180 min (one-way ANOVA for genotype: $F_{1,19} = 10.60$, $P < 0.01$ at 120 min; $F_{1,19} = 7.90$, $P < 0.05$ at 150 min; $F_{1,19} = 6.51$, $P < 0.05$ at 180 min). The study of the $PKC\gamma$ influence on the overall amplitude of the fentanyl-antinociceptive effects was not possible because the cut-off value was reached in both groups of mice during the peak effect time of the fentanyl injection (15 min).

3.2. Fentanyl induces $PKC\gamma$ -dependent delayed hyperalgesic/allodynic effects

The influence of the $PKC\gamma$ deletion on the delayed hyperalgesic effects of fentanyl was evaluated by using the tail-pressure (Fig. 2), Von Frey (Fig. 3) and formalin (Fig. 4) tests. Preliminary experiments revealed a long lasting and reliable decrease in nociceptive threshold in these experimental models 24 h after administration of $4 \times 60 \mu\text{g}/\text{kg}$ fentanyl (data not shown). In contrast, this delayed hyperalgesia was not observed in the tail-immersion

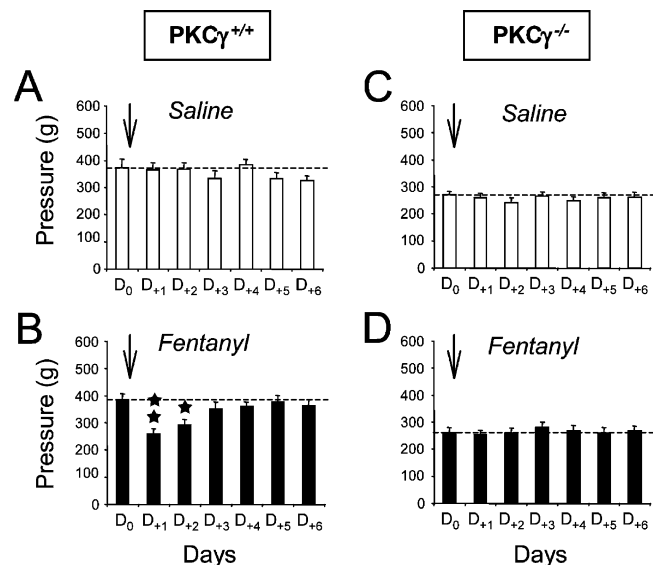


Fig. 2. Long-lasting effects of fentanyl in wild-type (A and B, $PKC\gamma^{+/+}$) and mutant (C and D, $PKC\gamma^{-/-}$) mice as measured by the tail-pressure test. Fentanyl was injected four times ($60 \mu\text{g}/\text{kg}$, s.c.) at 15 min intervals (arrows). The nociceptive threshold was evaluated on day 0 (D0) before fentanyl treatment and subsequently once-daily for 6 days. Number of mice per group: 18–23. Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ (Dunnett's test, comparison with the respective basal value on day 0).

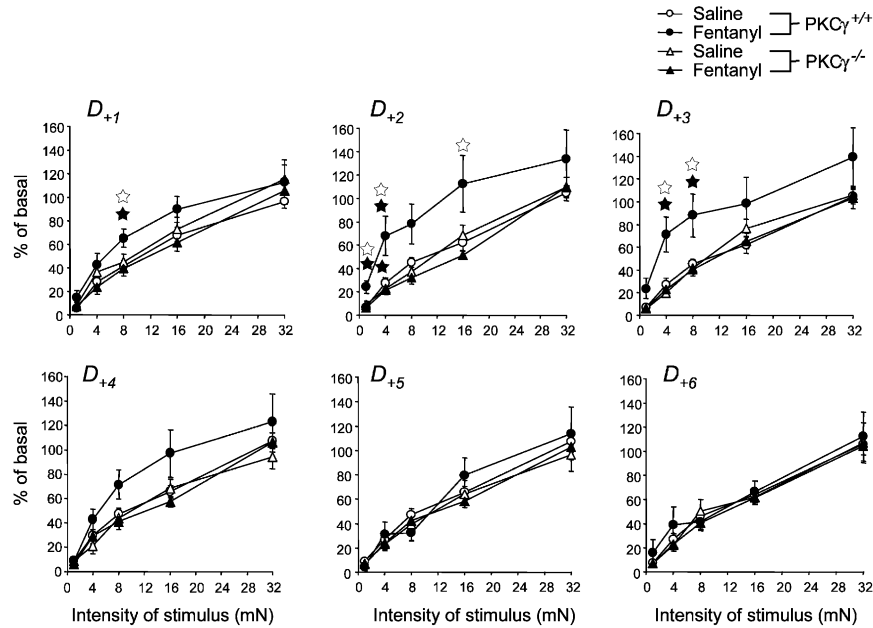


Fig. 3. Long-lasting effects of fentanyl in wild-type ($PKC\gamma^{+/+}$) and mutant ($PKC\gamma^{-/-}$) mice as measured by the Von Frey test. Fentanyl was injected four times ($60 \mu\text{g}/\text{kg}$, s.c.) at 15 min intervals (arrows). The nociceptive responses were evaluated on day 0 (D0) before fentanyl treatment and subsequently once daily for 6 days. Number of mice per group: 9–10. Data are expressed as mean \pm SEM. * $P < 0.05$ (one-way ANOVA, treatment effect) and $\ast P < 0.05$ (one-way ANOVA, genotype effect).

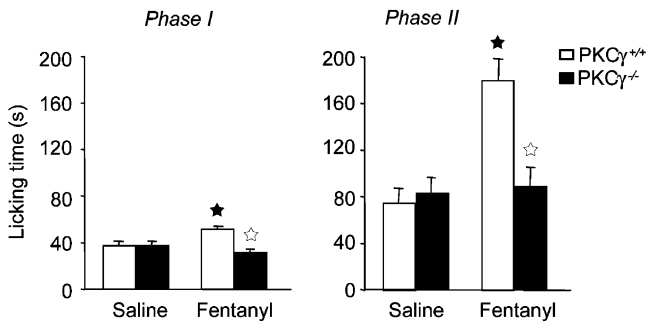


Fig. 4. Fentanyl effects of formalin-evoked licking behaviour in wild-type ($PKC\gamma^{+/+}$) and mutant ($PKC\gamma^{-/-}$) mice. Formalin (0.1%, s.c.) was administered 48 h after fentanyl treatment ($4 \times 60 \mu\text{g}/\text{kg}$ per injection, s.c.). Licking responses were recorded the first 5 min (phase I) and 10–40 min (phase II) after formalin administration. Number of mice per group: 17–18. Data are expressed as mean \pm SEM. * $P < 0.05$, (one-way ANOVA, treatment effect) and $\ast P < 0.05$ (one-way ANOVA, genotype effect).

sion test 24 h after the administration of 4×60 and $4 \times 100 \mu\text{g}/\text{kg}$ of fentanyl. This suggests that this test was not sensitive enough to detect opioid-induced hyperalgesia (data not shown).

3.2.1. Tail-pressure test

No variation in the nociceptive threshold was observed in wild-type mice that had previously received an injection of saline (Fig. 2A) or the lower dose of fentanyl ($4 \times 20 \mu\text{g}/\text{kg}$) on day 0 (data not shown). On the contrary a marked delayed decrease in the nociceptive threshold was observed following the injection of $4 \times$

$60 \mu\text{g}/\text{kg}$ of fentanyl (one-way ANOVA for time: $F_{6,119} = 5.59$, $P < 0.001$) (Fig. 2B). This delayed hyperalgesia started 24h after fentanyl administration and remained for 2 days (Dunnett's test; $P < 0.01$ at day 1 and $i < 0.05$ at day 2). Longer and more robust delayed hyperalgesic effects were found in the first 4 days after the administration of fentanyl at the dose of $4 \times 100 \mu\text{g}/\text{kg}$ (one-way ANOVA for time: $F_{6,91} = 5.70$, $P < 0.001$; Dunnett's test, $P < 0.001$ at day 1; $P < 0.01$ at days 2, 3 and 4) (data not shown).

In mutant mice, a greater sensitivity to mechanical stimulation in the tail-pressure test was observed in basal conditions (one-way ANOVA for genotype: $F_{1,106} = 28.04$, $P < 0.05$) (Fig. 2C). Saline in these mice did not produce changes in the nociceptive threshold for several days after administration. The injection of $4 \times 60 \mu\text{g}/\text{kg}$ fentanyl neither produced delayed changes in nociceptive threshold in mutant mice (Fig. 2D).

3.2.2. Von Frey test

The delayed allodynic effects of fentanyl, a nociceptive response to a normally non-noxious stimulus, were evaluated by using the mechanical Von Frey test (Fig. 3). In basal conditions, similar stimulus–response curve from subthreshold to suprathreshold range of Von Frey filament force was observed in mutant and wild-type mice (data not shown).

The administration of saline did not produce any modification in the stimulus-response curve to Von Frey hair stimulation when evaluated for 6 days after the treatment in wild-type and mutant mice (see Table 2 for

Table 2
PKC γ influence on fentanyl allodynic effects in the Von Frey test (three-way ANOVA)^a

	D+1		D+2		D+3		D+4		D+5		D+6	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
I	(4,136) = 67.23	< 0.001	(4,136) = 145.5	< 0.001	(4,136) = 131.7	< 0.001	(4,136) = 114.9	< 0.001	(4,136) = 118.1	< 0.001	(4,136) = 136.6	< 0.001
T	(1,34) = 0.15	NS	(1,34) = 2.64	NS	(1,34) = 3.36	NS	(1,34) = 1.97	NS	(1,34) = 0.03	NS	(1,34) = 0.04	NS
G	(1,34) = 2.04	NS	(1,34) = 4.73	< 0.05	(1,34) = 3.95	NS	(1,34) = 4.03	NS	(1,34) = 0.86	NS	(1,34) = 0.38	NS
I×T	(4,136) = 1.71	NS	(4,136) = 0.47	NS	(4,136) = 0.81	NS	(4,136) = 0.67	NS	(4,136) = 0.50	NS	(4,136) = 0.65	NS
I×G	(4,136) = 0.99	NS	(4,136) = 2.28	NS	(4,136) = 1.73	NS	(4,136) = 0.96	NS	(4,136) = 0.70	NS	(4,136) = 0.46	NS
T×G	(1,34) = 9.68	< 0.01	(1,34) = 4.84	< 0.05	(1,34) = 4.30	< 0.05	(1,34) = 1.67	NS	(1,34) = 0.01	NS	(1,34) = 0.29	NS
I×T×G	(4,136) = 40.51	NS	(4,136) = 1.88	NS	(4,136) = 0.80	NS	(4,136) = 1.49	NS	(4,136) = 0.94	NS	(4,136) = 0.06	NS

^a Factors of variation were intensity (within subject), treatment (between subject) and genotype (between subject); D + 1, D + 2 and D + 3, one, two and three days after fentanyl administration, respectively; NS, no significant.

three-way ANOVA). However, differences between genotypes were observed in the delayed effects of fentanyl. Thus, fentanyl induced an increase in the withdrawal responses for 3 days in wild-type mice. This increase was observed after application of the Von Frey filaments with a ranging force of 1–8 mN, indicative of allodynia (one-way ANOVA for treatment at day 1: $F_{1,18} = 7.52$, $P < 0.05$ for 8 mN; at day 2: $F_{1,18} = 8.94$, $P < 0.01$ for 1 mN and $F_{1,18} = 5.60$, $P < 0.05$ for 4 mN; at day 3: $F_{1,18} = 7.43$, $P < 0.05$ for 4 mN and $F_{1,18} = 4.98$, $P < 0.05$ for 8 mN) (Fig. 3). A tendency to increase the response was also observed after the application of the filaments with higher forces (12–32 mN) in wild-type mice from day 1 to day 3 post-fentanyl. The fentanyl-induced delayed allodynia was not observed in mutant mice and significant differences were found between genotypes (one-way ANOVA for genotype at day 1: $F_{1,17} = 5.87$, $P < 0.05$ for 8 mN; at day 2: $F_{1,17} = 8.34$, $P < 0.05$ for 1 mN, $F_{1,17} = 6.86$, $P < 0.05$ for 4 mN and $F_{1,17} = 5.61$, $P < 0.05$ for 16 mN; at day 3: $F_{1,17} = 8.66$, $P < 0.05$ for 4 mN and $F_{1,17} = 5.39$, $P < 0.05$ for 8 mN).

3.2.3. Formalin test

Similar biphasic responses were found in wild-type and mutant saline control mice after a formalin injection (0.1%), with an early phase (phase I) evaluated during the first 5 min and a late phase (phase II) extending from 10 to 40 min after the formalin injection (Fig. 4). Two-way ANOVA revealed a significant treatment effect in phase II ($F_{1,67} = 11.90$, $P < 0.01$) but not phase I. In addition, a genotype effect ($F_{1,67} = 5.94$, $P < 0.05$ in phase I; $F_{1,67} = 6.80$, $P < 0.05$ in phase II) and an interaction between these two factors ($F_{1,67} = 5.68$, $P <$

0.05 in phase I; $F_{1,67} = 9.43$, $P < 0.01$ in phase II) were observed in the two phases. In wild type mice, fentanyl pre-exposure, 48 h before the test, increased formalin-induced licking behaviour in both phase I (one-way ANOVA for treatment: $F_{1,34} = 4.99$, $P < 0.05$) and II (one-way ANOVA for treatment: $F_{1,34} = 17.50$, $P < 0.001$). Such an increase in formalin-induced licking behaviour was not observed in mutant mice. Significant differences in the response were found between genotype in both phase I (one-way ANOVA for genotype: $F_{1,34} = 13.93$, $P < 0.01$) and II (one-way ANOVA for genotype: $F_{1,34} = 11.13$, $P < 0.01$).

3.3. Naloxone test

The opioid receptor antagonist naloxone was administered 6 days after the fentanyl treatment, when the animals had recovered their pre-fentanyl nociceptive threshold. This was carried out in order to reveal significant fentanyl-induced long-term changes in opioid system activity by using the tail-pressure and Von Frey tests (Fig. 5).

3.3.1. Tail-pressure test

No significant effect of naloxone (1 mg/kg, s.c.) was found on day 6 in wild-type and mutant mice treated with saline (Fig. 5A) or the lower dose of fentanyl ($4 \times 20 \mu\text{g/kg}$) as compared to the pre-treatment value on day 0 (data not shown). However, naloxone induced a marked decrease in the nociceptive threshold of the wild-type mice previously treated with the dose of fentanyl of $4 \times 60 \mu\text{g/kg}$ as revealed by two-way ANOVA (treatment: $F_{1,77} = 7.61$, NS, genotype: $F_{1,77} = 8.49$, $P < 0.01$, treatment x genotype: $F_{1,77} = 7.61$, $P < 0.01$)

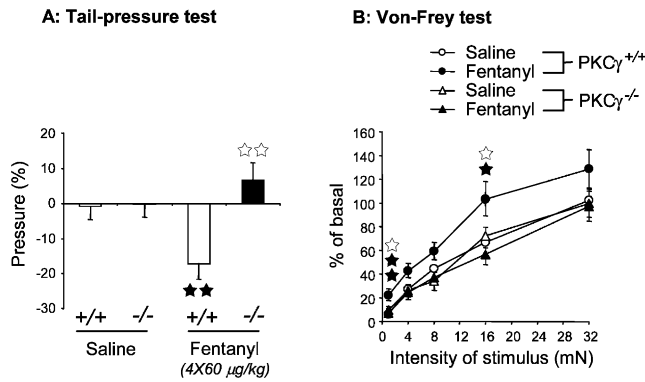


Fig. 5. Changes in basal nociceptive threshold induced by naloxone in wild-type ($PKC\gamma^{+/+}$) and mutant ($PKC\gamma^{-/-}$) mice evaluated 6 days after the fentanyl treatment by using tail-pressure (A) and Von Frey (B) tests. Fentanyl was injected four times ($60 \mu\text{g}/\text{kg}$, s.c.) at 15 min intervals. Naloxone ($1 \text{ mg}/\text{kg}$, s.c.) was injected six days later, when mice had recovered their pre-fentanyl nociceptive threshold values. The nociceptive threshold was measured 5 min after naloxone injection. Number of mice per group: 9–10. Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA, treatment effect) and * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA, genotype effect).

and subsequent one-way ANOVA for treatment ($F_{1,43} = 8.07$, $P < 0.01$) (Fig. 5A). Naloxone precipitated a stronger decrease in nociceptive threshold in wild-type mice previously treated with a high dose of fentanyl ($4 \times 100 \mu\text{g}/\text{kg}$) ($-22 \pm 5\%$) (data not shown). Such a naloxone-precipitated hyperalgesia was not observed in mutant mice previously treated by fentanyl ($4 \times 60 \mu\text{g}/\text{kg}$) and significant differences in the response were found between genotypes (one-way ANOVA for genotype: $F_{1,38} = 13.16$, $P < 0.01$) (Fig. 5A).

3.3.2. Von Frey test

Similar results were found in the Von Frey test (Fig. 5B). The administration of naloxone on day 6 did not produce any modification in the withdrawal threshold to Von Frey hair stimulation, as compared to pre-saline stimulus–response curve evaluated on day 0 in wild-type (two-way ANOVA, stimulus: $F_{4,72} = 247.1$, $P < 0.001$; time: $F_{1,18} = 0.03$, NS; stimulus \times time: $F_{4,72} = 1.08$, NS) and mutant (two-way ANOVA, stimulus: $F_{4,72} = 65.08$, $P < 0.001$; time: $F_{1,18} = 0.01$, NS, stimulus \times time: $F_{4,72} = 1.01$, NS) saline animals (data not shown). However, an increase in the withdrawal response was observed after naloxone in the wild-type mice previously treated with fentanyl ($4 \times 60 \mu\text{g}/\text{kg}$) (see Table 3 for three-way ANOVA). This increase was observed after application of the Von Frey filaments with a ranging force from 1 to 16 mN (one-way ANOVA for treatment: $F_{1,18} = 9.3$, $P < 0.01$ for 1 mN and $F_{1,18} = 5.80$, $P < 0.05$ for 16 mN) (Fig. 5B). Naloxone-precipitated allodynia was not observed in mutant fentanyl-treated mice as revealed by one-way ANOVA for treatment, and significant differences were found between genotypes (one-

Table 3

PKC γ influence on naloxone-precipitated allodynia by using the Von Frey test in mice that have received fentanyl 6 days before (three-way ANOVA)^a

	<i>F</i>	<i>P</i>
Intensity (I)	(4,136) = 67.23	<0.001
Treatment (T)	(1,34) = 0.15	NS
Genotype (G)	(1,34) = 2.04	NS
I \times T	(4,136) = 1.71	NS
I \times G	(4,136) = 0.99	NS
T \times G	(1,34) = 9.68	<0.01
I \times T \times G	(4,136) = 40.51	NS

^a Factors of variation were intensity (within subject), treatment (between subject) and genotype (between subject); NS, no significant.

way ANOVA for genotype, $F_{1,17} = 6.06$, $P < 0.05$ for 1 mN and $F_{1,17} = 7.19$, $P < 0.05$ for 16 mN).

4. Discussion

We evaluated the contribution of the PKC γ in the early and delayed nociceptive effects of fentanyl in mice. The appearance of spontaneous episodes of hyperalgesia after opioids has already been described in animals after both acute and chronic treatment (Simonnet and Rivat, 2003). In agreement with previous studies conducted in rats (C  lerier et al., 2000), we reported here for the first time in mice that an acute fentanyl exposure produced a biphasic time-dependent effect on nociception, i.e., an early antinociceptive response during several hours, followed by a delayed hyperalgesic/allodynic response for several days. This delayed pronociceptive effect was manifested by a sustained decrease of nociceptive threshold in the tail-pressure and Von Frey tests as well as by an increase in the response to the formalin injection two days after fentanyl administration. The duration of these fentanyl pronociceptive effects depends on the dose used, and can last four days after fentanyl administration (see also C  lerier et al., 2000). These results confirm in mice that opioid may recruit pronociceptive systems immediately following the first administration, and open the possibility to use genetically modified mice to study the mechanisms involved in such a paradoxical opioid effect.

The deletion of PKC γ gene in mice has produced an increase of the fentanyl antinociceptive effectiveness in the tail-immersion test and the complete prevention of fentanyl-induced delayed hyperalgesic/allodynic effects in the tail-pressure, Von Frey and formalin tests. In the tail-pressure test, wild-type and mutant mice showed different nociceptive sensitivity in basal conditions. This is the first time that a mechanical test with suprathreshold nociceptive stimulation has been used to evaluate nociception in PKC γ knock-out mice. Nevertheless,

although significantly lower than that of wild-type mice, the basal threshold found in knock-out mice did not represent any floor effect and did not constitute a limitation for detecting hyperalgesia in our experimental conditions. Moreover, this different basal nociceptive threshold has presumably no influence in the responses observed after fentanyl administration since a similar prevention of fentanyl pronociceptive effects was observed in mutant mice in the Von Frey and formalin tests where the basal nociceptive threshold was similar in both genotypes. Therefore, our results strongly suggest a key role for the PKC γ in the activation by fentanyl of pronociceptive systems associated with the appearance of delayed hyperalgesic/allodynic states.

The enhanced antinociceptive effects observed in PKC γ deficient mice suggest that this system is already activated after the first opioid exposure reducing the primary fentanyl antinociceptive effect. Indeed, it has been previously proposed that the appearance of hyperalgesic states after opioid administration could be the manifestation of the activation of opponent processes to analgesia that include NMDA receptors, with the consequence of a decrease of the antinociceptive effectiveness of opioids (Laulin et al., 1998, 1999; Célérier et al., 2001). Thus, the blockade of the PKC γ /NMDA-dependent pronociceptive effects by specific antagonists or gene deletion would allow the complete expression of the acute opioid antinociceptive response. In addition, opioid tolerance and abnormal pain sensitivity that develop after opioid chronic treatment may share common cellular mechanisms in part through the activation of the NMDA receptors (Mao et al., 1994, 1995a; Simonnet and Rivat, 2003). In this context, repeated opioid administration may induce sensitisation of the NMDA-dependent pronociceptive systems that are already activated from the first opioid exposure, leading to the development of tolerance as revealed by a progressive reduction of the opioid antinociceptive effects (Laulin et al., 1999; Célérier et al., 2001; Simonnet and Rivat, 2003). In agreement with this hypothesis, both the administration of NMDA receptor antagonists and the deletion of the PKC γ gene were able to attenuate the development of tolerance to opioid antinociceptive effects (Laulin et al., 1999; Mao et al., 1994; Mao, 1999; Zeitz et al., 2001).

Although the involvement of NMDA receptors in opioid pronociceptive effects and antinociceptive tolerance is now well accepted, it is unclear how the activation of NMDA receptors could be initiated after opioid administration. The allodynic/hyperalgesic states elicited in mice by short-term exposure of fentanyl, a selective μ -opioid receptor agonist, support a primary role for these μ -opioid receptors. This result confirms previous studies assessing fentanyl effects after acute administration in rats (Célérier et al., 2000). Thermal hyperalgesia and mechanical allodynia have also been observed in

mice after intermittent fentanyl administration for 6 days (Li et al., 2001). Furthermore, hyperalgesia was not observed after morphine treatment in CXBK mice, a strain which has reduced μ -opioid receptor expression (Li et al., 2001). Opioids have no detectable binding affinity for the NMDA receptors (Mao, 1999) and indirect mechanisms have been proposed. Thus, opioid treatment may up-regulate spinal dynorphin which evokes an increased release of excitatory transmitters from primary afferent fibres that promotes exaggerated pain through a NMDA dependent mechanism (Gardell et al., 2002; Vanderah et al., 2001). Here, PKC γ was identified as a possible key element that links μ -opioid receptor activation by fentanyl with the recruitment of the NMDA/glutamatergic systems implicated in the promotion of pain. In agreement with such a proposal, the subunit NR1 of the NMDA receptor seems to be directly associated with functionally active PKC γ through non-covalent interactions (Suen et al., 1998). Moreover, the μ -opioid receptor agonist DAMGO has been reported to increase in vitro NMDA receptor-mediated glutamate response via a PKC γ -mediated removal of the magnesium blockade of the NMDA receptor channel in nociceptive neurons (Chen and Huang, 1991, 1992). The subsequent increase of the intracellular Ca²⁺ concentration further stimulates the PKC γ activity leading to a long-lasting enhancement of glutamate synaptic efficiency by a positive feedback loop, a process resembling long-term potentiation (Nicoll and Malenka, 1999). Since PKC γ possesses this unique ability to become persistently active even following removal of the stimuli (Tanaka and Nishizuka, 1994; Braun and Schulman, 1995), similar PKC γ /NMDA receptor interaction could be proposed to explain the sustained activation of the pronociceptive systems observed after fentanyl in the present study. These long lasting PKC γ /NMDA-dependent changes could also explain our results revealing the effectiveness of the opioid receptor antagonist naloxone to precipitate hyperalgesia and allodynia in mice that recovered their pre-drug nociceptive value 6 days after the acute fentanyl treatment. As previously proposed (Célérier et al., 2001), the sustained and prolonged activation of the pronociceptive systems would be progressively opposed by a counteradaptation involving the recruitment of the endogenous analgesic systems. The naloxone-precipitated hyperalgesia/allodynia would then be the result of the sharp breakdown of a new equilibrium between endogenous opioid-dependent analgesic system and PKC γ /NMDA-dependent pronociceptive mechanisms.

In conclusion, this study in knock-out mice provides new data demonstrating the crucial role played by PKC γ in opioid-induced enhancement in nociceptive sensitivity. These results may shed light on the molecular mechanisms implicated in the activation of opposing processes to analgesia after opioid treatment. Consider-

ing the wide use of opioids in surgery and the management of chronic pain, our results represent an important advancement to better understand the mechanisms involved in their potential side effects. This will allow the proposition of molecular targets for the development of new analgesic agents and new strategies of combined medication in order to obtain an enhanced opioid analgesic effectiveness with reduced incidence of undesirable effects.

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