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## Supraspinal G $\beta\gamma$ -dependent stimulation of PLC $\beta_3$ originating from G inhibitory protein- $\mu$ opioid receptor-coupling is necessary for morphine induced acute hyperalgesia

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### Abstract

Although alterations in  $\mu$ -opioid receptor ( $\mu$ OR) signaling mediate excitatory effects of opiates in opioid tolerance, the molecular mechanism for the excitatory effect of acute low dose morphine, as it relates to  $\mu$ OR coupling, is presently unknown. A pronounced coupling of  $\mu$ OR to the  $\alpha$  subunit of G inhibitory protein emerged in periaqueductal gray (PAG) from mice systemically administered with morphine at a dose producing acute thermal hyperalgesia. This coupling was abolished in presence of the selective  $\mu$ OR antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub> administered at the PAG site, showing that the low dose morphine effect is triggered by  $\mu$ OR activated G inhibitory protein at supraspinal level. When G $\beta\gamma$  downstream signalling was blocked by intra-PAG co-administration of 2-(3,4,5-trihydroxy-6-oxoxanthen-

9-yl)cyclohexane-1-carboxylic acid, a compound that inhibits G $\beta\gamma$  dimer-dependent signaling, a complete prevention of low dose morphine induced acute thermal hyperalgesia was obtained. Phospholipase C  $\beta_3$ , an enzyme necessary to morphine hyperalgesia, was revealed to be associated with G $\beta\gamma$  in PAG. Although opioid administration induces a shift in  $\mu$ OR-G protein coupling from Gi to Gs after chronic administration, our data support that this condition is not realized in acute treatment providing evidence that a separate molecular mechanism underlies morphine induced acute excitatory effect.

**Keywords:**  $\mu$ -opioid receptor, G protein, hyperalgesia, M119, morphine, phospholipase C.

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Classically, morphine activates G inhibitory (Gi) protein coupled to  $\mu$ -opioid receptors ( $\mu$ OR) to inhibit adenylyl cyclase activity and decrease neuronal cAMP levels (Uhl *et al.* 1994). However, recent studies suggest that opioids can exert stimulatory effects either at doses well below those producing neuronal inhibition or after chronic exposure. In cultured dorsal root ganglion neurons, nanomolar concentrations of opioid agonists increase action potential duration, whereas micromolar concentrations produce the opposite effect (Chen *et al.* 1988; Shen and Crain 1989). This dual action of opioids has been explained on the basis of a bimodal opioid receptor model. In this model, ultra-low doses of an agonist activate a Gs-coupled mode of the opioid receptor to activate adenylyl cyclase and increase neuronal excitability. In contrast, higher doses of opioids activate a

Gi/Go-coupled mode of the receptor to inhibit adenylyl cyclase activity and reduce neuronal excitability. This bimodal model of morphine action has been invoked to explain paradoxical hyperalgesia after chronic opioid administration. Accordingly, the predominance of the Gs-coupled mode of the  $\mu$ OR during chronic treatment opposes the

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**Abbreviations used:**  $\mu$ OR,  $\mu$ -opioid receptors; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>; Gi, G inhibitory; M119, 2-(3,4,5-trihydroxy-6-oxoxanthen-9-yl)cyclohexane-1-carboxylic acid; PAG, periaqueductal gray; PLC, phospholipase C.

analgesic response eliciting tolerance associated-hyperalgesia through adenylyl cyclase activation (Crain and Shen 1990, 1992). Alternatively, different authors showed that excitatory signaling of opioid receptors in chronic morphine treatment can also occur by G $\beta\gamma$  activation of adenylyl cyclase (Wang and Gintzler 1997; Gintzler and Chakrabarti 2001). Yet, extremely low doses of morphine can induce acute excitatory effects. Peripheral application of a very low dose of morphine induces a flexor response (Ono *et al.* 2002). The stimulation of sensory nerve endings induced by morphine through peripheral  $\mu$ OR activation and its downstream mechanisms has been ascribed to activation of phospholipase C (PLC) through substance P release from polymodal C fibers (Ono *et al.* 2002). Extremely low doses of systemic morphine can elicit acute hyperalgesia in animal model of pain such as tail flick (Crain and Shen 2001; Esmacili-Mahani *et al.* 2008), Freund's adjuvant-induced arthritic rats (Kayser *et al.* 1987), and hot plate tests (Galeotti *et al.* 2006). A specific signaling pathway for morphine-induced acute thermal hyperalgesia has been shown via  $\mu$ OR activation of PLC/protein kinase C inositol-lipid signalling pathway (Galeotti *et al.* 2006). Among the large PLC family, the  $\beta_3$  isoform of PLC appeared to be implicated in inducing this excitatory effect whereas adenylyl cyclase levels remained unmodified in CNS after low dose morphine exposure (Galeotti *et al.* 2006; Esmacili-Mahani *et al.* 2008). Although alterations in  $\mu$ OR signaling mediate excitatory effects of opiates in opioid tolerance and dependence, these excitatory effects have not been directly examined in an *in vivo* acute treatment paradigm with respect to  $\mu$ OR-G protein coupling. Thus, this work investigates  $\mu$ OR-G protein coupling as well as coupling between G $\beta\gamma$  and PLC at supraspinal level in low dose morphine-induced acute hyperalgesia.

## Methods and materials

### Animals

Pathogen free sexually mature male albino Swiss Webster mice (Charles River, Wilmington, MA, USA) weighing 23–30 g were used. Five-six mice were housed per cage. The animals were fed a standard laboratory diet and water *ad libitum* and kept at  $23 \pm 1^\circ\text{C}$  with a 12-h light/dark cycle. All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 for experimental animal care. All efforts were made to minimize the number of animals used and their suffering. All the animals were previously habituated to the laboratory according to Abbott *et al.* (1986).

### Drug treatment

The following drugs were used: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub> (CTOP), morphine HCl (Sigma Chemicals, St Louis, MO, USA); 2-(3,4,5-trihydroxy-6-oxoxanthene-9-yl)cyclohexane-1-carboxylic acid (M119). Drugs were administered in a volume of 0.2  $\mu\text{L}$  per mouse by intracranial infusion, and 10 mL/kg by

intraperitoneal (i.p.) injection. Morphine and CTOP were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. M119 was initially solubilized in dimethylsulfoxide and subsequently diluted in distilled water. Morphine was administered i.p.; CTOP and M119 were administered into the periaqueductal gray (PAG) immediately before morphine administration; saline was administered i.p. and dimethylsulfoxide vehicle intra-PAG.

Different groups of mice received: i.p. control saline or intra-PAG vehicle; 1  $\mu\text{g}/\text{kg}$  morphine in presence or absence of CTOP (80 ng/mouse) administered intra-PAG immediately before morphine; 1  $\mu\text{g}/\text{kg}$  morphine or saline in the presence or absence of intra-PAG M119 (5–40 ng/mouse). CTOP and M119 were also administered alone. Other different groups received i.p. injection of saline, morphine (7 mg/kg) or twice daily morphine (10 mg/kg) for 7 days.

### Surgery and microinjection

Cannula implantation was performed as previously described (Carvalho-Netto *et al.* 2007). Briefly, mice were implanted with stainless steel guide cannula (32-gauge) under anesthesia. Stereotaxic coordinates (Paxinos and Franklin 2001) for the PAG were, respectively, 4.2 mm posterior to bregma, 1.3 mm lateral to the midline, and 2.2 mm ventral to the skull surface, with the guide cannula angled  $26^\circ$  to the vertical. A dummy cannula was inserted into each guide-cannula immediately after each surgery to reduce the incidence of occlusion. Five to seven days after surgical recovery, solutions were injected into the PAG by microinjection unit which extended 1.0 mm beyond the tips of the guide cannula. Each microinjection unit was attached to a 5- $\mu\text{L}$  Hamilton microsyringe via polyethylene tubing and administration was controlled by an infusion pump programmed to deliver a volume at rate of 0.1  $\mu\text{L}$  over a period of 30 s. At the conclusion of the experiments 1% Evans blue dye was administered to mice according to the microinjection procedure described above for intra-PAG administration. A post-mortem histological control of the injection site was performed on cryostat sections of unfixed brains previously frozen. The data of any mice were excluded from statistical analysis if the cannula tip was outside the PAG or if the region had sustained extensive damage. The brains from mice which were further submitted to western blot were extracted leaving the cannula implanted in the brain. The location of cannula inside PAG was observed under stereomicroscope (Leica MZ12.5, Leica, Solms, Germany). Only specimens from mice with cannula path inside the PAG were used for western blot experiments.

### Hot plate test

Mice were placed inside a stainless steel container, which was set thermostatically at  $52.5 \pm 0.1^\circ\text{C}$  in a precision water-bath. Here we have used lower temperature in hot plate test ( $52^\circ\text{C}$  instead of  $54^\circ\text{C}$ ) to reveal possible subtle alterations that may occur in basal nociception. The licking latency was measured immediately prior i.p. morphine injection. Hot plate test started 15 min after morphine administration and licking latencies were measured at 15-min intervals for 60 min after starting time. A 30 s cut-off to prevent tissue damage was used. The endpoint for the licking response was the first paw lick of the front paw. Increased nociception was seen as shorter latencies to the responses evaluated. The test was performed in a blind fashion. Mice pre-treated with drugs administered as previously described, were evaluated for licking latency basal value.

### Rota-rod and hole-board test

For both tests, animals were pre-treated with saline (i.p.), intra-PAG vehicle or M119 (40 ng/mouse), 1  $\mu$ g/kg morphine (i.p.) in presence or absence of M119 and submitted to rota-rod and hole-board (Ghelardini *et al.* 2008). Twelve mice per group were tested. The rota-rod test apparatus consists of a base platform and a rotating rod (3  $\times$  30 cm) placed at a height of 15 cm from the base and divided into five equal sections by six disks. Up to five mice were tested simultaneously on the apparatus, with a rod-rotation speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s, according to Vaught *et al.* (1985). Performance time was measured before and 15, 30 and 45 min after the administration of the investigated compounds. The hole board test consisted of a 40 cm square plane with 16 flush-mounted cylindrical holes (3 cm diameter) distributed four by four in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (spontaneous motility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (inspection activity) by the mice. Mice pre-treated with 1  $\mu$ g/kg morphine (i.p.) in presence or absence of intra-PAG CTOP (80 ng/mouse) were previously submitted to both tests (Ghelardini *et al.* 2008).

### $\mu$ OR-G protein coupling assay

Mice used for these experiments were killed 15 min after 1  $\mu$ g/kg or 7 mg/kg morphine administration at which time maximum thermal hyperalgesic/analgesic effect is obtained (Galeotti *et al.* 2006) or 7 days after repeated morphine administration at the doses previously described.  $\mu$ OR-G protein coupling assay data after acute and chronic high-dose morphine administration are largely known (Sánchez-Blázquez *et al.* 2001; Wang *et al.* 2005; Askari *et al.* 2008) but were used to support the method utilized in this study. The animals were anesthetized with CO<sub>2</sub>, cervically dislocated, decapitated and the brain dissected, put immediately in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$ . PAG brain area from control and treated mice was dissected on a cold plate. Enriched synaptic membranes were prepared from PAG of mice from each treatment group as described by Gray and Whittaker (1962). Protein concentration was determined according to Lowry *et al.* (1951). The association of G protein coupled receptors with G proteins was investigated using co-immunoprecipitation procedure as previously described (Wang *et al.* 2005). Briefly, PAG membranes were incubated with Krebs-Ringer and then solubilized in immunoprecipitation buffer (25 mM HEPES, pH 7.5, 200 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2% 2-mercaptoethanol, 50  $\mu$ g/mL leupeptin, 25  $\mu$ g/mL pepstatin A, 0.01 U/mL soybean trypsin inhibitor, 0.04 mM phenylmethylsulfonyl fluoride) containing 0.5% digitonin, 0.2% sodium cholate and 0.5% NP-40 (Sigma Chemicals, St Louis, MO, USA) emulsifying agent with end-over-end rotation for 60 min at  $4^{\circ}\text{C}$  and further centrifugated at 50 000 *g*. The supernatant was divided for separate passage through immunoaffinity columns containing immobilized antibodies to G $\alpha$ s, G $\alpha$ i, G $\alpha$ o, G $\alpha$ 11, G $\alpha$ q or G $\beta$  proteins. Anti-G protein antibodies (Santa Cruz Biotechnology,

Santa Cruz, CA, USA) were covalently cross-linked to protein-G-conjugated resin in Seize-X protein G immunoprecipitation kit (Pierce-ENDOGEN, Rockford, IL, USA) according to the manufacturer instructions. G protein complexes in solubilized brain lysates were isolated by immunoprecipitation in which 200  $\mu$ g solubilized brain membrane extracts were incubated with immobilized anti-G-protein G-resin at  $4^{\circ}\text{C}$  overnight. After centrifugation and three washes with phosphate-buffered saline (pH 7.2) at  $4^{\circ}\text{C}$ , the G protein complexes were eluted with 190  $\mu$ L of antigen elution buffer (pH 2.8) and immediately neutralized by adding 20  $\mu$ L of 1.5 M Tris buffer (pH 8.8). The neutralized G complexes were combined with 180  $\mu$ L of 2 $\times$  polyacrylamide gel electrophoresis sample preparation buffer (62.5 mM Tris-HCl, pH 6.8; 20% glycerol, 4% sodium dodecyl sulfate; 10% 2-mercaptoethanol, 0.1% Bromophenol Blue), boiled for 5 min and then submitted to western blotting using a specific antibody directed against the amino-terminal region of the  $\mu$ OR for G $\alpha$  and PLC $\beta$ <sub>1-4</sub> for G $\beta$  (Santa Cruz Biotechnology). 18% and 4–15% or 4–20% Tris-HCl gels were used respectively for G-protein- $\mu$ OR complex and specificity assay of antibodies or G $\beta$ -PLC $\beta$ <sub>1-4</sub> co-immunoprecipitation experiments. The specificity of the anti-G $\alpha$ , anti-G $\beta$  and PLC $\beta$ <sub>1-4</sub> antibodies was determined by western blotting using 100  $\mu$ L of mouse whole brain homogenate with or without antigen peptide (25  $\mu$ g) pre-absorption for 30 min. The specificity of anti- $\mu$ OR antibody was assayed on brain tissue from knockout brain mouse (GR21 $^{-/-}$ ; a generous gift of Dr Brigitte Kieffer, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Département Neurobiologie et génétique, Illkirch, France). A highly acidic (pH 2.8) or neutral elution buffer was used to elute antigens from the G $\alpha$  immuno-complexes in order to establish if the experimental procedure yielded  $\mu$ OR with an approximate molecular weight of respectively 53 and 67 kDa (Chen *et al.* 1995; Wang *et al.* 2005).

### Western blot analysis

Western blot was performed as previously described in detail (Pan *et al.* 1995). In summary, immunoprecipitates (from 1  $\mu$ g/ $\mu$ L protein lysate) of G $\alpha$ i, G $\alpha$ o, G $\alpha$ q, G $\alpha$ 11, G $\alpha$ s and G $\beta$  protein from PAG of morphine, CTOP, M119, saline and vehicle pre-treated mice were solubilized in sodium dodecyl sulfate buffer and separated on polyacrylamide gels (1.5 mm). Proteins were transferred to nitrocellulose (1.5 h at 190 mA) and the membranes were blocked in phosphate-buffered saline containing 3% bovine serum albumin for 1 h before addition of anti- $\mu$ OR or anti-G $\beta$  antibody at 1 : 500 dilution. The blots were stripped and reprobed with antibodies against various G proteins using the antisera against G $\alpha$ i, G $\alpha$ o, G $\alpha$ q, G $\alpha$ 11, G $\alpha$ s and G $\beta$  protein as probes at 1 : 1000 dilution. The blotting was visualized using a chemiluminescence detection system (Super Signal West Fento, Pierce Biotechnology Inc.) and quantified with the Versa Doc 1000 Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Three independent experiments were done at the same protein concentration for each experimental condition. Specific bands were quantitated by densitometric scanning.

### Immune complex PLC activity measurement

Phospholipase C enzyme activity in anti-G $\beta$  immunoprecipitates was measured as described in Allan *et al.* (1997). Immune complexes for G $\beta$  (30  $\mu$ L) were assayed for 15 min at  $37^{\circ}\text{C}$  in

the presence of 35 mM sodium phosphate, pH 6.8, 70 mM KCl, 0.8 mM EGTA, 0.8 mM CaCl<sub>2</sub>, 0.20 mM [<sup>3</sup>H]phosphatidylinositol 4,5-bisphosphate (8Ci/nmol, Perkin-Elmer, Waltham, MA, USA), and 2.86 mM (0.18%, v/v) Triton X-100 in a final volume of 50 μL. Enzyme activity was quantitated as the release of [<sup>3</sup>H] Ins(1,4,5)P<sub>3</sub> measured by liquid scintillation spectroscopy. Isozyme-specific activity was calculated by subtracting background [<sup>3</sup>H] Ins(1,4,5)P<sub>3</sub> (release present in no antibody control samples) from the activity measured in immune complexes. Data were calculated as nanomoles Ins(1,4,5)P<sub>3</sub> product formed per minute per milligram protein present in the fraction from which the enzyme was immunoprecipitated. All conditions were run in triplicate.

### Statistical analysis

All experimental results are given as mean ± SEM. Analysis of variance followed by Fisher protected least significant difference procedure for *post hoc* comparisons was used to verify significance between two means for data obtained from co-immunoprecipitation of μOR-G protein or Gβγ-PLCβ complexes after hyperalgesic/analgesic dose and chronic morphine administration. Unpaired repeated measures ANOVA followed by Scheffé test paired *post hoc* was applied to hot plate and rota-rod test results. Data were analysed with the Statview Software for the Macintosh (1992, Apple, Cupertino, CA, USA).

## Results

### Co-immunoprecipitation of μOR-G protein complexes after hyperalgesic or analgesic dose and chronic morphine administration

Under non-denaturing conditions that keep μOR-G protein complexes intact, specific G proteins (Gi, Go, Gq, G11 and Gs) together with their coupled receptors were immunoprecipitated with selective anti-Gα antibodies from solubilized synaptic membranes obtained from PAG of the different treatment groups under both basal and morphine-stimulated conditions. μOR coupling to each of the G protein subtypes in the different treatment groups is shown in western blots of the Gα immunoprecipitates probed with the anti-μOR-specific antibody (Fig. 1a). In PAG, μOR coupled exclusively to Go in saline and CTOP treated mice, and to both Go and Gi in low dose morphine-treated mice. In PAG of mice treated with morphine in presence of CTOP, coupling to Gi was markedly decreased from that in the morphine-treated animals (Fig. 1a). Morphine i.p. administration at analgesic dose dramatically increased μOR-Gi coupling (Fig. 1a). Go and Gs coupling by μOR weakly increased with respect to control; however these were not statistically significant (Fig. 1a). Chronic morphine decreased Gi coupling by μOR. A pronounced coupling to Gs appeared in PAG from mice submitted to chronic morphine treatment (Fig. 1a). Densitometric scanning of immunoprecipitated Gα proteins was used as loading control. The relative amount of each of these proteins was not significantly different with respect to saline in all experimental conditions (Fig. 1a). Pre-absorption

with 25 μg of their respective antigen peptides drastically abolished the detection of targeted G proteins by western blotting in mouse brain homogenate (Fig. 1b). No immunoreactive band was observable in brain extracts from μOR<sup>-/-</sup> mice in presence of anti-μOR antibody (Fig. 1b). Elution with the highly acetic antigen elution buffer yielded μOR with an apparent molecular weight of 53 kDa (Fig. 1b). Elution with a neutral pH predominantly yielded μOR with an apparent molecular weight of 67 kDa (Fig. 1b).

### Effect of M119 compound in morphine induced hyperalgesia

Morphine hyperalgesia induced by i.p. 1 μg/kg dose in the mouse hot plate test was completely prevented by intra-PAG pre-treatment with M119 at 5–40 ng/mouse (Fig. 2a). This effect appeared to be dose dependent. The M119 compound, when administered alone at the same doses, did not induce any significant change in licking latency (Fig. 2b).

### Rota-rod test and spontaneous activity meter

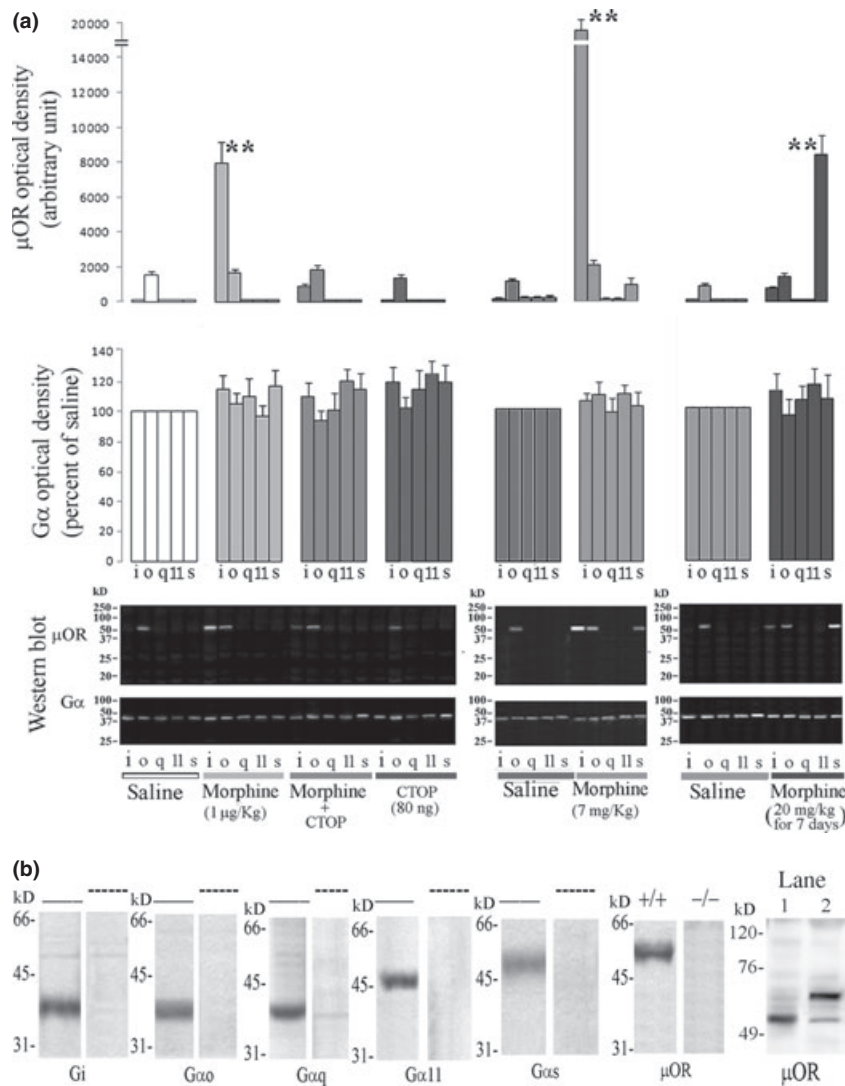
The spontaneous motility as well as inspection activity of mice were unmodified by pre-treatment with drugs in comparison with control group (Fig. 3a). The number of falls from the rotating rod evaluated before and 15, 30 and 45 min after the beginning of the rota-rod test showed the lack of any impairment in motor coordination of mice submitted to the above treatments in comparison with control group (Fig. 3b).

### Gβγ co-immunoprecipitation with PLCβ

In order to test the hypothesis that Gβγ associates with PLCβ in mouse PAG, anti-Gβ immune complexes were isolated from PAG of mice 15 min following 1 μg/kg morphine administration and probed for PLCβ<sub>1-4</sub> immunoreactivity. We found that Gβ immunoprecipitates with PLCβ<sub>3</sub> whereas immunoreactivity associated with anti-PLCβ<sub>1</sub>, β<sub>2</sub> and β<sub>4</sub> was not different from the saline background (Fig. 4a). Pre-absorption with 25 μg of their respective antigen peptides drastically abolished the detection of PLCβ<sub>1-4</sub> and Gβ by western blotting in mouse brain homogenate (Fig. 4b). Anti-Gβ immune complexes were isolated from PAG of mice 15 min following low dose morphine administration and assayed for associated PLC activity. These results demonstrate that Gβ associated with PLCβ<sub>3</sub> in low dose morphine administration and this was catalytically active (Fig. 5). PLC activity could not be detected in anti-Gβ immune complexes isolated from PAG of mice previously administered with low morphine dose in presence of M119 or CTOP at the higher effective doses (Fig. 5).

### Histology

Histology confirmed that 95% mice used in the experiments had cannula placement within the PAG. Only data from mice in which the cannula was correctly placed within the PAG were considered.



**Fig. 1** (a) G protein-μOR coupling in PAG from mice treated with morphine – A representative western blots of the presence of μOR protein in immunoprecipitates of i, o, q, 11, and s subunits of Gα protein in PAG from mice submitted to acute or chronic morphine treatments in presence or absence of CTOP is shown at the bottom of the figure. The blots stripped and reprobed with antibodies against the above G proteins, are shown for the different treatments. Band optical density for Gα protein subunits after different treatments is represented at the middle of the figure. Each bar represents the mean density of each Gα subunit obtained from three independent experiments and expressed as percent of corresponding saline. Statistics were applied to the raw data prior to transformation to percent. Mean value of μOR density detected in immunoprecipitates of considered

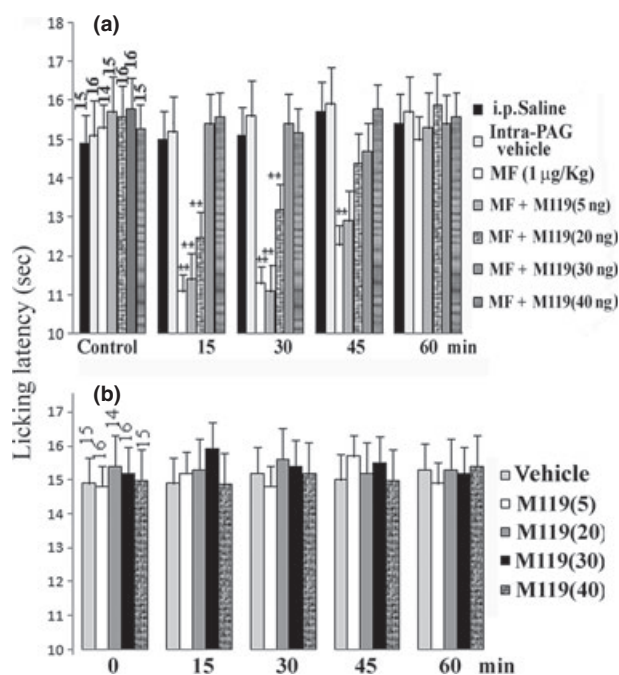
Gα subunits is represented at the top of the figure. Single values were normalized to surrounding background and expressed as arbitrary units. \*\*α < 0.01 significant difference in comparison with corresponding saline value. Vertical lines represent SEM. (b) Specificity assay of anti-G protein and anti-μOR antibodies – Homogenates from whole mouse brain were submitted to western blotting after incubation with the proper antibody in presence or absence of respective immunogen sequence in excess as shown in the figure. Western blotting was performed on extracts of brain tissue from normal (+/+) and μOR knockout (-/-) mice with anti-μOR antibody. Molecular weight of μOR immunoprecipitated by the anti-μOR antibody after elution with an acidic (lane 1) versus a neutral (lane 2) buffer is reported in the last column. —, not pre-absorbed; - - -, pre-absorbed.

## Discussion

### μOR-G protein coupling after morphine administration to mice

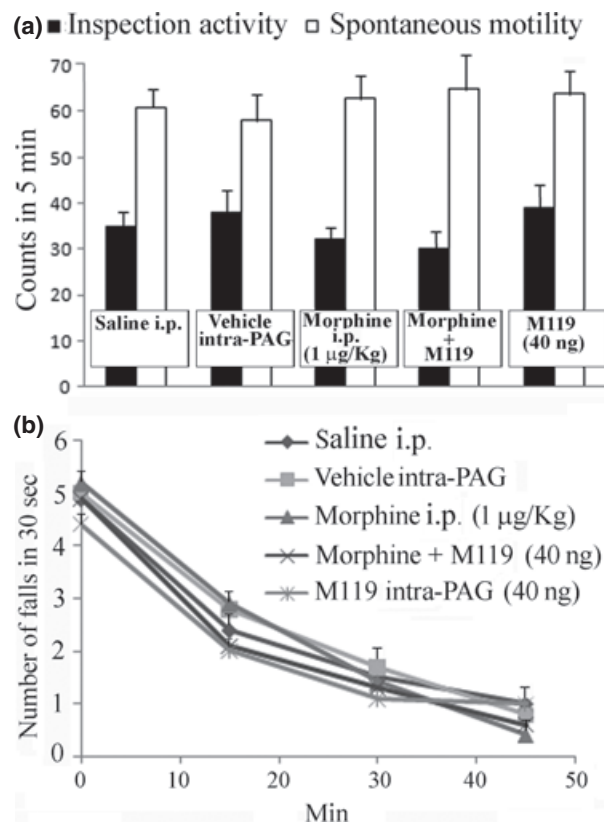
Although changes in μOR signaling have been previously demonstrated in the excitatory effects of opiates throughout

opioid tolerance and dependence, alterations in μOR signaling that mediate excitatory effects in an *in vivo* treatment acute paradigm have not been yet studied. To determine whether alterations in μOR-G protein coupling occur at μOR-expressing CNS tissue after morphine low dose systemic administration, we isolated the PAG from mice



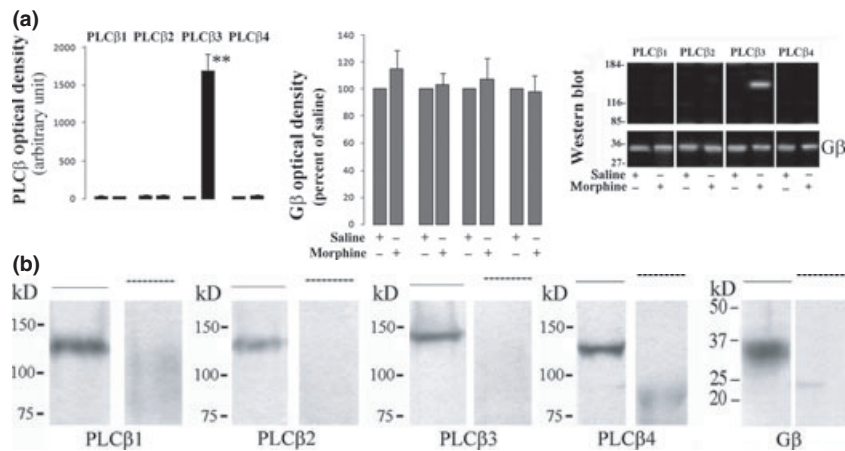
**Fig. 2** Effect of M119 co-administration on morphine induced hyperalgesic response—Licking latencies were measured before and after (15, 30, 45 and 60 min) i.p. morphine administration (1 µg/kg) in presence or absence of M119 intra-PAG co-administration at different doses (a). Licking latencies measured after M119 intra-PAG administration to mice are represented in (b). Each value represents the mean  $\pm$  SEM of licking latencies. Vertical bars represent SEM; \*\* $\alpha < 0.01$  significant difference in comparison with corresponding basal value. MF, morphine. The number of animals used for each experimental condition is reported at the top of control bars.

receiving systemic morphine at a dose which produces acute thermal hyperalgesia (Crain and Shen 2001; Galeotti *et al.* 2006; Esmaeili-Mahani *et al.* 2008). PAG is an important site of opioid analgesia (Yaksh *et al.* 1976) and tolerance to the antinociceptive effects of both systemic and locally applied morphine (Siuciak and Advokat 1987; Lane *et al.* 2004). In mouse PAG,  $\mu$ OR activation hyperpolarizes most neurons *via* activation of G-protein-gated inwardly rectifying potassium channels (Vaughan *et al.* 2003), and also inhibits GABA release from nerve terminals (Hack *et al.* 2003), consistent with the disinhibitory mechanisms proposed to be responsible for PAG-mediated opioid analgesia. Otherwise, opioid effect may extend beyond inhibition in the PAG. In brain slices, the excitatory action of NMDA on PAG neurons is potentiated by a  $\mu$ OR agonist at low nanomolar concentration (Kow *et al.* 2002). Exposure of PAG neurons to selective  $\mu$ OR antagonist CTOP completely reversed the morphine low dose induced acute hyperalgesic response showing that  $\mu$ OR activation in PAG is necessary to the excitatory response (Ghelardini *et al.* 2008). Under non-denaturing conditions that keep  $\mu$ OR-G protein complexes intact,



**Fig. 3** (a) Morphine and M119 administration do not induce any significant difference with respect to saline or vehicle on inspection activity and spontaneous mobility evaluated in the mouse hole board test. (b) Lack of effect of morphine and M119 administration on motor co-ordination evaluated in the mouse rota rod test. Vertical lines represent SEM.

specific  $G\alpha$  proteins together with their coupled receptors were immunoprecipitated with selective anti- $G\alpha$  antibodies from solubilized synaptic membranes obtained from the PAG under both basal and morphine-stimulated conditions. In our experiments,  $\mu$ OR coupled exclusively to Go in PAG from control mice. When mice were administered with systemic low morphine dose, a pronounced coupling of  $\mu$ OR to  $\alpha$  subunit of Gi protein emerged in PAG area; this coupling was markedly decreased from that in the morphine-treated animals in presence of selective  $\mu$ OR antagonist CTOP administered at the PAG site showing that low dose morphine effect is triggered by  $\mu$ OR activated Gi protein at supraspinal level. Morphine administration to mice at analgesic dose induced a dramatic increase in  $\mu$ OR coupling to  $\alpha$  subunit of Gi and a small, non-significant increase in  $\mu$ OR coupling to Go and Gs with respect to control, in agreement with previous results (Sánchez-Blázquez *et al.* 2001; Askari *et al.* 2008). When mice were submitted to chronic morphine administration, the coupling of  $\mu$ OR to Gs protein emerged in PAG whereas Gi protein- $\mu$ OR coupling



**Fig. 4** (a) Representative blot of co-immunoprecipitation of Gβ proteins with PLCβ<sub>1-4</sub> in PAG from low dose morphine administered mice is shown in figure. The blots stripped and reprobbed with Gβ antibody are shown at the bottom of the figure. Band optical density for Gβ protein subunits after saline or morphine is represented in the middle of the figure. Each bar represents the mean density of Gβ subunit obtained from three independent experiments and expressed as percent of corresponding saline. Statistics was applied to the raw data prior to transformation to percent. Mean value of PLCβ subunit density

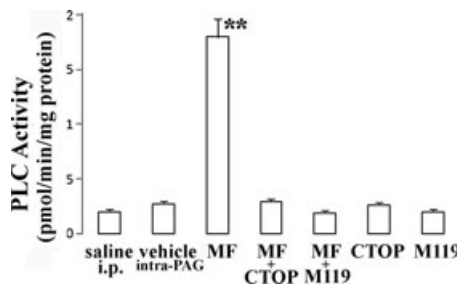
detected in immunoprecipitates of Gβ is represented at the top of the figure. Single values were normalized to surrounding background and expressed as arbitrary units. \*\* $\alpha < 0.01$  significant difference in comparison with corresponding saline value. Vertical lines represent SEM. (b) Specificity assay of anti-PLCβ<sub>1-4</sub> and anti-Gβ protein antibodies – western blot of whole mouse brain tissue pre-incubated with the proper antibody in presence or absence of respective immunogen sequence in excess are shown in figure.

dramatically decreased as previously obtained by different authors (Crain and Shen 1998; Wang *et al.* 2005). In the classic G protein signaling cascade, Gβγ subunits are released as a complex from the α subunit after activation of an associated receptor at the cell surface (Smrcka 2008). Bonacci *et al.* identified a compound, M119, that bound to Gβγ subunit and selectively inhibited Gβγ downstream signaling from the Gβγ subunit (Bonacci *et al.* 2006; Mathews *et al.* 2008). In our experiments, we used a hot plate test for evaluating thermal nociception in mice. Intra-PAG co-administration of M119 at the higher dose caused complete prevention of low dose morphine-induced acute

thermal hyperalgesia demonstrating that thermal hyperalgesia is dependent on Gβγ at the supraspinal level.

**Gβγ-PLCβ interaction in low dose morphine induced hyperalgesia**

Opioids are known to generate different neurochemical adaptations as inhibition of adenylyl cyclase (Childers 1991), activation of inwardly rectifying K<sup>+</sup> channels (North *et al.* 1987), and inhibition of voltage-activated calcium channels (Schroeder *et al.* 1991). Additionally, there is growing evidence that modulation of phosphoinositide-specific PLC, and consequently altered formation of inositol 1,4,5trisphosphate/diacylglycerol/Ca<sup>2+</sup> signaling, plays a key role in mediating excitatory opioid effects (Smith *et al.* 1999). PLC is one of only two signaling effector enzymes, the other being adenylyl cyclase, whose activity is directly modulated by opioids and several physiological studies have implicated PLC-linked pathways in a diverse range of opioid-modulated events as *in vivo* pain regulation (Bonacci *et al.* 2006; Galeotti *et al.* 2006; Esmaeili-Mahani *et al.* 2008; Mathews *et al.* 2008) and opioid tolerance (Smith *et al.* 1999). Among the large PLC family, the PLCβ<sub>3</sub> isoform was localized in regions of the brain important for nociceptive transmission, including PAG, and was activated in this region after morphine administration to mice at a hyperalgesic dose (Bianchi *et al.* 2009). Galeotti *et al.* (2006) recently demonstrated that low dose morphine induced hyperalgesic effect is mediated by the activation of PLCβ<sub>3</sub>. Studies *in vitro* showed that G protein mediation, pertussis toxin-insensitive (via the



**Fig. 5** Phospholipase C activity present in anti-Gβ immunoprecipitates from PAG of mice previously administered with morphine (1 μg/kg) in presence or absence of CTOP (80 ng) or M119 (40 ng). Each bar represents PLC activity obtained from three independent experiments under different treatment conditions. Immune complex PLC activity was measured and expressed as pmol Ins(1,4,5)P<sub>3</sub> product formed/min/mg protein. Vertical lines represent SEM;\*\* $\alpha < 0.01$ .



$\alpha$  subunit of Gq) or sensitive (via the G $\beta\gamma$  subunit of Gi/Go) is a prerequisite for receptor activation of PLC $\beta$  isoforms (Wu *et al.* 1992; Smrcka and Sternweis 1993). In order to test the hypothesis that G $\beta\gamma$  associates with PLC $\beta$  subfamily in PAG after low morphine dose administration, anti G $\beta$ -immune-complex were isolated from PAG area and probed for anti-PLC $\beta$  immunoreactivity after saline or low dose morphine administration. Our results show that, at 15 min after morphine administration which corresponds at the time of the maximum hyperalgesic effect, PLC $\beta_3$  was associated with G $\beta$  and appeared catalytically active; this effect was reversed by supraspinal administration of the G $\beta\gamma$  blocker, M119. When  $\mu$ OR was blocked at supraspinal site by CTOP, phospholipase C activity remained unmodified showing that G $\beta\gamma$ -dependent PLC activation is dependent on  $\mu$ OR.

## Conclusion

Collectively, our data support that hyperalgesic doses of morphine exposure induces a G $\beta\gamma$ -dependent stimulation of PLC $\beta_3$  triggered from Gi through its coupling to  $\mu$ OR at supraspinal level. Previous findings demonstrated that the  $\alpha$  subunit of the Gi triggered the analgesic effect at higher morphine doses (Sánchez-Blázquez *et al.* 2001), suggesting a bimodal opioid receptor induced activation of the same protein. A similar pattern was previously proved in smooth muscle where the  $\beta\gamma$  subunit of the Gi protein has been showed to activate PLC signaling whereas the  $\alpha$  subunit inhibits adenylyl cyclase activity (Murthy and Makhoulf 1996). The  $\beta_3$  isoform of PLC appears to be implicated also in analgesic morphine effects. When an acute analgesic morphine dose is administered to PLC $\beta_3$  knockout or down-regulated mice, a potentiation in the analgesic effect was obtained by different authors (Xie *et al.* 1999; Bonacci *et al.* 2006; Galeotti *et al.* 2006). G $\beta\gamma$  blocker M119 co-administration resulted in a dramatic increase in acute thermal analgesic potency of morphine (Mathews *et al.* 2008). This led to the proposal that  $\mu$ OR activation by morphine might trigger separate stimulatory and inhibitory effects linked to different effector systems. Assuming that the overall pharmacological effect of morphine is equal to the sum of these two processes, the high dose morphine inhibitory analgesic system would not be opposed by the excitatory nociceptive component when the PLC $\beta_3$ -dependent stimulatory pathway is switched off. It has been reported that systemic injection of morphine produces a rebound hyperalgesia after the antinociceptive effect was terminated (Ossipov *et al.* 2005). The residual low concentration of morphine that remains after cessation of its administration might elicit the stimulatory withdrawal hyperalgesia which was shown both after single or chronic morphine administration when nociceptive facilitatory systems are not overwhelmed by the opponent antinociceptive inhibitory systems. The blockage of withdrawal hyperalgesia by naloxone confirmed the involvement

of opioid receptors in the phenomena supporting that withdrawal hyperalgesia is a direct effect of a residual, low concentration of morphine. A large dose of intra-operative opioids before the onset of noxious stimuli, that is pre-emptive analgesia, could lead to the development of abnormal pain sensitivity post-operatively (Guignard *et al.* 2000). The patients treated intra-operatively with opioids reported more post-operative pain than the matched non-opioid control subjects (Guignard *et al.* 2000). Prolonged hyperalgesia following short term morphine exposure, when the opiate concentration is expected to be as low as after low dose administration, may explain why the results of clinical studies of pre-emptive analgesia as a means of reducing post-surgical pain have been sometimes disappointing. Increasing the opioid dose to restore the analgesic effect may not always be the answer to morphine decreased efficacy. Knowledge of the molecular mechanism of possible excitatory action of opiates may allow the development of new chemical approaches that can prevent these effects as well as change the way in which these drugs are used clinically. Selectively inhibiting excitatory signaling represents a novel approach to target opioid-induced abnormal pain sensitivity confirming the potential use of M119 in clinical management of pain.

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