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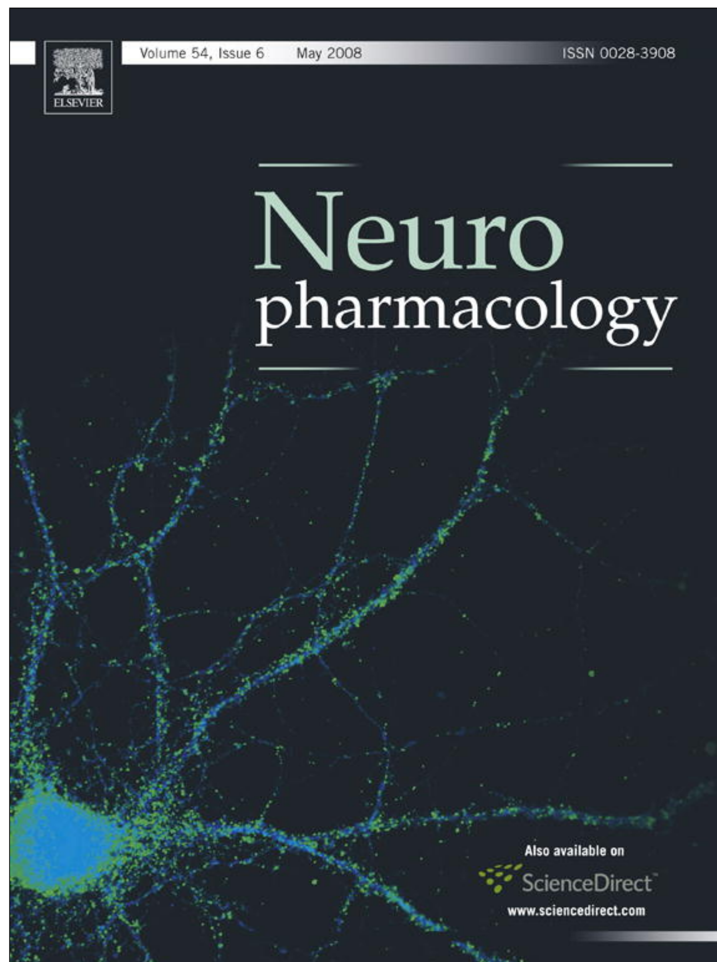

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Supersensitivity to μ-opioid receptor-mediated inhibition of the adenylyl cyclase pathway involves pertussis toxin-resistant Gα protein subunits

Ricardo Mostany [a,1], Álvaro Díaz [a,1], Elsa M. Valdizán [a,1], María Rodríguez-Muñoz [b], Javier Garzón [b], María A. Hurlé [a,c,*]

[a] Departamento de Fisiología y Farmacología, Facultad de Medicina, Universidad de Cantabria, Av. Herrera Oria s/n, E-39011 Santander, Spain
[b] Unidad de Neurofarmacología, Instituto Cajal, CSIC, Madrid, Spain
[c] IFIMAV, Av. Herrera Oria s/n, E-39011 Santander, Spain

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Abstract

Sustained administration of opioids leads to antinociceptive tolerance, while prolonged association of L-type Ca^{2+} channel blockers (e.g. nimodipine) with opioids results in increased antinociceptive response. Herein, we investigated the changes in μ-opioid receptor signalling underlying this shift from analgesic tolerance to supersensitivity. Thus, the interaction of μ-opioid receptors with G proteins and adenylyl cyclase was examined in lumbar spinal cord segments of rats. In control animals, the μ-opioid selective agonists, sufentanil and DAMGO, stimulated $[^{35}S]$5′-(gamma-thio)-triphosphate ($[^{35}S]$GTPγS) binding and inhibited forskolin-stimulated adenylyl cyclase activity, through a mechanism involving pertussis toxin (PTX) sensitive $G\alpha_{i/o}$ subunits. Seven days of chronic sufentanil treatment developed antinociceptive tolerance associated with a reduction in μ-agonist-induced $[^{35}S]$GTPγS binding, μ-agonist-induced adenylyl cyclase inhibition, and co-precipitation of $G\alpha_o$, $G\alpha_{i2}$ $G\alpha_z$ and $G\alpha_{q11}$ subunits with μ-opioid receptors. In contrast, combined nimodipine treatment with sufentanil over the same period increased the sufentanil analgesic response. This antinociceptive supersensitivity was accompanied by a significant increase of μ-agonist-induced inhibition of adenylyl cyclase that was resistant to the antagonism by PTX. In good agreement, co-precipitation of the PTX-resistant, $G\alpha_z$ and $G\alpha_{q/11}$ subunits with μ-opioid receptors was not lowered. On the other hand, the PTX-sensitive subunits, $G\alpha_{i2}$ and $G\alpha_o$, as well as agonist-stimulated $[^{35}S]$GTPγS binding were still reduced. Our results demonstrate that μ-opioid analgesic tolerance follows uncoupling of spinal μ-opioid receptors from their G proteins and linked effector pathways. Conversely, the enhanced analgesic response following combined nimodipine treatment with sufentanil is associated with adenylyl cyclase supersensitivity to the opioid inhibitory effect through a mechanism involving PTX-resistant G protein subunits.
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Keywords: Nociception; Analgesia; Opioid tolerance; Opioid supersensitivity; G-protein; Adenylyl cyclase; μ-Opioids; Calcium antagonist

1. Introduction

Opioid drugs are the most efficacious drugs available in clinical practice to relieve pain. It is accepted that there are three major types of opioid receptors: μ, δ and κ (Snyder and Pasternak, 2003). The antinociceptive effect of morphine and other opioids is induced primarily through μ-opioid receptor activation in spinal and supraspinal regions of the CNS (Matthes et al., 1996). Opioid receptor signalling is transmitted to intracellular effectors through G transducer proteins from different subfamilies. Several lines of evidence indicate that μ-opioid receptors predominantly couple to G_i and G_o

Abbreviations: AC, adenylyl cyclase; DAMGO, D-Ala(2)-MePhe(4)-Gly-ol(5) enkephalin; DTT, dithiothreitol; FK, forskolin; $[^{35}S]$GTPγS, $[^{35}S]$guanylyl-5′-*O*-(gamma-thio)-triphosphate; IBMX, 3-isobutyl-1-methyl-xanthine; OD, optical density; PTX, pertussis toxin; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; TBS, Tris-buffered saline.

* Corresponding author. Departamento de Fisiología y Farmacología, Facultad de Medicina, Universidad de Cantabria, Av. Herrera Oria s/n, E-39011 Santander, Spain. Tel.: +34 942 201961; fax: +34 942 201903.
E-mail address: hurlem@unican.es (M.A. Hurlé).

[1] These authors contributed equally to this paper.

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classes (Garzón et al., 2000; Sánchez-Blázquez et al., 1995; Standifer et al., 1996; Yoburn et al., 2003). The downstream effectors regulated include the cyclic AMP (cAMP) pathway, the phosphoinositides metabolism and phospholipase C pathway, and the mitogen-activated protein kinases pathway, as well as voltage-gated calcium channels and inwardly rectifying potassium channels (Law et al., 2000). The preferential pattern of G-protein isoforms activated depends upon the particular agonist that activates the receptor, and on specific cellular or tissular factors, conferring particular pharmacological profiles to different agonists (Gaibelet et al., 1999; Garzón et al., 1998).

It is well known that potency and efficacy of opioid agonists can be modified by a number of pharmacological procedures. In animal models and in humans, repeated administration of opioids leads to tolerance development, whereby increasing concentrations of these drugs are necessary to achieve a given effect. In clinical practice, tolerance reduces the analgesic efficacy of opioids and contributes to the need of dose escalation in chronic painful conditions (Ballantyne and Mao, 2003). Conversely, prolonged association of L-type Ca^{2+} channel blockers (e.g. nimodipine) with opioids results in antinociceptive supersensitivity instead of tolerance in rats (Dierssen et al., 1990). In humans, it has been reported that cancer patients who developed morphine dose escalation significantly reduced their morphine requirements to relieve pain following the association of nimodipine to the treatment (Santillán et al., 1998).

Among the adaptive mechanisms produced by chronic treatment with μ-opioid agonists, uncoupling of receptors from G-proteins (Garzón and Sánchez-Blázquez, 2001), receptor down-regulation (Díaz et al., 2000), and a counteradaptive up-regulation of the cAMP-signalling pathway (Liu and Anand, 2001; Nestler, 2004) have been proposed to play an essential role in μ-opioid receptor desensitization and tolerance. Indeed, it is deemed of interest to unravel the mechanisms underlying opioid supersensitivity, since it could be useful in developing strategies to prevent tolerance and to improve the therapeutic benefits of opioid drugs. Therefore, this study was aimed at analysing the neurochemical adaptations associated with opioid supersensitivity versus tolerance in the spinal cord of rats, a paradigmatic structure where μ-opioid receptors play a key role in processing nociceptive information (Dickenson and Kieffer, 2006). As previously described (Dierssen et al., 1990), tolerance was induced by chronic treatment with the μ-selective opioid agonist, sufentanil, whereas supersensitivity was induced by the simultaneous administration of the calcium antagonist, nimodipine, and sufentanil. We have assessed the adaptive changes in the coupling of μ-opioid receptor to Gα-transducer proteins and the subsequent modifications in the adenylyl cyclase pathway under both experimental conditions.

2. Materials and methods

2.1. Animals

Male albino Wistar rats (Harlan, Barcelona, Spain), weighing 250–300 g, were used. They were housed in a room kept at 22 °C with a 12:12 h light/dark cycle. Food and water were provided ad libitum. This study was approved by the Cantabria University Institutional Laboratory Animal Care and Use Committee and all experiments adhered to the Declaration of Helsinki and the European Communities Council Directive (86/609/EEC).

2.2. Drugs and chemicals

The μ-opioid agonist, sufentanil, and the L-type Ca^{2+} channel blocker, nimodipine, were kindly provided by Janssen Cilag S.A. (Madrid, Spain) and Química Farmacéutica Bayer S.A. (Barcelona, Spain), respectively. DAMGO, naloxone, forskolin (FK), 3-isobutyl-1-methyl-xanthine (IBMX), EGTA, EDTA, GDP, ATP, GTPγS, dithiothreitol (DTT), NAD, thymidine, sodium dodecyl sulphate (SDS), Tween 20, adenosine deaminase, phosphocreatine, creatine phosphokinase, myokinase, biotin and streptavidin agarose were purchased from Sigma (Madrid, Spain). [^{35}S]GTPγS (1250 Ci/mmol) was purchased from Perkin Elmer (Madrid, Spain). Pertussis toxin (PTX) was purchased from Calbiochem (Roche Diagnostics, Barcelona, Spain), Nonidet P-40 was purchased from Roche Applied Science (Roche Diagnostics). Leupeptin was obtained from Amersham Biosciences (Barcelona, Spain). The following antibodies were used: affinity purified IgG against μ-opioid receptors (Garzón et al., 1995), antiserum to $G\alpha_{q/11}$, $G\alpha_o$ (Du Pont–New England Nuclear Research Products, Boston, MA, USA), $G\alpha_z$ and $G\alpha_{i2}$ subunits (Sánchez-Blázquez et al., 2003). The chronic delivery of saline or drug-containing solution was carried out using Alzet 2001 osmotic minipumps (Alza Corp., Palo Alto, CA, USA), implanted subcutaneously under light ether anaesthesia. These pumps deliver solutions at a constant rate of 1 μl/h, for a period of 7 days.

2.3. Experimental groups

Four different experimental groups were designed for the present study (Dierssen et al., 1990). Group I: the control group received a chronic infusion of saline (1 μl/h) for 7 days. Group II: the tolerant group was treated with the μ-opioid agonist sufentanil (2 μg/h) for 7 days. Group III: the supersensitive group received chronic and simultaneous administration of sufentanil (2 μg/h) and the Ca^{2+} channel blocker, nimodipine (1 μg/h), for 7 days. Group IV: the nimodipine group received nimodipine alone (1 μg/h) for 7 days. Animals used for the neurochemical studies were euthanized under pentobarbital anaesthesia and their spinal cords were rapidly removed, dissected at the lumbar level, frozen on dry ice and stored at −80 °C until use.

2.4. Evaluation of nociception

The nociceptive threshold was assessed by using the tail-flick test in eight animals per group. The tail-flick response was elicited by applying a radiant heat to the dorsal surface of the tail (analgesimeter LI-7100, Leica, Barcelona, Spain). The intensity of the stimulus was adjusted so that, the control latency was within 3–5 s. A cut-off time of 10 s was set to prevent blistering. The tail-flick latency was measured before and 15 min after the injection of a challenging dose of sufentanil (0.5 μg/kg, s.c.). Analgesic end-point was defined as an increase of 100% in the individual reaction time in relation to the pre-drug control latency. The antinociceptive effect was expressed as the percentage of animals that reached the analgesic end-point. Statistical analysis was carried out by the Mann–Whitney U-test.

2.5. Cyclic AMP assays

Adenylyl cyclase assays were performed in lumbar spinal cord samples from six animals per group following the method described previously (Mato et al., 2002). Samples were homogenized in ice-cold homogenization buffer (20 mM Tris–HCl, 1 mM EGTA, 5 mM EDTA, 5 mM DTT, 25 μM leupeptin and 300 mM sucrose, pH 7.4). The homogenates were centrifuged at 500 × g for 5 min at 4 °C and, subsequently, the supernatants were centrifuged at 13,000 × g for 15 min at 4 °C. The pellets were resuspended to a final concentration of 120 μg protein/ml of assay buffer (80 mM Tris–HCl, 0.2 mM EGTA, 1 mM EDTA, 2 mM $MgCl_2$, 100 mM NaCl, 60 mM sucrose, 1 mM

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DTT, 10 μM GTP, 0.5 mM IBMX, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase, 5 U/ml myokinase, pH 7.4) without (basal adenylyl cyclase activity) or with 5 μM forskolin (FK-stimulated cAMP accumulation). Opioid receptor mediated inhibition of FK-stimulated cAMP accumulation was determined at different concentrations of the agonists sufentanil and DAMGO (10^{-9} to 10^{-4} M). The specificity of the response was confirmed in separated experiments (data not shown) by incubation with the opioid agonists (10 μM) in the presence of the antagonist naloxone (10 μM). After a pre-incubation period of 5 min at 37 °C, ATP was added to a final concentration of 200 μM, then, the mixture was incubated for another 10 min at 37 °C. The reaction was stopped by boiling for 5 min, and cAMP concentration was determined in 50 μl supernatants by using a commercial kit (Cyclic AMP [^{3}H] assay system, Amersham Biosciences, Barcelona, Spain).

In parallel experiments, cAMP assays were performed using homogenates pre-treated with PTX. For this purpose, PTX was pre-activated for 30 min at 30 °C in 50 mM Tris–HCl, pH 7.5 20 mM dithiothreitol and 0.1% of BSA. Spinal cord samples from each experimental group were homogenized in 25 mM Tris–HCl buffer containing 0.05% SDS, 10 mM DTT, 1 mM EDTA, 2.5 μM NAD, and 10 mM thymidine, plus 1 μg/ml of the pre-activated PTX. The homogenates were incubated with the activated toxin for 30 min at 30 °C. After that, the samples were extensively washed, centrifuged and processed as above. Basal adenylyl cyclase activity was expressed as pmol cAMP/min/mg protein, and FK-stimulated cAMP accumulation was expressed as percentage of increase over the basal value. The effect of opioids was expressed as the percentage of inhibition of FK-stimulated activity (considered as 100% of the effect). In each assay, triplicate samples from each experimental group were run in parallel.

2.6. [^{35}S]GTPγS autoradiography assays

The experiments of [^{35}S]GTPγS binding were performed in transverse lumbar spinal cord sections from five animals per group, following the method previously described (Maher et al., 2001). Sections (20 μm) were cut on a cryostat at −20 °C, mounted on gelatin-subbed slides and stored at −80 °C. On the day of the experiment, sections were thawed for 30 min, rinsed in assay buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) for 15 min at 25 °C and pre-incubated for 15 min in the assay buffer containing 2 mM GDP and 10 mU/ml adenosine deaminase. Sections were then incubated for 2 h at 25 °C in the assay buffer containing 1 mM DTT and 0.04 nM [^{35}S]GTPγS, without (basal binding) or with an opioid agonist (stimulated binding). Non-specific binding was determined in the presence of 10 μM GTPγS. The μ-opioid agonists, sufentanil and DAMGO, were tested at concentrations ranging from 10^{-10} to 10^{-4} M. The specificity of the response was confirmed in consecutive sections incubated with the opioid agonist (10 μM) in the presence of the antagonist naloxone (10 μM). After incubation, slides were rinsed twice in cold Tris buffer (50 mM Tris–HCl, pH 7.4) for 5 min, dipped in deionized water and, finally, dried under an ice-cold air stream. Tissue sections and [^{14}C] radioactive microscales (Amersham Biosciences) were exposed to Kodak-MR films (Amersham Biosciences) for 48 h.

Autoradiographic densities were determined by densitometry using the Scion Image software (Scion Corp., Frederick, MD, USA). Polynomial calibration curves were fitted to relative optical density values measured from ^{14}C-labelled microscales. Relative optical density (OD) values of dorsal horns were averaged over three consecutive spinal cord sections per rat (bilateral readings) and converted to nCi/g of tissue. Net agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal binding from agonist-stimulated binding. Data are expressed as percentage of agonist-stimulated binding over basal activity (100%).

2.7. Co-immunoprecipitation studies

The opioid receptors and the Gα proteins were co-immunoprecipitated from the solubilized synaptosomal P2 fraction obtained from lumbar spinal cord lysates from four animals per group, as described previously (Sánchez-Blázquez et al., 2003). The solubilized fraction was incubated at 4 °C, overnight, with 3 μg of the antibody raised against the second extracellular loop of μ-opioid receptors (Garzón et al., 1995). Fifty microlitres of streptavidin agarose were added and incubated for 90 min at 4 °C. The samples were centrifuged at 3000 × g for 5 min and the supernatant removed. The agarose pellets underwent five cycles of washing, followed by centrifugation and resuspension in 1 ml Nonidet P-40 buffer. At the end of this process, the proteins contained in the immune complexes were separated and denatured by heating the agarose pellets in 300 μl 40 mM Tris–HCl, 1% SDS buffer, for 10 min, at 100 °C. The mixture was cooled to room temperature and, then streptavidin agarose was separated in centrifugal filter devices with a 0.45-μm pore (Ultrafree-MC #UFC30GV, Millipore Iberica S.A.). To prevent interference in visualizing the Western blots, the biotinylated IgGs detached during the initial heating were selectively removed by adding octylthioglucoside to a final percentage of 0.65% in 400 μl, plus 30 μl of fresh streptavidin agarose. After 2 h at 4 °C, the samples were centrifuged for 5 min at 10,000 × g and the streptavidin agarose with the attached biotinylated IgGs was discarded. Proteins from the soluble fraction were concentrated in centrifugal filter devices (10,000 nominal molecular weight limit, Amicon Microcon YM-10 #42407, Millipore) and then solubilized in Laemmli 2× buffer with mercaptoethanol.

Identical amounts of protein from each sample were loaded on 10–16% sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE), electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Labs, Hercules, CA, USA). Non-specific binding of antibodies was prevented by incubating membranes in 10% dried milk in TBS-T buffer (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween). The blots were incubated with antisera against μ-opioid receptors or antisera anti-$G\alpha_o$, $G\alpha_z$ $G\alpha_{q/11}$ or $G\alpha_{i2}$ subunits in TBS-T with 2% dried milk, overnight, at 4 °C. After extensive washing with TBS-T, blots were incubated for 1 h, at room temperature, with a peroxidase-labelled secondary antibody at 1:10,000 dilution. Immunoblots were visualized with a ChemiImager IS-5500 (Alpha Innotech, San Leandro, CA, USA) and analyzed by densitometry (AlphaEase v3.2.2). Control experiments were performed to establish that the changes of the intensity of the bands were linear with respect to the amount of sample loaded. Gels were loaded with samples from each experimental group. All immunoprecipitation samples were assayed for each Gα subunit in three independent experiments. Relative variations between the bands of problem and control samples were calculated in the same image. Data are expressed as mean of four animals per group ± S.E.M.

2.8. Data analysis

The data analyses were performed using the GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) statistical software package. The antinociceptive effect was expressed as the percentage of animals that reached the analgesic end-point. The Mann–Whitney U-test was used to compare the antinociceptive effect of each given acute sufentanil probe dose with the equivalent dose of the control group. Data from [^{35}S]GTPγS binding and adenylyl cyclase assays were fitted to sigmoidal concentration–response curves to determine potency (EC_{50} and IC_{50}, respectively) and efficacy (E_{max} and I_{max}, respectively). The EC_{50} and IC_{50} values were normalized as $-\log EC_{50}$ (pEC_{50}) and $-\log IC_{50}$ (pIC_{50}) for statistical comparison. All values are reported as mean ± standard error of the mean (S.E.M.). Statistical analysis was made by Student t-test and one-way ANOVA followed by Student–Newman–Keuls test, where appropriate. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Chronic treatment with sufentanil produces tolerance whereas its association with nimodipine produces supersensitivity to the opioid antinociceptive effect

The antinociceptive response induced by a challenging dose of sufentanil (0.5 μg/kg) was tested in eight rats per group. As shown in Fig. 1, in the control group sufentanil produced an analgesic response in 50% of the animals, whereas it produced an antinociceptive response only in 12.5% of the rats chronically

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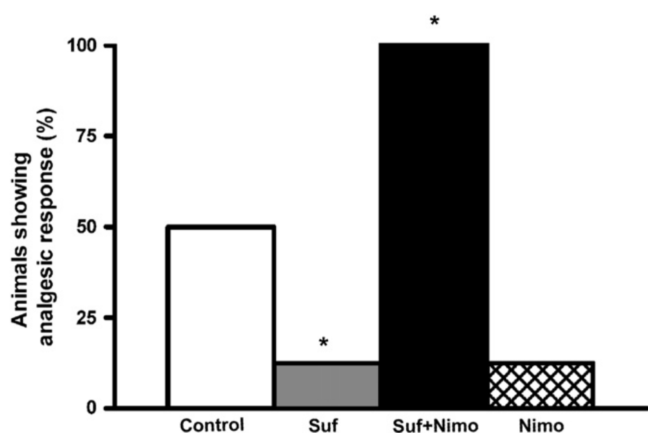

Fig. 1. Antinociceptive effect of sufentanil assessed in the tail-flick test. Animals received a challenging dose of sufentanil (0.5 μg/kg, s.c.) on the 7th day of chronic administration of 1 μl/h saline (control), 2 μg/h sufentanil (suf), 2 μg/h sufentanil plus 1 μg/h nimodipine (suf + nimo) or 1 μg/h nimodipine (nimo). The antinociceptive effect was expressed as the percentage of animals reaching the analgesic end-point, defined as an increase of 100% in the individual reaction time in relation to the pre-drug control latency. The tail-flick latency was measured before and 15 min after sufentanil injection. $^*P < 0.05$ vs. the control group (Mann–Whitney U-test).

treated with sufentanil (2 μg/h for 7 days) ($P < 0.05$ vs. the control group; Mann–Whitney U-test), indicating tolerance development. On the other hand, after 7 days of chronic and simultaneous treatment with nimodipine (1 μg/h) and sufentanil (2 μg/h), the challenging dose of the opioid reached the antinociceptive threshold in 100% of animals ($P < 0.05$ vs. the control group; Mann–Whitney U-test). Chronic nimodipine (1 μg/h) administered alone for 7 days failed to modify the antinociceptive response induced by the probe dose of sufentanil.

3.2. The combined sufentanil–nimodipine treatment increased μ-opioid-mediated inhibition of adenylyl cyclase and switched it to a predominantly PTX-resistant response

In control rats, incubation of spinal cord membranes with increasing concentrations (10^{-9}–10^{-4} M) of sufentanil or DAMGO produced a concentration-dependent inhibition of FK-stimulated cAMP accumulation (Fig. 2).The maximal inhibitions induced by sufentanil and DAMGO were similar (I_{max} values = −21.6 ± 2.9% and −24.9 ± 1.8%, respectively) as well as their potencies (pIC_{50} values = 6.5 ± 0.1 and 6.3 ± 0.3, respectively).

Basal level of adenylyl cyclase activity was significantly increased in sufentanil (35.5 ± 5.2 pmol/min/mg protein) and sufentanil plus nimodipine-treated animals (42.1 ± 5.0 pmol/min/mg protein) in comparison with the control group (18.6 ± 2.8 pmol/min/mg protein). In rats chronically treated with sufentanil, the inhibitory response of FK-induced cAMP accumulation to both opiates was almost abolished (sufentanil group I_{max} = −8.3 ± 1.6%; DAMGO group I_{max} = −12.0 ± 1.1%). However, in the group of animals chronically treated with sufentanil plus nimodipine, the efficacies of sufentanil (I_{max} = −45.6 ± 7.1%) and DAMGO

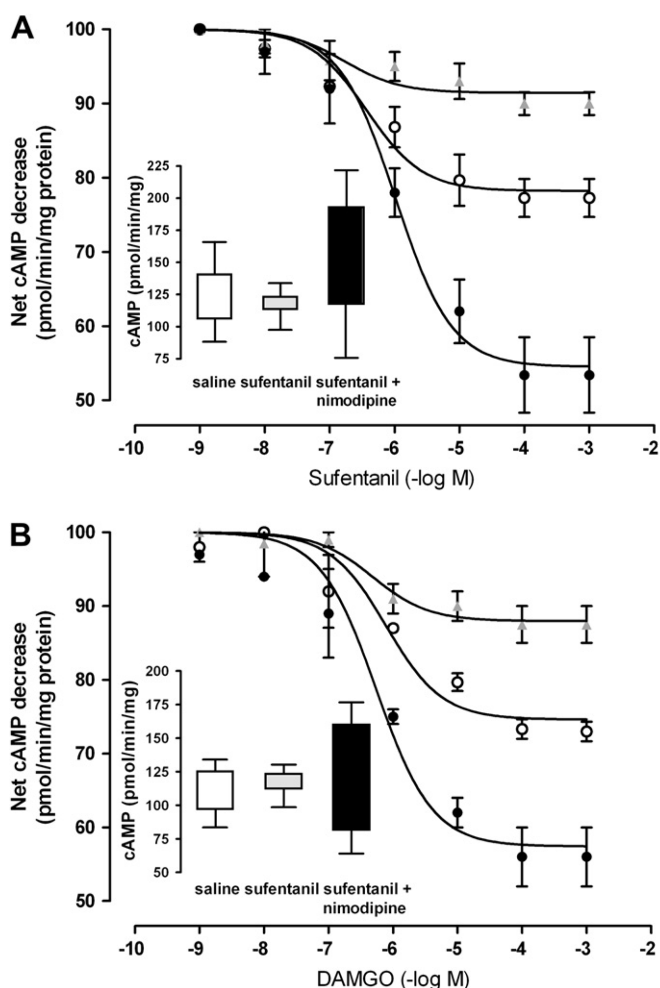

Fig. 2. Concentration–response curves of sufentanil (A) and DAMGO (B) inhibition on FK-induced cAMP accumulation in spinal cord homogenates from animals chronically treated for 7 days with saline (○), sufentanil (2 μg/h) (▲) and sufentanil (2 μg/h) plus nimodipine (1 μg/h) (●). Values represent the percentage (mean ± S.E.M.) of cAMP accumulation vs. FK-stimulated cAMP accumulation (100%). Inset: Bars represent cAMP accumulation, in pmol/min/mg. Top values express the mean ± S.E.M accumulation of cAMP in the presence of forskolin. Bottom values are the mean ± S.E.M of I_{max} (maximal inhibition) achieved by each agonist on the FK-induced cAMP accumulation. The bar length represents the net cAMP inhibition induced by the opioid agonists in the saline (open bars), sufentanil (grey bars) and sufentanil plus nimodipine (black bars) groups.

(I_{max} = −42.6 ± 6.5%) to inhibit FK-induced cAMP accumulation were significantly augmented when compared with both control and tolerant groups. In animals chronically treated with nimodipine alone, the maximal inhibitory effects of sufentanil (I_{max} = −27.3 ± 3.7%) and DAMGO (I_{max} = −28.9 ± 2.1%) were similar to the control group values.

Pertussis toxin causes the ADP-ribosylation and, thereby, inactivation of $G\alpha_{i/o}$ proteins with the exception of $G\alpha_z$ (Casey et al., 1990). The presence of PTX in the medium did not modify either basal or FK-stimulated cAMP levels in any experimental group (data not shown). As shown in Fig. 3, the maximal inhibitory effect of 10^{-4} M DAMGO (−23.1 ± 0.2%) was almost completely prevented by PTX

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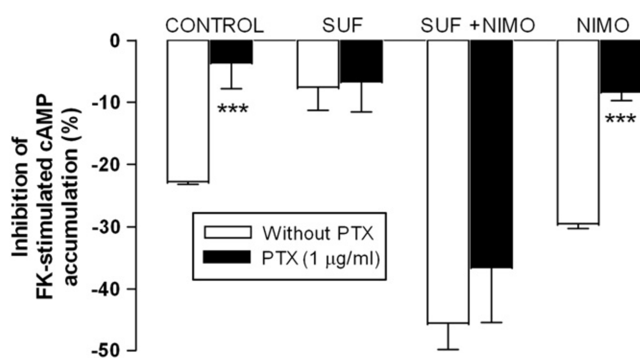

Fig. 3. Effect of pertussis toxin (PTX) on the inhibition of DAMGO (10 μM) on FK-induced cAMP accumulation in spinal cord homogenates from animals chronically treated for 7 days with saline, sufentanil (2 μg/h), sufentanil (2 μg/h) plus nimodipine (1 μg/h) and nimodipine alone (1 μg/h). In each assay, spinal cord samples from all the experimental groups were incubated, in parallel, with an aliquot from the same pool of pre-activated PTX. Data are mean ± S.E.M of six animals per group. Statistical analysis was carried out by the Student's t-test; *** $P < 0.001$.

pre-treatment both, in control animals ($-3.9 \pm 4.0\%$) and in rats chronically treated with nimodipine alone ($-8.5 \pm 2.6\%$). However, in the group of animals receiving sufentanil plus nimodipine, PTX only partially antagonized the inhibitory effect of 10^{-4} M DAMGO on FK-induced cAMP accumulation (suf + nimo group without PTX = $-45.8 \pm 4.5\%$ vs. suf + nimo group with PTX = $-36.8 \pm 8.7\%$). In the group of animals chronically treated with sufentanil, the presence of PTX in the media did nor modify the effect of DAMGO (chronic sufentanil group without PTX: $-7.8 \pm 3.5\%$; suf with PTX: $-6.8 \pm 4.7\%$).

3.3. Seven days of chronic sufentanil treatment reduced co-precipitation of all Gα subunits with μ-opioid receptors, whereas co-precipitation of Gα$_z$ subunits was not reduced following the combined treatment with sufentanil and nimodipine

Co-precipitation of Gα subunits with the immunoprecipitated μ-opioid receptors was analyzed in the synaptosomal P2 fraction obtained from lumbar spinal cord lysates. The precipitated μ-opioid receptor was detected by Western blot in bands of 55–65, 70–80 and 100–110 kDa (Fig. 4E). The diversity of sizes was probably due to the presence of glycosylated isoforms of the receptor (Garzón et al., 1995). No differences in the pattern of μ-opioid receptor immunoprecipitation were observed under the different pharmacological treatments. As shown in Fig. 4, $G\alpha_o$, $G\alpha_{i2}$, $G\alpha_z$ and $G\alpha_{q11}$ subunits were present in the immunoprecipitated material. Chronic administration of sufentanil substantially decreased the association of all Gα subunits (from 40% to 60% vs. the control group) with μ-opioid receptors. Notably, co-administration of nimodipine concurrently with sufentanil prevented the reduction of PTX-insensitive $G\alpha_z$ (from $-53.3 \pm 3.1\%$ to $-2.1 \pm 1.0\%$) and $G\alpha_{q/11}$

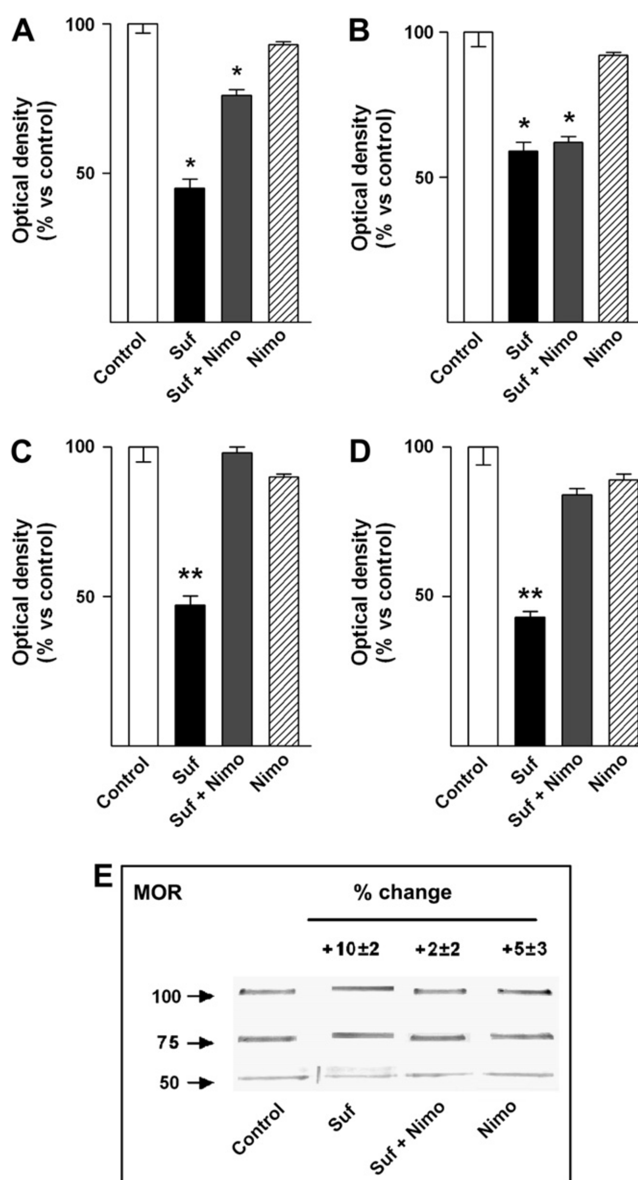

Fig. 4. Co-immunoprecipitation of $G\alpha_o$ (A), $G\alpha_{i2}$ (B), $G\alpha_z$ (C), and $G\alpha_{q/11}$ (D) subunits with μ-opioid receptors. Equal loading of μ-opioid receptor protein was confirmed in parallel blots with the same samples (E). The antisera against the N-terminus of μ-opioid receptors recognize three major bands at 55–65, 70–80 and 100–110 kDa. Rat spinal cord synaptosomal preparations were incubated with affinity-purified biotinylated IgGs anti-second external loop of μ-opioid receptors overnight at 4 °C. Immunocomplexes were precipitated with streptavidin agarose, resolved by SDS–PAGE and revealed in Western blots probed with antibodies anti-$G\alpha_o$, $G\alpha_{i2}$, $G\alpha_z$ and $G\alpha_{q/11}$. Data are mean ± S.E.M of four animals per group. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA followed by Bonferroni's test).

(from $-57.2 \pm 2.0\%$ to $-15.5 \pm 1.5\%$) subunits. On the other hand, $G\alpha_{i2}$ co-precipitation remained decreased (from $-41.2 \pm 3.2\%$ to $-38.5 \pm 2.1\%$) and $G\alpha_o$ subunits only partially recovered (from $-55.4 \pm 3.2\%$ to $-24.8 \pm 1.9\%$). Chronic treatment with nimodipine alone produced no changes in the immunosignals of all the Gα subunits tested.

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3.4. Seven days of chronic sufentanil treatment caused a reduction of μ-agonist-induced [^{35}S]GTPγS binding, and the combined treatment with sufentanil-and nimodipine still decreased μ-agonist-induced [^{35}S]GTPγS binding

Basal and agonist-induced μ-opioid receptor activation of G-proteins was determined in spinal cord sections by [^{35}S]GTPγS autoradiography (Figs. 5 and 6). No differences between groups were detected in the basal levels of [^{35}S]GTPγS binding. In the control group, the selective μ-opioid agonists sufentanil and DAMGO significantly stimulated [^{35}S]GTPγS binding in dorsal horn laminae I and II. Sufentanil produced a concentration-dependent stimulation of [^{35}S]GTPγS binding ($pEC_{50} = 7.9 \pm 0.2$), achieving a maximal effect of $+60.6 \pm 3.4\%$ over basal (100%). DAMGO also stimulated [^{35}S]GTPγS binding in a concentration-dependent manner, exhibiting lower potency ($pEC_{50} = 5.9 \pm 0.1$) but higher efficacy ($E_{max} = +119.7 \pm 11.4\%$ over basal) than sufentanil. The specificity of the effects was confirmed by addition of the opiate antagonist naloxone (10 μM) to the assay buffer (data not shown). In animals chronically treated with sufentanil, the efficacy of both agonists was significantly reduced (sufentanil $E_{max} = +16.7 \pm 1.1\%$ over basal; DAMGO $E_{max} = +74.9 \pm 5.2\%$ over basal; $P < 0.01$ vs. control group for both agonists). Following chronic administration of sufentanil plus nimodipine, the ability of both agonists to stimulate [^{35}S]GTPγS binding was still decreased. In the case of sufentanil a null response was obtained, even at the highest concentration tested (sufentanil $E_{max} = +2.4 \pm 9.8\%$ over basal; DAMGO $E_{max} = +69.6 \pm 5.9\%$ over basal; $P < 0.01$ vs. control group for both agonists). Chronic treatment with nimodipine alone did not significantly modify the efficacy of both agonists to stimulate [^{35}S]GTPγS binding (sufentanil $E_{max} = +46.1 \pm 12.4\%$ over basal; DAMGO $E_{max} = +114.3 \pm 11.8\%$ over basal).

4. Discussion

Repeated administration of opioid drugs leads to tolerance development (Ballantyne and Mao, 2003), whereas association of the Ca^{2+} channel blocker, nimodipine, with opioids results in increased antinociceptive response, instead of tolerance (Dierssen et al., 1990; Santillán et al., 1998). In this study, we have analysed the spinal cord changes in μ-opioid receptor signalling involved in this switch from antinociceptive tolerance to supersensitivity.

Opioid-mediated analgesia involves $G\alpha_o$, $G\alpha_{i1-3}$, $G\alpha_z$, and $G\alpha_{q/11}$ (Sánchez-Blázquez et al., 1995; Standifer et al., 1996; Yoburn et al., 2003), but the coupling profile of μ-opioid receptors to the different Gα protein subunits displays regional differences (Chalecka-Franaszek et al., 2000; Wang et al., 2005). In the present study, $G\alpha_o$, $G\alpha_{i2}$, $G\alpha_z$ and $G\alpha_{q11}$ subunits were detected in association with the immunoprecipitated receptor in spinal cord membranes from control rats, indicating their potential involvement in signal transduction. Further evidence for a functional coupling between μ-opioid receptors and $G\alpha_i$/$G\alpha_o$ subunits was derived from the analysis of the guanine nucleotide exchange in [^{35}S]GTPγS binding assays (Milligan, 2003). Here we observed that, following μ-opioid

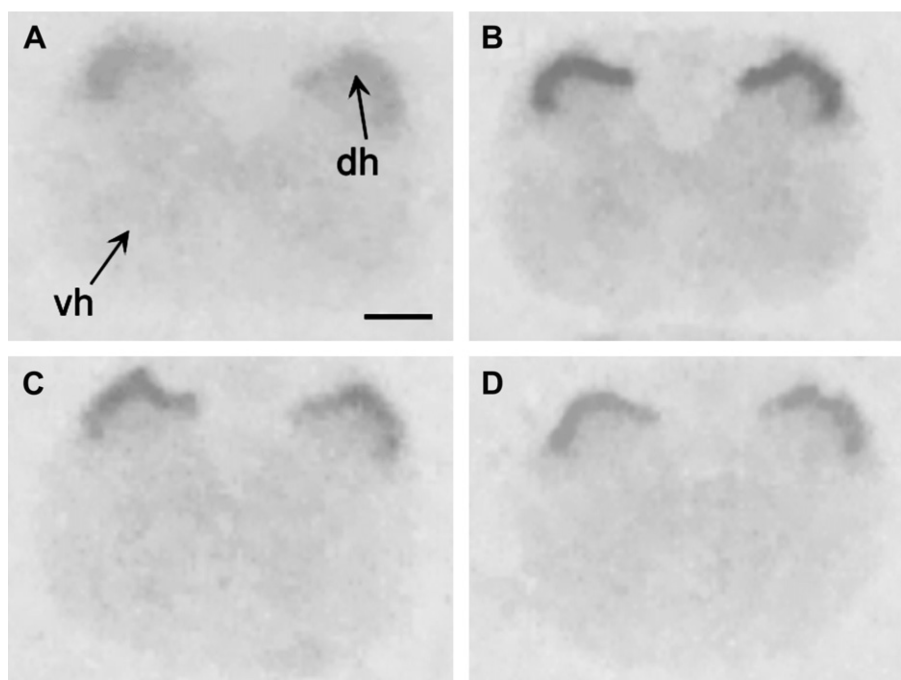

Fig. 5. Representative autoradiographic illustration showing basal (A) and DAMGO (10 μM) stimulated [^{35}S]GTPγS binding in spinal cord sections from animals chronically treated for 7 days with saline (B), sufentanil (2 μg/h) (C) and sufentanil (2 μg/h) plus nimodipine (1 μg/h) (D). dh, dorsal horn; vh, ventral horn. Bar = 0.5 mm.

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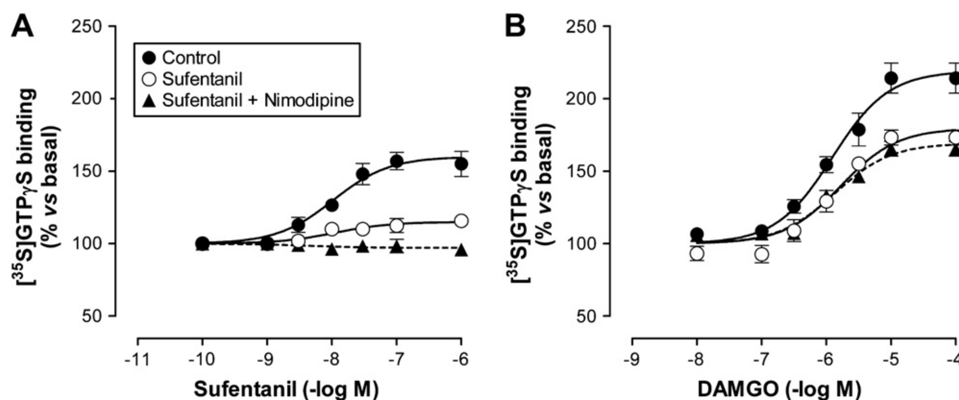

Fig. 6. Concentration–response curves of sufentanil-stimulated (A) and DAMGO-stimulated (B) [^{35}S]GTPγS binding in spinal cord sections from animals chronically treated for 7 days with saline (●), sufentanil (2 μg/h) (○), sufentanil (2 μg/h) and plus nimodipine (1 μg/h) (▲). Values are expressed as mean ± S.E.M. of percentage of stimulated [^{35}S]GTPγS binding with respect to basal value (100%) from five animals per group.

receptor occupation by DAMGO and sufentanil, $G\alpha_i$/$G\alpha_o$ subunits activation was localized in laminae I and II of the dorsal horn, as previously described (Maher et al., 2001).

The AC/cAMP pathway is probably the best characterized effector system linked to the μ-opioid receptor signalling cascade (Law et al., 2000). Following opioid receptor activation, the inhibitory signal to the AC is mainly transduced by $G\alpha_i$ subunits, resulting in a reduced cAMP production (George et al., 2000). Our results indicate that, in control animals, sufentanil and DAMGO produced a concentration-dependent inhibition of FK-stimulated AC activity in spinal cord homogenates. The antagonism by PTX revealed that, under basal conditions, the preferential pathway for the opioid inhibitory effect on AC activity is through PTX-sensitive $G_{i/o}$ proteins.

Rats receiving chronic sufentanil developed antinociceptive tolerance in the course of the treatment, as previously described (Dierssen et al., 1990; Hurlé et al., 2000). It is generally accepted that opioid tolerance courses with receptor phosphorylation and desensitization (Marie et al., 2006) without changing the total amount of receptor protein present in the membrane (Law et al., 2000), although the active fraction of μ-opioid receptors, as labelled by agonists, may undergo down-regulation (Díaz et al., 2000). In good agreement, in our study the amount of synaptosomal μ-opioid receptors immunodetected remained stable. The existence of receptor desensitization following chronic sufentanil treatment was manifested as a significant reduction in the maximal stimulatory effect of both sufentanil and DAMGO on [^{35}S]GTPγS binding. Similar data were reported for animals chronically treated with morphine or other opioid drugs (Maher et al., 2001; Marie et al, 2006; Sim et al., 1996). In our study, the functional uncoupling between μ-opioid receptors and G proteins was reflected also as an overall reduction of immunoprecipitated Gα subunits accompanying similar amounts of receptor protein.

Regarding the cAMP signalling pathway, in tolerant animals we observed two adaptive mechanisms: first, basal cAMP levels were increased, and second, the inhibitory effect of sufentanil and DAMGO on FK-induced cAMP accumulation was almost abolished. Up-regulation of cAMP and other components of the cAMP pathway has been extensively observed in cultured cells and in several brain regions following sustained exposure to opioid drugs (Liu and Anand, 2001; Sharma et al., 1977; Watts and Neve, 2005). This compensatory increase in AC activity has been classically associated to tolerance and dependence development (Liu and Anand, 2001; Nestler, 2004). Thus, our present data indicate that antinociceptive tolerance following chronic sufentanil administration courses with cellular adaptations at the spinal cord level which include: uncoupling of μ-opioid receptors to Gα inhibitory subunits, reduction of the inhibitory effect of opioids on AC activity and up-regulation of the AC/cAMP pathway. Although not analysed in this study, the reduced co-precipitation of $G\alpha_o$ and $G\alpha_{q/11}$ with the receptor allows the suggestion of the existence of alterations affecting also the signalling pathways linked to these subunits. Indeed, voltage sensitive Ca^{2+} channels, protein kinase C or extracellular signal-regulated kinase are fundamental determinants of opioid effects (Law et al., 2000).

Combined administration of nimodipine with sufentanil for 7 days led to a switch from antinociceptive tolerance to enhanced analgesic response, as previously reported (Dierssen et al., 1990; Hurlé et al., 2000). Interestingly, in this group of animals, the inhibitory effect on FK-induced cAMP accumulation induced by both sufentanil and DAMGO was also augmented. The ability of DAMGO to inhibit AC after PTX treatment indicated the involvement of a PTX-insensitive G protein. Although the best characterized inhibitory influences on AC are mediated by $G\alpha_i$ and $G\alpha_z$ proteins (Ho and Wong, 2001), some reports suggest that following opioid-receptor activation, $G_{\beta\gamma}$ subunits can also inhibit several AC isoforms (Steiner et al., 2005, 2006). Thus, our data indicate that the PTX-insensitive proteins, possibly $G\alpha_z$ and/or $G\alpha_{q/11}$-associated βγ dimers, are involved in the increased inhibitory response of adenylyl cyclase to μ-opioid-agonists. Consistently, following combined sufentanil and nimodipine treatment, we observed that $G\alpha_z$ and $G\alpha_{q/11}$ subunit co-precipitation with μ-opioid receptors was not reduced as occurred in animals treated with sufentanil alone. On the other hand, $G\alpha_{i2}$ subunits remained decreased and $G\alpha_o$ co-precipitation was only

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partially recovered. Considering that [^{35}S]GTPγS binding assay is, in practice, restricted to the analysis of guanine nucleotide exchange on $G\alpha_i/G\alpha_o$ subunits (Harrison and Traynor, 2003; Milligan, 2003), the absence of a recovery of μ-opioid stimulated [^{35}S]GTPγS binding following the combined treatment with sufentanil and nimodipine is not surprising.

The present study provides no information about the mechanism involved in the switch of receptor-associated G-protein transduction pathway for adenylyl cyclase inhibition. However, it is interesting to emphasize that some reports in the literature described a switch from $G\alpha_i$ to $G\alpha_z$ proteins in opioid-receptor G-protein coupling following the formation of μ–δ heterodimeric receptors (George et al., 2000; Fan et al., 2005). The consecutive change in opioid-receptor transduction leads to increased μ-opioid receptor binding and signalling activity, and to an enhancement of morphine antinociceptive potency (Gomes et al., 2004).

In summary, our results suggest that μ-opioid analgesic tolerance, induced by chronic sufentanil, follows uncoupling of spinal μ-opioid receptors from their linked G proteins and effector pathways, while the enhanced analgesic response following combined nimodipine with sufentanil treatment is associated with supersensitivity of the adenylyl cyclase pathway to the opioid inhibitory effect and with a shift from PTX-sensitive to PTX-resistant G-protein transduction pathways.

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