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Contribution of G inhibitory protein alpha subunits in paradoxical hyperalgesia elicited by exceedingly low doses of morphine in mice

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ABSTRACT

Aims: Although morphine, at higher doses, induces analgesia, it may also enhance sensitivity to pain at extremely low doses as shown in studies for testing an animal's sensitivity to pain. We used an antisense approach capable of selectively down-regulating in vivo G_i (G inhibitory protein), G_o and G_s members of the G_α sub-family protein subunits in order to establish if these proteins might be implicated in the effects induced by extremely low morphine doses on acute thermociception.

Main methods: Mice pretreated with a morphine hyperalgesic dose (1 μ g/kg) were submitted to hot plate test after pre-treatment with antisense oligodeoxynucleotides (aODNs) targeting $G_{i\alpha}$, $G_{o\alpha}$ and $G_{s\alpha}$ regulatory proteins. The association of G-protein (guanine nucleotide-binding regulatory protein) coupled receptors with G protein was investigated using co-immunoprecipitation procedure.

Key findings: The downregulation of the $G_{i\alpha 1-3}$ and $G_{o\alpha 1}$ proteins reversed the licking latency responses induced by 1 μ g/kg morphine administration toward the basal value whereas downregulation of the $G_{o\alpha 2}$ and $G_{s\alpha}$ proteins did not significantly modify the hyperalgesic response.

Significance: These results suggest that G inhibitory proteins play a role in the production of low dose evoked morphine hyperalgesia in mouse. Immunoprecipitation studies revealed that both μ opioid receptor (μ OR) and α_2 adrenoreceptor (α_2 AR) are bound to G inhibitory proteins in hyperalgesic response to morphine extremely low dose. Both μ OR and α_2 AR appear to be necessary for low morphine dose induced hyperalgesic response through G inhibitory proteins.

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Introduction

Hyperalgesic and analgesic actions, as well as biphasic dose–response relationships, have been demonstrated in animals and humans following administration of opioids (Kayan et al., 1971; Jacquet and Lajtha, 1973; Levine et al., 1978; Woolf, 1981; Gracely et al., 1983). Different authors demonstrated that extremely low doses of morphine (less than 1 μ g/kg) can elicit acute thermal hyperalgesia in animal model of pain as tail flick and Freund's adjuvant-induced arthritic rats (Kayser et al., 1987). The intraplantar application of micromolar doses of morphine has been proved to induce a flexor response in mice (Ono et al., 2002). Depending on the type of cell used and dose applied, opioids can induce hyperpolarization or depolarization and either inhibit or stimulate

neuronal cells. On the basis of electrophysiological studies, it was suggested that either stimulatory or inhibitory influence on neurones by opioid agonists depends on the dose applied (North and Uchimura, 1989; Crain and Shen, 1990; Smart and Lambert, 1996; Keren et al., 1999). Studies in primary dorsal root ganglion cultures showed that neuronal stimulation occurred at nanomolar concentrations of opioids whereas inhibition occurred at micromolar concentrations. This led to the proposal that there might be separate stimulatory and inhibitory effects linked to different effector systems indicating that opioid receptor coupling switch from G inhibitory (G_i) protein to G stimulatory (G_s) protein under certain conditions (Crain and Shen, 1998). Different G protein (guanine nucleotide-binding regulatory protein) classes activated by morphine in the production of spinal and supraspinal antinociception have been identified: $G_{i\alpha 2}$, $G_{o\alpha}$ and $G_{s\alpha}$ proteins appeared to have a main role in supraspinal μ OR opioid analgesia tested in animal model of thermal pain whereas $G_{i\alpha 2}$ was determinant in mediating spinal analgesia (Standifer et al., 1996; Garzon et al., 2000). Therefore we used an antisense approach capable of selectively down-regulate in vivo G_i and G_o members of the G_α sub-family protein subunits in order to establish if also these proteins might be implicated in the effects induced by morphine acute administration at extremely low doses.

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Table 1
Sequences of antisense, mismatch and degenerate oligodeoxynucleotides.

aODN	Sequences
Anti-G _{1α1}	5'-G* ^C T GTC CTT CCA CAG TCT CTT TAT GAC GCC G* ^G C-3'
Anti-G _{1α2}	5'-A* ^T G GTC AGC CCA GAG CCT CCG GAT GAC GCC C* ^G A-3'
Anti-G _{1α3}	5'-G* ^C C ATC TCG CCA TAA ACG TTT AAT CAC GCC T* ^G C-3'
Anti-G _{0α1}	5'-A* ^G G CAG CTG CAT CTT CAT AGG TG* ^T T-3'
Anti-G _{0α2}	5'-G* ^A G CCA CAG CTT CTG TGA AGG CA* ^C T-3'
Anti-G _{5α}	5'-T* ^T G TTG GCC TCA CGC* ^T G-3'
mODN	Sequences
Anti-G _{1α1}	5'-G* ^C T GTC CTT CAC CAG TCT TCT TAT GAC CGC G* ^G C-3'
Anti-G _{1α2}	5'-A* ^T G TGC AGC CCA AGG CCT CCG GAT GAC CGC C* ^G A-3'
Anti-G _{1α3}	5'-G* ^C C TAC TCG CCA ATA ACG TTT AAT ACC GCC T* ^G C-3'
Anti-G _{0α1}	5'-A* ^G G ACG CTG CTA CTT CAT GAG TG* ^T T-3'
Anti-G _{0α2}	5'-G* ^A G CAC CAG CTT TCG TGA AGG AC* ^C T-3'
Anti-G _{5α}	5'-T* ^T G TGT GCC TAC CGC* ^T G-3'
dODN	Sequences
Anti-G ₁	5'-N*N*N NNN NNN NNN NNN NNN NNN NNN N*N*N-3'
Anti-G ₀	5'-N*N*N NNN NNN NNN NNN NNN NNN NNN N*N*N-3'
Anti-G ₅	5'-N*N*N NNN NNN NNN NNN* ^T N-3'

N = G, C, A or T.

Methods and materials

Animals

Sexually mature male albino Swiss Webster mice (Morini, S. Polo d'Enza, Italy) weighing 30–40 g were used. Three to four 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.

Habituation procedure

All the animals were previously habituated to the laboratory according to Abbot (Abbott et al., 1986). Mice were handled on each of the two days preceding the start of the experiments. Mice were allowed to habituate to the testing room for 30 min before hot plate session.

Drugs and antisense oligonucleotide

Morphine HCl, naloxone and yohimbine were purchased from Sigma Chemicals (St Louis, MO, USA). Phosphodiester oligonucleotides (ODNs) protected from terminal phosphorothioate double substitution (capped ODNs) and degenerate ODN (dODNs) were purchased by Tib Molbiol (Berlin, Germany); ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Sigma).

Intracerebroventricular administration

Antisense oligodeoxynucleotides (aODNs), mismatch sequence ODN (mODN), 33-mer fully degenerated ODN (33dODN) and 25mer fully degenerated ODN (25dODN) were injected in a 5- μl final volume of artificial cationic lipid (DOTAP, Sigma). aODNs, mODNs and dODNs

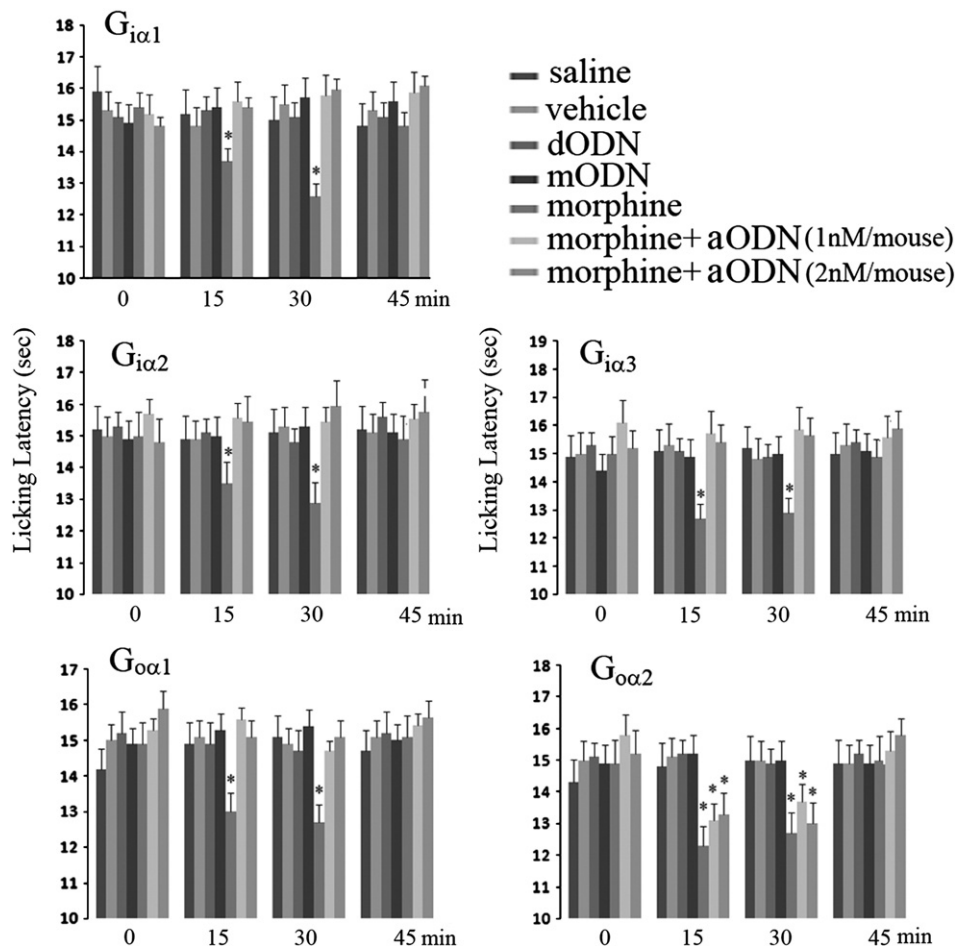


Fig. 1. Effects of aODNs, mODNs and dODNs against G_{1α1-3} and G_{0α1-2} on hyperalgesia induced by 1 $\mu\text{g/kg}$ morphine administration—licking latencies were measured before and after pre-treatment with ODNs in presence of 1 $\mu\text{g/kg}$ morphine. Vertical bars represent S.E.M. * = $\alpha < 0.01$ vs saline. Each value represents the mean \pm S.E.M. of at least 15 mice.

were preincubated at 37 °C for 30 min with 13 μ M DOTAP. The i.c.v. (intracerebroventricular) injections were performed into the right and left cerebral ventricle according to the method described by Haley and McCormick (1957) injecting 2.5 μ l in each side of the brain.

Antisense oligodeoxynucleotides

We have used aODNs against $G_{i\alpha 1-3}$, $G_{o\alpha 1-2}$ and $G_{s\alpha}$ proteins. The sequences of the above ODNs and their characterization are described in a previous paper (Galeotti et al., 2002) and base composition is reported in Table 1. As previously established (Standifer et al., 1996; Sanchez-Blazquez and Garzon, 1998; Galeotti et al., 2002), we used the lowest effective aODN doses (25 μ g) to ensure the selectivity of the response in hot plate test. The aODNs, mODNs and dODNs were supplied to mice by i.c.v. injection 18 and 24 h before the starting of the test. Three base pairs in the antisense sequence were reversed to obtain the mODN respectively for $G_{i\alpha}$, $G_{o\alpha}$ and $G_{s\alpha}$ proteins, as reported in Table 1. 33mer dODN, 25mer dODN and 17mer dODN were used as control respectively for anti- $G_{i\alpha}$, $G_{o\alpha}$ and $G_{s\alpha}$ proteins.

Hot plate test

According to O'Callaghan (O'Callaghan and Holtzman, 1975) mice were placed inside a stainless steel container, which was set

thermostatically at 52.5 ± 0.1 °C in a precision water-bath from KW mechanical workshop (Siena, Italy). Here we have used lower temperature in hot plate test (52 °C instead of 54 °C). As previously shown, increased temperatures result in a decrease of the response latencies (Tjølsen et al., 1991). Therefore, lower temperatures are useful to reveal possible subtle alterations that may occur in basal nociception. The licking latency was measured immediately prior s.c. (subcutaneous) morphine (1 μ g/kg) injection. Hot plate test started 15 min after morphine administration and licking latencies were measured at 15 min intervals for 45 min after starting time. Mice i.c.v. pretreated with aODNs, mODNs and dODNs, were injected with a single s.c. morphine dose (1 μ g/kg) and submitted to hot plate test 15 min after. Other different group received s.c. injection of saline or morphine (10 mg/kg) twice daily for 7 days, supplied with aODNs, mODNs and dODNs by i.c.v. injection at the 6th and 7th days and submitted to hot plate test 24 h after the end of treatments. The licking latencies were measured at the same times as above. A 30 s cut-off to prevent tissue damage was used. The endpoint for the licking response was the first paw lick of the rear paw. Anti-nociception was seen as increased latencies to the responses evaluated while increased nociception was seen by shorter latencies. The analgesic tests were performed in a blind fashion.

Rota-rod test and spontaneous activity meter

For both tests, animals were i.c.v. pretreated 18–24 h before the tests with dODNs or aODNs. Up to 5 mice were tested simultaneously on the apparatus, with a rod rotating 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 (Vaught et al., 1985). Performance was measured before treatment and 15, 30 and 45 min after the starting of the experiments. Locomotor activity was quantified using a type S Animex activity meter (LKB, Farad, Sweden) set to maximum sensitivity. Five mice were placed on the meter. The instrument transformed movements into digital signals. Activity counts were made every 15 min for 45 min.

Receptor-G protein coupling assay

Mice used for these experiments were sacrificed 15 min after 1 μ g/kg morphine administration at which time maximum thermal hyperalgesic effect was obtained in hot plate test (Galeotti et al., 2006). The animals were anesthetized with CO₂, cervically dislocated, decapitated and the brain dissected, put immediately in liquid nitrogen and then stored at –80 °C. Enriched synaptic membranes were prepared from brain of mice treated with morphine as described by Gray and Whittaker (Gray and Whittaker, 1962). Protein concentration was determined according to Lowry (Lowry et al., 1951). The association of G protein coupled receptors with G proteins was investigated

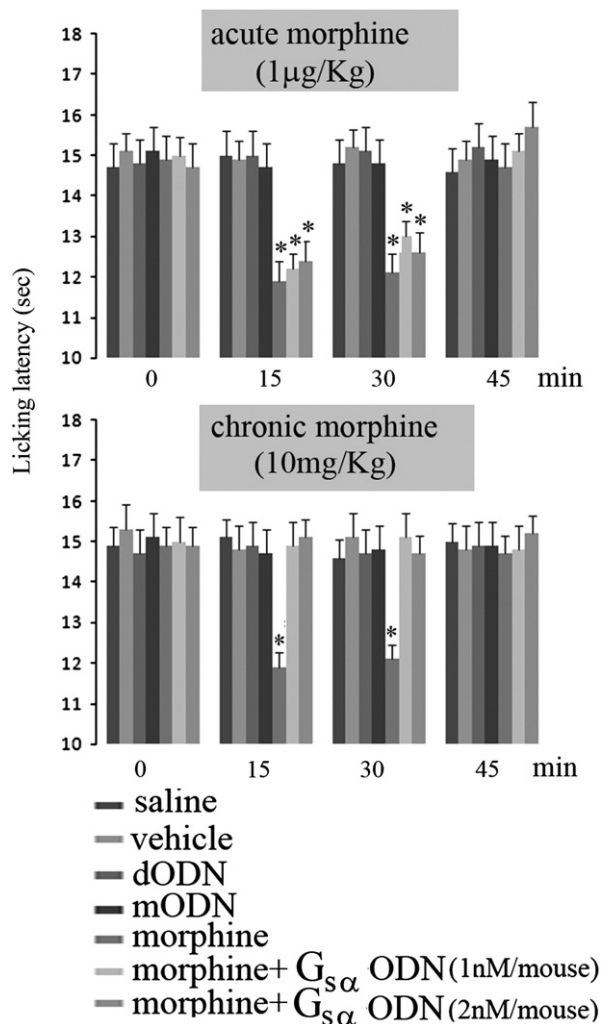


Fig. 2. Effects of aODNs, mODNs and dODNs against $G_{s\alpha}$ on thermal hyperalgesia—licking latencies were measured before and after pre-treatment with ODNs in presence of 1 μ g/kg morphine or after twice daily morphine administration for seven days. Vertical bars represent S.E.M. * = $P < 0.01$ vs saline. Each value represents the mean \pm S.E.M. of at least 15 mice.

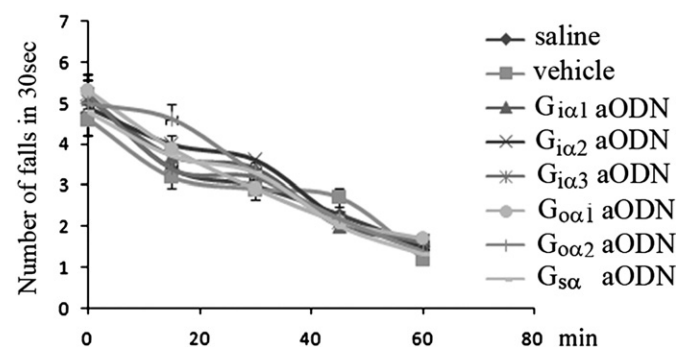


Fig. 3. Lack of effect by saline, vehicle and aODNs against $G_{i/o}$ or G_s proteins administered to mice at 2 nM/mouse, on mouse spontaneous mobility evaluated in the mouse rotarod test. Vertical lines represent S.E.M.

using co-immunoprecipitation procedure as previously described (Wang et al., 2005). The specificity of the anti- G_{α} and anti- α_2 AR antibodies was determined by Western blotting using 100 μ l of mouse whole brain homogenate with or without antigen peptide (25 μ g) pre-adsorption for 30 min. The specificity of anti- μ OR antibody was previously assayed (Bianchi et al., 2009).

Western blot analysis

Western blot was performed as previously described in detail (Pan et al., 1995). In summary, immunoprecipitates (from 1 μ g/ μ l protein lysate) of $G_{i\alpha 1}$, $G_{i\alpha 2}$, $G_{i\alpha 3}$, and $G_{o\alpha}$ protein from brain of morphine and saline pretreated mice were solubilized in SDS 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 PBS containing 3% BSA for 1 h before addition of anti- μ OR or anti- α_2 AR antibody at 1:500 dilution. The blots were stripped and

reprobed with antibodies against various G proteins using the antisera against $G_{i\alpha 1}$, $G_{i\alpha 2}$, $G_{i\alpha 3}$, and $G_{o\alpha}$ protein as probes at 1:1000 dilution. The blotting was visualized using a chemiluminescence detection system (Super Signal West Fento, Pierce Biotechnology Inc., Rockford, IL, USA) 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.

Controls

The mice which underwent the hot plate test were submitted to paw temperature measurement 24 h, 10 min and 1 min before testing. The temperature was measured with an infrared thermometer (Omega, Stamford, CT).

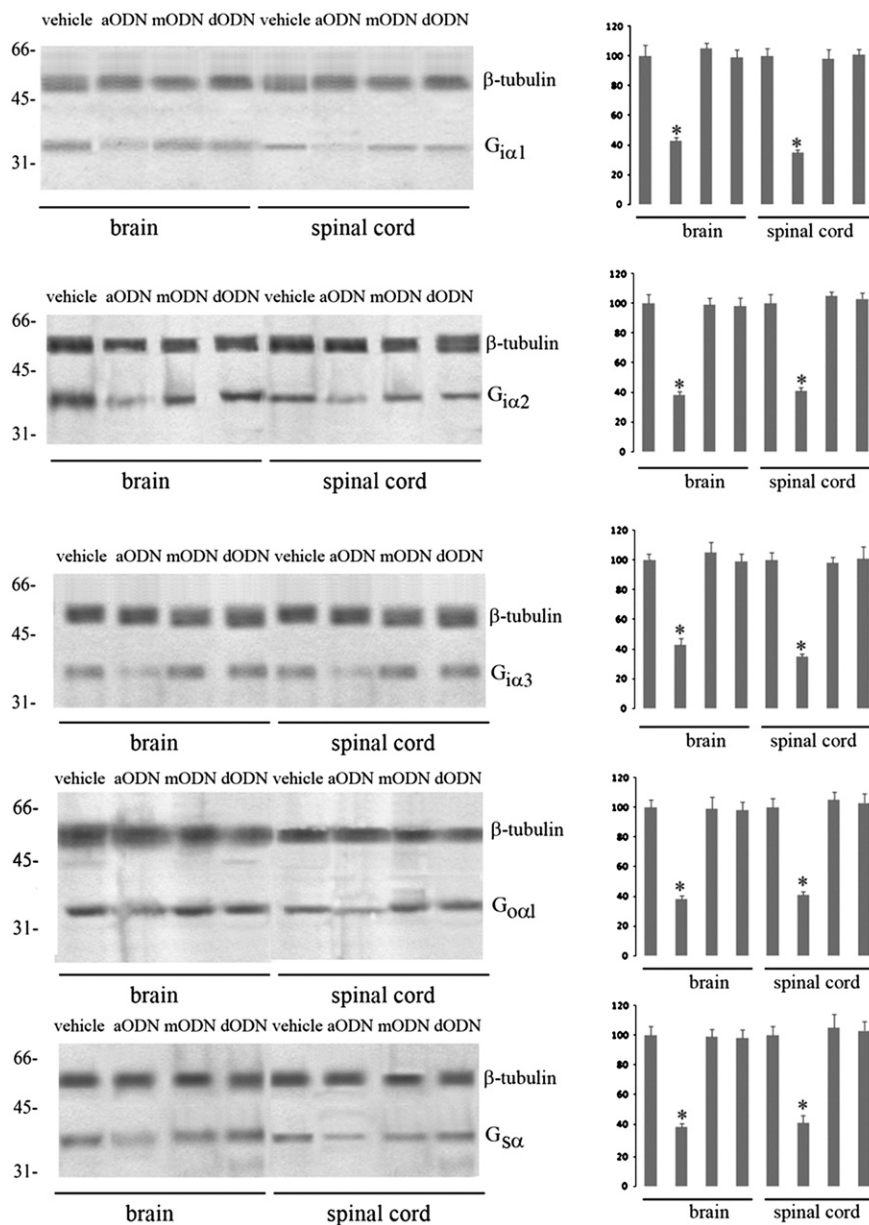


Fig. 4. Downregulation of $G_{i/o}$ proteins—a representative immunoblot is shown with β -tubulin as loading control. The mean \pm S.E.M. of density values obtained in brain and spinal cord from five independent experiments are reported at the right side. Values are expressed as percentage of density obtained in saline treated mice; * = difference at $\alpha < 0.01$ significance level in comparison with control value. Vertical lines represent S.E.M.

At the conclusion of the experiments, the mice were anesthetized, their brains were excised and cut coronally to check the visible path of i.c.v. injection. Only data from mice in which the injection was correctly located within ventricles were considered.

Statistical analysis

All experimental results are given as mean \pm S.E.M. Analysis of variance followed by Fisher protected least significant difference (PLSD) procedure for post-hoc comparisons was used to verify significance between two means. Data were analysed with the Statview Software for the Macintosh (1992).

Results

Role of G protein subunits in morphine induced thermal hyperalgesia

Morphine hyperalgesia induced by s.c. 1 μ g/kg dose in the mouse hot plate test was blocked by pre-treatment with aODNs against $G_{i\alpha 1-3}$ and $G_{o\alpha 1}$ (Fig. 1). The probes anti- $G_{o\alpha 2}$ did not significantly modify the hyperalgesic response induced by 1 μ g/Kg morphine dose (Fig. 1). Antisense ODN pre-treatment against $G_{s\alpha}$ could reverse the hyperalgesic effect induced 24 h after the end of chronic morphine treatment whereas was ineffective in presence of acute low morphine dose administration (Fig. 2).

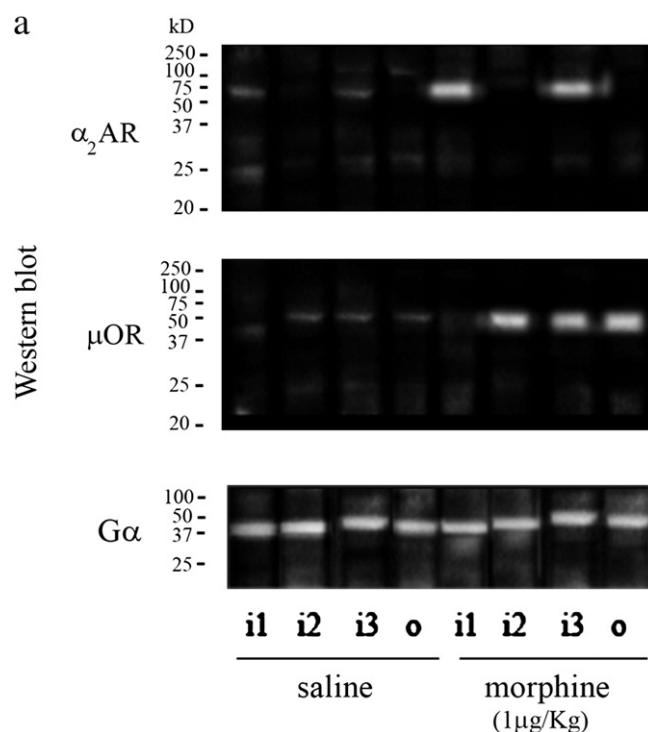


Fig. 5. G protein-receptor coupling in brain from mice treated with morphine—a representative Western blot of the presence of μ OR and α_2 AR protein in immunoprecipitates of i_{1-3} and o subunits of G_α protein in brain from mice treated with saline (control) or morphine at 1 μ g/kg is shown in (a). The blots stripped and reprobed with antibodies against the above G proteins are shown for morphine treatment and control (a). Band optical density for G_α protein subunits is represented in (b). Each bar represents the mean density of each G_α subunit obtained from three independent experiments and is expressed as percent of corresponding saline. Statistics were applied to the raw data prior to transformation to percent. Mean value of μ OR and α_2 AR density detected in immunoprecipitates of considered G_α subunits are represented in (b). Single values were normalized to surrounding background and expressed as arbitrary units. * = $\alpha < 0.01$ vs saline. Vertical lines represent S.E.M.

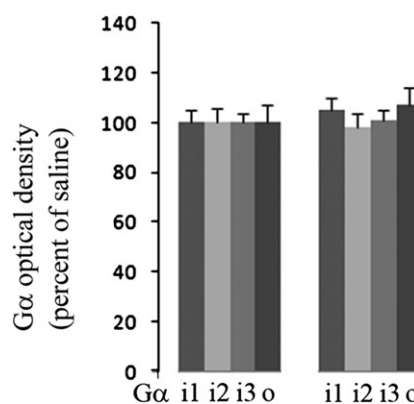
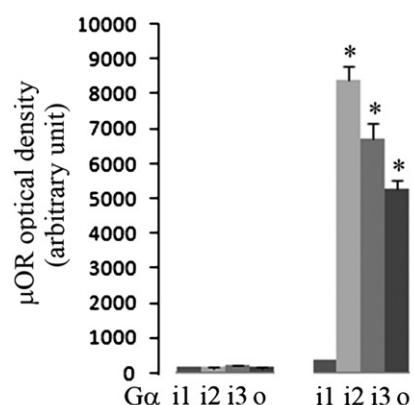
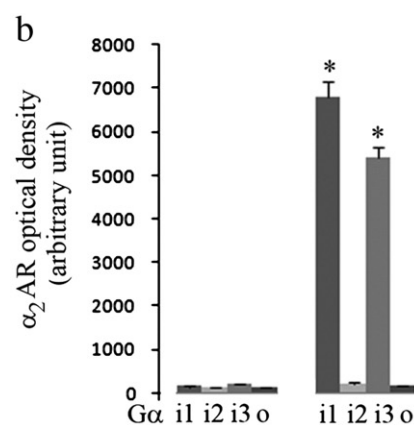


Fig. 5. (continued)

Rota-rod test

No significant differences were observed in the number of falls at different times between saline, vehicle, aODN, mODN and dODN pretreated mice. Therefore, no motor disturbances were observed in rotarod test. Data are shown only for aODN against G protein (Fig. 3).

ODN immunoblotting

Immunoblotting revealed a significant decrease of $G_{i\alpha 1-3}$, $G_{o\alpha 1-2}$ and $G_{s\alpha}$ expression in brain and spinal cord from mice previously treated with corresponding aODN 18 and 24 h before sacrifice, with respect to mODN treated mice (Fig. 4). Immunoblot was reprobed for a non-regulatory protein, β -tubulin, and no significant difference was revealed for this protein between samples from brain or spinal cord.

Co-immunoprecipitation of μ OR and α_2 AR G protein complexes after acute low morphine dose administration

Under non-denaturing conditions, specific G proteins ($G_{i\alpha 1-3}$ and G_o) together with their coupled receptors were immunoprecipitated with selective anti- G_{α} antibodies from solubilized synaptic membranes obtained from brain of the different treatment groups under basal and morphine-stimulated conditions. In our experiments, μ OR coupled to $G_{i\alpha 2}$, $G_{i\alpha 3}$ and $G_{o\alpha}$ protein in low (1 μ g/kg) dose morphine-treated mice. α_2 AR coupling to both $G_{i\alpha 1}$ and $G_{i\alpha 3}$ could be detected after acute 1 μ g/Kg dose morphine administration (Fig. 5a). Densitometric scanning of immunoprecipitated proteins is represented in Fig. 5b.

Effect of μ OR and $AR_{\alpha 2}$ antagonists in morphine induced hyperalgesia

Morphine hyperalgesia induced by s.c. 1 μ g/kg dose in the mouse hot plate test was completely prevented by i.c.v. pre-treatment with selective μ OR antagonist CTOP at 0.5 ng/kg or selective $AR_{\alpha 2}$ antagonist yohimbine (0.4 ng/kg) (Fig. 6).

Controls

When untreated or aODN administered or previously submitted to immobilization procedure mice underwent hot plate test, no significant difference could be detected as respect to saline controls (Fig. 7).

The skin temperature of the paws remained unchanged after administration of the different pre-treatments (data not shown).

Discussion

Opioids are generally thought to produce inhibitory responses evoking analgesia through inhibition of adenylyl cyclase activation of K^+ channels or inhibition of Ca^{++} channels. In some neuronal clonal cultures, induced Ca^{++} increase was observed from cell grown in sparse culture in presence of micromolar opioid concentration (Jin et al., 1992). Direct excitatory effects of opioids have been also described in sensory neurons in presence of morphine low

dose. Nanomolar concentrations of opioids prolonged the duration whereas micromolar concentrations shortened the duration of action potential in mouse dorsal root ganglion neurons (Shen and Crain, 1989). The idea that high affinity excitatory effects are distinct from low affinity inhibitory ones was supported by the observation that nanomolar concentrations of opioids enhance enkephalin release from the guinea pig myenteric plexus via cholera toxin sensitive process whereas higher concentrations (10–100 nM) inhibited release by a pertussis toxin sensitive pathway. It is well known that treatment with pertussis toxin uncouples inhibitory receptors linked to the regulatory G_i and G_o proteins whereas cholera toxin interferes with ligand activation of G_s linked excitatory receptors. Crain and Shen (1998) showed, in primary dorsal root ganglion cultures, that neuronal stimulation at nanomolar concentrations of opioids was coupled with opioid receptor stimulatory G_s protein. In our experiments, when groups of mice pretreated with 1 μ g/kg morphine in presence of aODNs targeting $G_{i\alpha 1-3}$, $G_{o\alpha 1-2}$ were submitted to acute thermal nociceptive test, the downregulation of the $G_{i\alpha 1-3}$, $G_{o\alpha 1}$ proteins reversed the decreased licking latency responses induced by 1 μ g/kg morphine administration toward the basal licking latency value whereas downregulation of $G_{s\alpha}$ protein was ineffective to reverse the hyperalgesic effect. When mice were submitted to repeated twice daily morphine administration, the downregulation of $G_{s\alpha}$ protein could reverse the hyperalgesic effect induced by the interruption of morphine treatment. Although $G_{s\alpha}$ protein appears to be implicated in hyperalgesia induced in morphine abstinent mice, our data support that this condition is not realized in acute morphine treatment, providing evidence that low dose morphine hyperalgesic effects are supported by a separate molecular mechanism. Our results establish that G_i proteins can play a role in the transduction mechanism responsible for the hyperalgesic effect produced in Swiss Webster mice by low morphine dose. Previously, co-expression study in transfected cells (Chan et al., 1995) indicated that opioid receptors can induce excitatory effects by G_i protein activation of type II adenylyl cyclase which appeared to be expressed primarily in the brain (Feinstein et al., 1991); DAMGO induced stimulation of type II adenylyl cyclase was increased in cells co-expressing either $G_{i\alpha 1-3}$ and $G_{o\alpha 1-2}$ proteins. Therefore, an excitatory system might be activated also through G_i .

No hyperalgesic effect was induced by low dose morphine administration in completely untreated mice excluding that stress condition associated with experimental procedure injections might induce an analgesic effect counteracting the hyperalgesic effect induced by low morphine doses.

The hyperalgesic effect obtained in presence of a low morphine dose could be reverted in presence of selective μ OR antagonist CTOP whereas δ OR and κ OR antagonists were ineffective. Interestingly, the morphine induced acute hyperalgesic effect could be reversed in presence of selective α_2 AR antagonist yohimbine. In co-immunoprecipitation experiments, a pronounced coupling of μ OR and α_2 AR to α subunits of $G_{i/o}$ emerged in brain from mice systemically administered with low morphine dose. These results suggest that both the descending opioid and noradrenergic system appear as important components in spinal/supraspinal interactive mechanisms that may mediate the hyperalgesic action induced by low morphine dose. Although α_2 ARs do not seem involved in the opioid withdrawal-induced hyperalgesia (Bie et al., 2003), our data show that these receptors are implicated in morphine hyperalgesia through G inhibitory proteins.

Opioids such as morphine remain the most efficacious and widely used analgesics for moderate to severe pain. Accumulating evidence suggests that the administration of opioid analgesics might lead not only to analgesia but also to a paradoxical sensitization to noxious stimuli. Numerous clinical studies indicate that sustained opioid treatment can also paradoxically cause hyperalgesia. A recent prospective trial in which sustained-acting morphine was given to patients with chronic low back pain demonstrated measurable

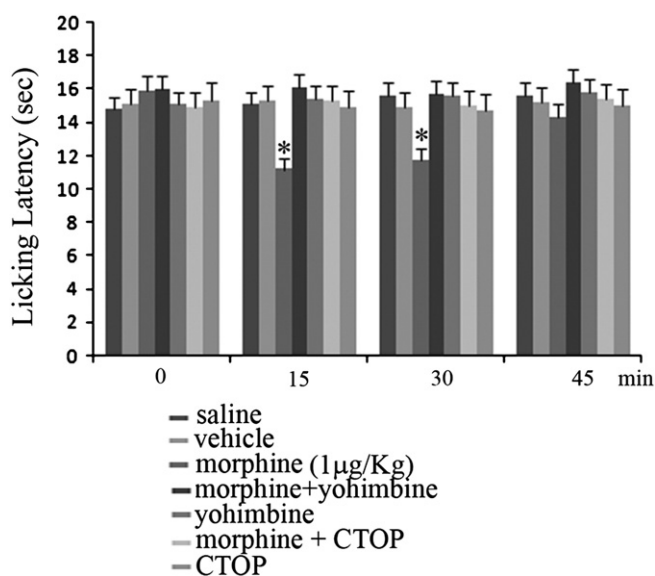


Fig. 6. Effect of CTOP and yohimbine coadministration on morphine induced hyperalgesic response—licking latencies were measured before and after (15, 30 and 45 min) s.c. morphine administration (1 μ g/kg) in presence or absence of i.c.v. CTOP or yohimbine coadministration. Each value represents the mean \pm S.E.M. of licking latencies. Vertical bars represent S.E.M. * = $\alpha < 0.01$ vs saline. Each value represents the mean \pm S.E.M. of at least 15 mice.

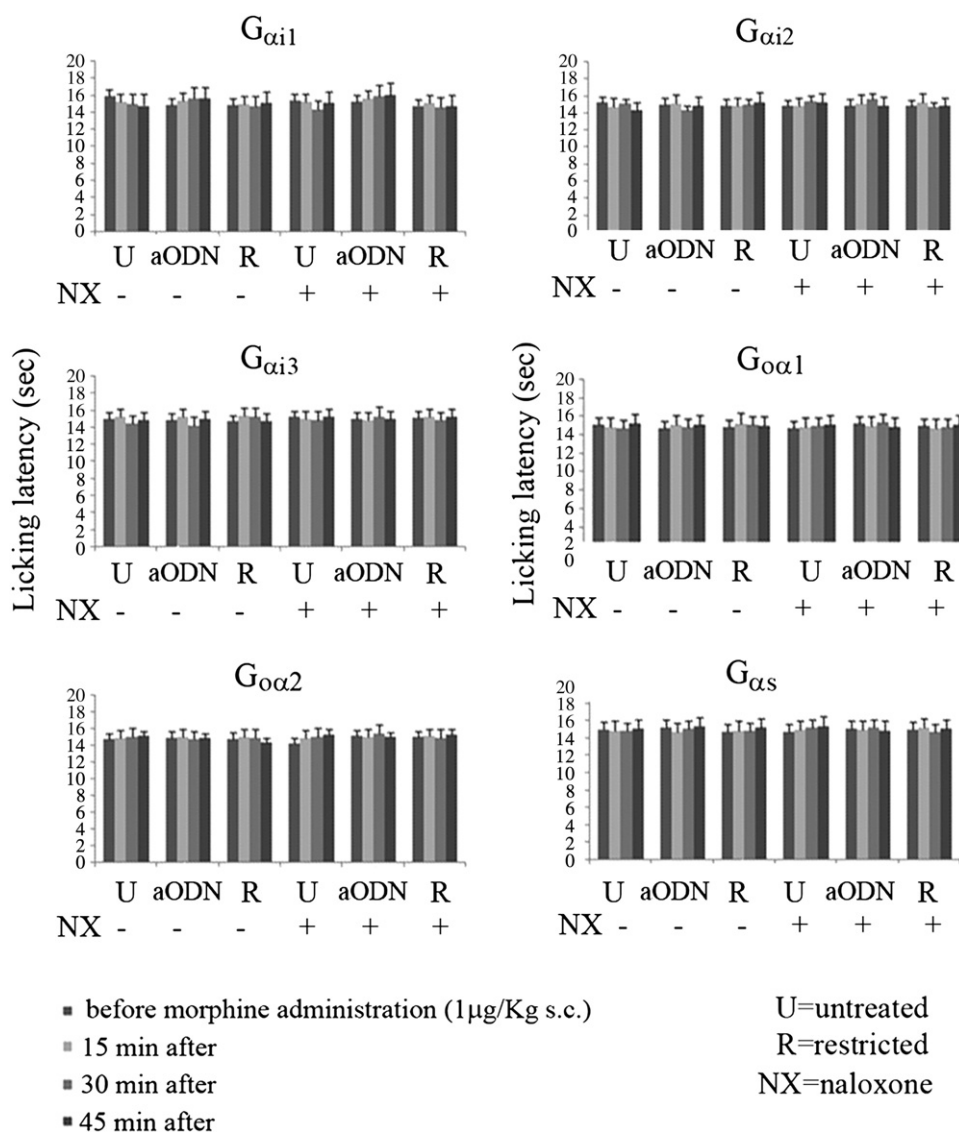


Fig. 7. Hot plate test in non-injected and aODN treated mice—licking latencies were measured before and after (15, 30 and 45 min) in untreated, restricted and aODN treated mice in presence or absence of naloxone. Each value represents the mean \pm S.E.M. of licking latencies. Vertical bars represent S.E.M. * = $P < 0.01$ vs saline. Each value represents the mean \pm S.E.M. of at least 15 mice.

hyperalgesia within one month of beginning therapy (Chu et al., 2006). Other human data suggest that hyperalgesia and allodynia have been observed in human volunteers after opioid analgesia (Guignard et al., 2000). The conventional practice of opioid therapy in presence of diminishing analgesic efficacy is based on a dose escalation to restore analgesic effects. Otherwise, the present data add a possible new mechanism for previously explained observations further corroborating the assumption that opioid induced hyperalgesia can presently considered to be a physiological antagonist to analgesia. These considerations offer a novel approach for the development of strategies that could improve the use of opioids for pain.

Conclusions

In summary, our data showed that both opioid and adrenergic systems are implicated in morphine induced excitatory effect through G inhibitory protein activation.

Conflict of interest statement

The authors of the present paper declare that no financial or personal relationship with other people or organizations inappropriately influenced our work.

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