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RGS-Rz and RGS9-2 proteins control mu-opioid receptor desensitisation in CNS: the role of activated Gαz subunits

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Abstract

Two consecutive icv administrations of analgesic doses of mu-opioid receptor agonists lead to a profound desensitisation of the latter receptors; a third dose produced less than 20% of the effect obtained with the first administration. Desensitisation was still effective 24 h later. Impairing the activity of Gαz but not Gαi2 subunits prevented tolerance developing after the administration of three consecutive doses of morphine. Further, the icv injection of Gαi2 subunits potentiated morphine analgesia and abolished acute tolerance, whereas icv-administered Gαz subunits produced a rapid and robust loss of the response to morphine. The RGSZ1 and RGSZ2 proteins selectively deactivate GαzGTP subunits, and their knockdown increased the effects produced by the first dose of morphine. However, impairing their activity also accelerated tachyphylaxis following successive doses of morphine, and facilitated the development of acute morphine tolerance. In contrast, inhibiting the RGS9-2 proteins, which bind to GαoGTP and GαiGTP but only weakly deactivates them, preserved the effects of consecutive morphine doses and abolished the generation of acute tolerance. Therefore, desensitisation of mu-opioid receptors can be achieved by reducing the responsiveness of post-receptor elements (via the possible action of activated Gαz subunits) and/or by depleting the pool of receptor-regulated G proteins that agonists need to propagate their effects, e.g., through the activity of RGS9-2 proteins.
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1. Introduction

The beneficial antinociceptive effects of opioids are unfortunately overshadowed by the loss of potency that accompanies their repeated administration. Such tolerance is observed even after a single dose of an opioid agonist and can persist for about 3 days (Huidobro et al., 1976; Garzón et al., 2001). In the CNS, mu-opioid receptors play an important role in the antinociceptive action of opioids, but become desensitised by a cell membrane-limited process in which a series of signalling elements are involved. Indeed, tachyphylaxis and acute tolerance at mu receptors can be influenced by agents targeted towards elements of the receptor transduction system, – such as G proteins (Garzón et al., 2001), RGS proteins – the specific regulators of activated Gα subunits (Garzón et al., 2001, 2003, 2004; Sánchez-Blázquez et al., 2003; Zachariou et al., 2003), and phosducin-like proteins – the regulators of Gβγ dimers (Garzón et al., 2002).

Our understanding of the internalisation and processing of the extracellular signals has greatly improved in recent years. Receptors, G proteins, RGS proteins

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and effectors all appear to be tightly packed together to co-operate in the internalisation of signals from extracellular agonists. Opioid receptors are directly coupled to heterotrimeric Gαβγ proteins, which connect the extracellular signals to intracellular effectors. While the mu, delta and kappa receptors activate the Gi/o and Gq/11 proteins, mu receptor signalling mainly involves the Gz proteins (Sánchez-Blázquez et al., 1995; Standifer et al., 1996; Garzón et al., 1997a,b; Belcheva et al., 2000). Indeed, during the production of supraspinal antinociception, the majority of mu receptor agonists activate Gz proteins (Garzón et al., 1997a,b, 2000; Sánchez-Blázquez et al., 2001). However, the situation is complicated since agonists show different affinities to the mu receptors depending on whether they are coupled to Gi, Go or Gz proteins (Garzón et al., 1998; Stanasila et al., 2000; Massotte et al., 2002). Moreover, this coupling determines the agonist pattern of G protein activation (see Sánchez-Blázquez et al., 2001, and references therein).

The pertussis toxin-insensitive Gz transducer protein is regulated by a large number of receptors (Fields and Casey, 1997; Ho and Wong, 2001) and can provoke the inhibition of adenylyl cyclase activity (Wong et al., 1992) as well as the stimulation of K^+ channels (Jeong and Ikeda, 1998). While Gαi and Gαo hydrolyse bound GTP with a $t_{1/2}$ of about 10–20 s, Gαz does so extremely inefficiently ($t_{1/2} = 7$ min) (Casey et al., 1990; Wang et al., 1997). This unique feature greatly influences the regulatory activity of Gz on cellular effectors (Ho and Wong, 2001). Furthermore, it indicates that receptor-activated GαzGTP subunits are difficult to switch off unless other specific regulatory proteins promote GTP hydrolysis. This activity is undertaken by the Rz subfamily of RGS proteins, which includes neural RGSZ1, RGSZ2 and GAIP (Glick et al., 1998; Wang et al., 1998; Ho and Wong, 2001). RGS-Rz proteins bind GαzGTP, and by increasing the rate of hydrolysis by 200- to 400-fold they can produce the effector-inactive GαzGDP form in just a few seconds (Glick et al., 1998; Wang et al., 1998). Therefore, RGS-Rz proteins control the influence that activated GαzGTP subunits have on effectors, which could desensitise mu receptors. Indeed, a recent study indicated that mu receptor-mediated activation of Gz proteins is highly effective at initiating adaptive mechanisms, leading to the dampening of mu-opioid signalling (Garzón et al., 2004).

In contrast to the RGS-Rz proteins, other RGS proteins have negative effects on mu receptor signalling. For example, the members of R7 subfamily of RGS proteins, RGS6, RGS7, RGS9-2 and RGS11, are thought to participate in the onset of agonist tachyphylaxis and acute tolerance at mu receptors (Garzón et al., 2003; Sánchez-Blázquez et al., 2003). In support of this, the knockdown or knockout of the RGS9-2 protein leads to an increase in the potency of mu opioid agonists and prevents or delays the appearance of tachyphylaxis/acute tolerance (Garzón et al., 2001; Zachariou et al., 2003). In these circumstances, the receptor-activated Gα subunits acted upon by RGS-R7 proteins become responsible for prolonging the actions of mu opioids. Among the pertussis toxin-sensitive G proteins activated by opioids, Gi2 is one of the most abundant in the mouse CNS and is regulated by mu opioid receptors in the production of supraspinal antinociception (see e.g., Garzón et al., 2000). In fact, the administration of Gαi2 subunits restores the efficacy of morphine in tolerant mice and also delays the appearance of tachyphylaxis at mu receptors (Garzón et al., 1999, 2001).

The aim of this study was to further characterise the role of Gz and of Gi2 proteins on the activity of opioids at mu receptors. Mu receptor desensitisation was promoted by single or repeated administrations of morphine. The activity of both Gαz and Gαi2 subunits was altered either by inhibiting their expression with antisense oligodeoxynucleotides, through the administration of exogenous Gα subunits or by reducing the expression of RGS-Rz and RGS9-2 proteins. The results confirmed that Gz proteins facilitate the desensitisation of mu receptors. However, administering Gi2 proteins or impairing RGS9-2 function prevented the extinction of morphine effects after repeated doses, and stopped acute tolerance from developing.

2. Methods

2.1. Downregulation of Gαz, Gαi2, RGSZ1, RGSZ2 and RGS9-2 expression

Previously characterised synthetic end-capped phosphorothioate (indicated as *) antisense oligodeoxynucleotides (ODN) were used to reduce the expression of the target proteins. These included the 16 base ODN 5′-C*T*CGAATCAGTTCG*C*T-3′ corresponding to nucleotides 1044–1059 of the murine RGS9-2 mRNA expressed in the CNS (AF125046) (Garzón et al., 2003), the 17 base ODN 5′-T*T*CCGTCCGCTCAGA*T*C-3′ corresponding to nucleotides 127–143 of the murine RGSZ1 gene (AF191552; NM_021374) (Garzón et al., 2004), the 17 base ODN 5′-C*C*GAAGAGTCTCCT C*T*T-3′ corresponding to nucleotides 281–297 of the murine RGSZ2 gene (AF191555) (Garzón et al., submitted for publication), the 33 base ODN T*G*TAATCTCACCCTTGCTCTCTGCTGGGCC-A*G*T corresponding to nucleotides 1033–1065 of the murine Gαz subunit gene (NM_010311) (Sánchez-Blázquez et al., 1995), and the 33 base ODN G*T*GGTCAGCCCAGAGCCTCCGGATGACGC-CC*G*A corresponding to nucleotides 477–502 of the murine Gαi2 subunit gene (NM_008138) (Raffa et al.,

1994). As a control, antisense ODN contained five mismatched bases were also generated: ODN-RGS9-2M, 5′-C*T***GC**AAT**G**AGTT**GC***T*C-3′; ODN-RGSZ1M, 5′-T*T***A**GT**C**A**G**TGC**A**GC**G**G*T*T-3′: ODN-RGSZ2M 5′-C***G***GAA**C**AG**A**CTC**G**TC***A***T-3′. A random ODN (ODN-RD) that did not alter the analgesic activity of the opioids was also used as a control (Sánchez-Blázquez et al., 1995).

These ODNs were injected into the lateral ventricle of animals lightly anaesthetised with ether as previously described (Sánchez-Blázquez et al., 1995; Garzón et al., 2000). Each series of ODN injections was performed on a distinct group of mice according to the following 5-day schedule: 1 nmol on days 1 and 2, 2 nmol on days 3 and 4, and 3 nmol on day 5. Functional studies were usually started on the sixth day.

2.2. Animals and the evaluation of antinociception

Male albino mice CD-1 (Charles River) weighing 22–25 g were housed and used in accordance with European Community Guidelines for the Care and Use of Laboratory Animals (Council Directive 86/609/EEC). Animals were lightly anaesthetised with ether and all substances were injected into the lateral ventricles in 4 μl volumes as previously described (Sánchez-Blázquez et al., 1995). The response of the animals to nociceptive stimuli was determined by the warm water (52 °C) tail-flick test. Baseline latencies ranged from 1.5 to 2.2 s. Treatment with the selected active and mismatched ODNs did not alter the baseline latencies. Since the mismatched ODNs produced no changes in opioid activity when compared to saline-treated mice, the results obtained with saline are presented as controls. The opioids used (sufentanyl, fentanyl, methadone, levorphanol, oxymorphine, normorphine and morphine) were obtained from the "*Control de Estupefacientes*" (Ministry of Health, Spain). Antinociception was expressed as a percentage of the maximum possible effect: MPE = 100(test latency − baseline latency)/(cut-off time (10 s) − baseline latency).

Groups of 10–15 mice received an opioid agonist dose and antinociception was assessed at different intervals. The areas under the time–course curves were determined by the trapezoidal rule using the Sigmaplot 8.0 software package (SPSS Science Software, Erkrath, Germany). Statistical analysis of the results included analysis of variance (ANOVA) followed by the Student–Newman–Keuls test (SigmaStat, SPSS Science Software). Significance was set at $P < 0.05$.

2.3. Production and evaluation of delayed tolerance to morphine

To study tolerance to an acute dose, or after three consecutive doses, an icv test dose of the same opioid was given 24 h later when the previous dose(s) no longer affected baseline latencies in the analgesic test. This effect of the opioid was now compared with that promoted in control mice that received saline instead of the opioid priming dose(s). The antinociceptive activity was determined 10 min later for sufentanyl, fentanyl and methadone, or 30 min later for levorphanol, oximorphone, normorphine and morphine. Delayed tolerance was determined by a decrease in the antinociceptive potency. Each treatment was performed on a different group of 8 or 12 mice.

2.4. Preparation of membranes from mouse PAG

This study was conducted in membranes from periaqueductal grey (PAG) matter. The PAG plays a major role in mediating the effects of opioids when given by the icv route (Yaksh et al., 1976). Male albino mice CD-1 weighing 22–25 g were killed by cervical dislocation and the PAG obtained. About 1 mm tissue around the aqueduct was taken from 2-mm-thick coronal sections (MP-600 Micropunch, Activational Systems Inc) and pooled in 25 mM Tris–HCl buffer (pH 7.7), supplemented with 1 mM EGTA, 0.32 M sucrose, protease inhibitor cocktail (Sigma, Madrid, Spain, #P8340), phosphatase inhibitor mixture (Sigma, #P2850) and H89 (Sigma, #B1427). Homogenates were prepared using a Polytron homogeniser (PT 10-35) and centrifuged at $1000 \times g$ for 10 min to remove the nuclear fraction (Sorvall RC5C, rotor SS-34, Newton, CT, USA). After the pellet was discarded, the supernatant was centrifuged at $20,000 \times g$ for 20 min to obtain the crude synaptosomal pellet P2. After two cycles of washing, resuspension in buffer and centrifugation, the final pellet was diluted in Tris buffer and processed for the corresponding assays.

2.5. Co-immunoprecipitation of signalling proteins

Affinity purified IgGs against the second extracellular loop of mu-opioid receptors (Garzón et al., 2000) were labelled with biotin (Sigma #B1022) according to the manufacturer's instructions. The opioid receptors were immunoprecipitated from the P2 fraction of mouse solubilised PAG as described, with minor modifications (Sánchez-Blázquez et al., 2003). The amount of PAG solubilised protein, IgGs and the period of incubation were adjusted to precipitate all of the desired protein in a single run. PAG membranes containing about 1 mg protein were sonicated (2 cycles of 5 s each) in 400 μl of 50 mM Tris–HCl (pH 7.7), 50 mM NaCl, 1% Nonidet P-40, 50 μl protease and phosphatase inhibitor mixtures, H89, and solubilisation continued overnight at 4 °C. Afterwards, the samples were cleared with 20 μl of

streptavidin agarose (Sigma, #S1638) pre-equilibrated for 1 h at 4 °C, and centrifuged at 3000 × g for 5 min. The solubilised material was incubated overnight at 4 °C with 3 µg of affinity purified biotinylated IgGs against mu-opioid receptors. Streptavidin agarose (50 µl) was then added and incubation continued for an additional 90 min at 4 °C. The samples were then centrifuged for 5 min at 3000 × g, and the agarose pellets subjected to three cycles of washing and resuspension in 1 ml Nonidet P-40 buffer, followed by further centrifugation. At the end of this process, the agarose pellets were heated in 300 µl 40 mM Tris–HCl, 1% SDS buffer for 10 min at 100 °C. The mixture was cooled at room temperature and the streptavidin agarose separated in centrifugal filter devices (pore size 0.45 µm) (Ultrafree-MC #UFC30GV, Millipore Iberica S.A.). The proteins in the soluble fraction were concentrated in centrifugal filter devices (10,000 nominal molecular weigh limit, Amicon Microcon YM-10 #42407, Millipore) and then solubilised in Laemmli 2× buffer plus mercaptoethanol by heating at 100 °C for 3 min. The quantities of protein obtained were sufficient for the loading of four to six gel lanes.

2.6. Detection of RGS and Gαz proteins: electrophoresis and immunoblotting

At the end of ODN treatment, groups of mice were sacrificed by decapitation and their PAG structures collected. For each determination, PAG P2 membranes from four mice (or the immunoprecipitates as obtained above) were solubilised in SDS and resolved by SDS/polyacrylamide gel electrophoresis (PAGE) in 8 cm × 11 cm × 1.5 cm gel slabs (10–20% total acrylamide concentration/2.6% bisacrylamide cross-linker). About 60 µg protein/lane were used for immunodetection. The separated proteins were transferred to 0.2 µm polyvinylidene difluoride membranes (BioRad). The polyclonal antisera against RGSZ1, RGSZ2, RGS9-2, Gαi2 and Gαz were typically diluted 1:1000 in TBS–0.05% Tween 20 (TTBS) and incubated with the transfer membranes at 6 °C for 24 h. The RGSZ1, RGSZ2, Gαi2 and Gαz antibodies (Garzón et al., 1997b, 2004; Sánchez-Blázquez et al., 1995) were detected with a goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate antiserum (BioRad, #170-6515). RGS9-2 (Santa Cruz Biotech., Inc, SC-8143) antibody was detected with a donkey anti-goat IgG horseradish peroxidase conjugate antiserum (SC-2020). Secondary antisera were diluted 1:5000 in TTBS, incubated for 3 h and visualized with the ECL + plus Western Blotting Detection System (RPN2132, Amersham Biosciences). Immunoblots were analysed by densitometry with a ChemiImager IS-5500 (AlphaEase v3.2.2, Alpha Innotech, San Leandro, California).

3. Results

3.1. Desensitisation of mu-opioid receptors in response to successive agonist doses

Initial icv administrations of mu receptor agonists brought about a reduction in the response to successive administrations of the same dose of that opioid. For all opioids tested (sufentanyl, fentanyl, methadone, levorphanol, oxymorphine, normorphine and morphine), this was observed when doses were administered that produced peak analgesic effects greater than 75% of the maximum measured in naïve mice (Figs. 1 and 2). Three identical doses of each opioid were given consecutively at 180 min intervals. Since agonist administration produced desensitisation in each case, the analgesic effect of the third dose was always less intense than that of the second. Even 24 h after receiving the third dose, mice showed tolerance to the analgesic actions of the opioids (Figs. 1 and 2).

3.2. Downregulating Gαz and Gαi2 subunits affects the morphine-induced desensitisation of mu receptors

Gz and Gi2 proteins are critical for the propagation of the effects of morphine (Sánchez-Blázquez et al., 1995; Standifer et al., 1996; Garzón et al., 2000). The active ODNs produced a decrease in PAG Gαi2- and Gαz immunoreactivity of 58 ± 3%* and 52 ± 3%*, respectively (mean ± SEM from three independent experiments). * Significantly different from the ODN-RD control group, ANOVA, Student–Newman–Keuls test, $P < 0.05$). The specificity of the treatments was confirmed since no changes were produced by either ODN-Gαi2 or ODN-Gαz on non-target Gα subunits (Fig. 3). The depletion of either Gαi2 or Gαz subunits caused a significant reduction in the analgesic effects of morphine over the entire time–course of its activity (see Figs. 2 and 3; Garzón et al., 2004). Thus, to match the effects produced by morphine in control mice with those obtained in Gαi2/z knockdown mice, the opioid was used at 3 nmol and 10 nmol, respectively (Fig. 3). Impairment of Gαi2 function did not prevent desensitisation of the receptors with the three consecutive doses of 10 nmol morphine, and tolerance was still observed 24 h later. However, downregulation of Gαz proteins extended the period over which morphine produced analgesic effect, accompanied by a virtual absence of desensitisation to successive doses. Moreover, no tolerance to morphine effect was observed 24 h after injecting the third dose of the opioid (Fig. 3).

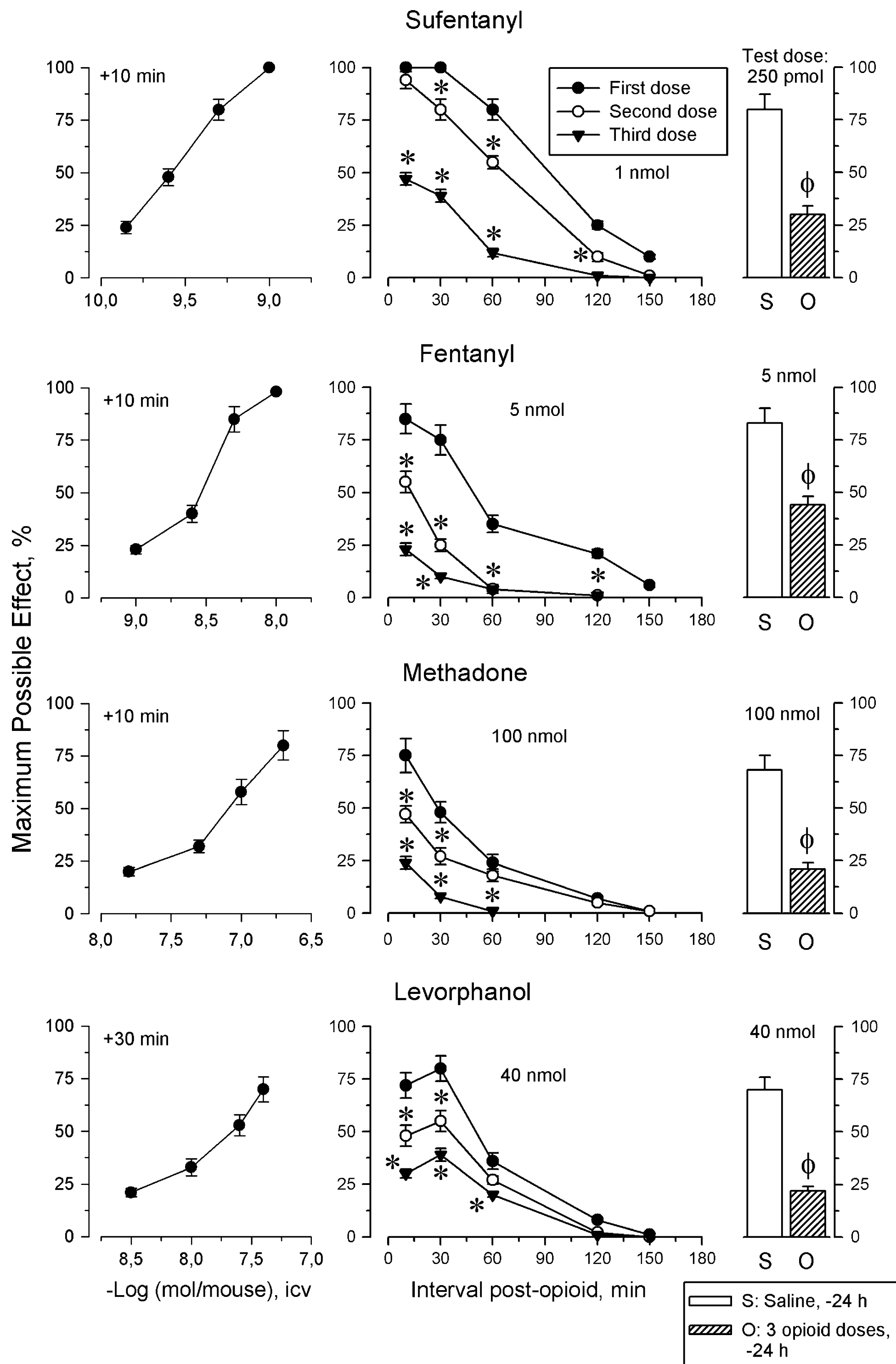

Fig. 1. Dose–response and time–course curves of mu-opioid agonists. Long-term desensitisation is promoted by three successive doses of the opioids. Left column: opioid dose–effect curves constructed at the interval post-icv injection indicated. Middle column: time–course for the analgesic effects of three administrations of the same dose of the opioid given 180 min apart. Right column: effect of test doses of the opioids icv-administered to the mice 24 h after they received either saline (S) or the three doses of the opioid (O). * Significantly different to the first dose of the opioid; ϕ significantly different to the control group that received saline instead of the opioid, ANOVA, Student–Newman–Keuls test; $P < 0.05$.

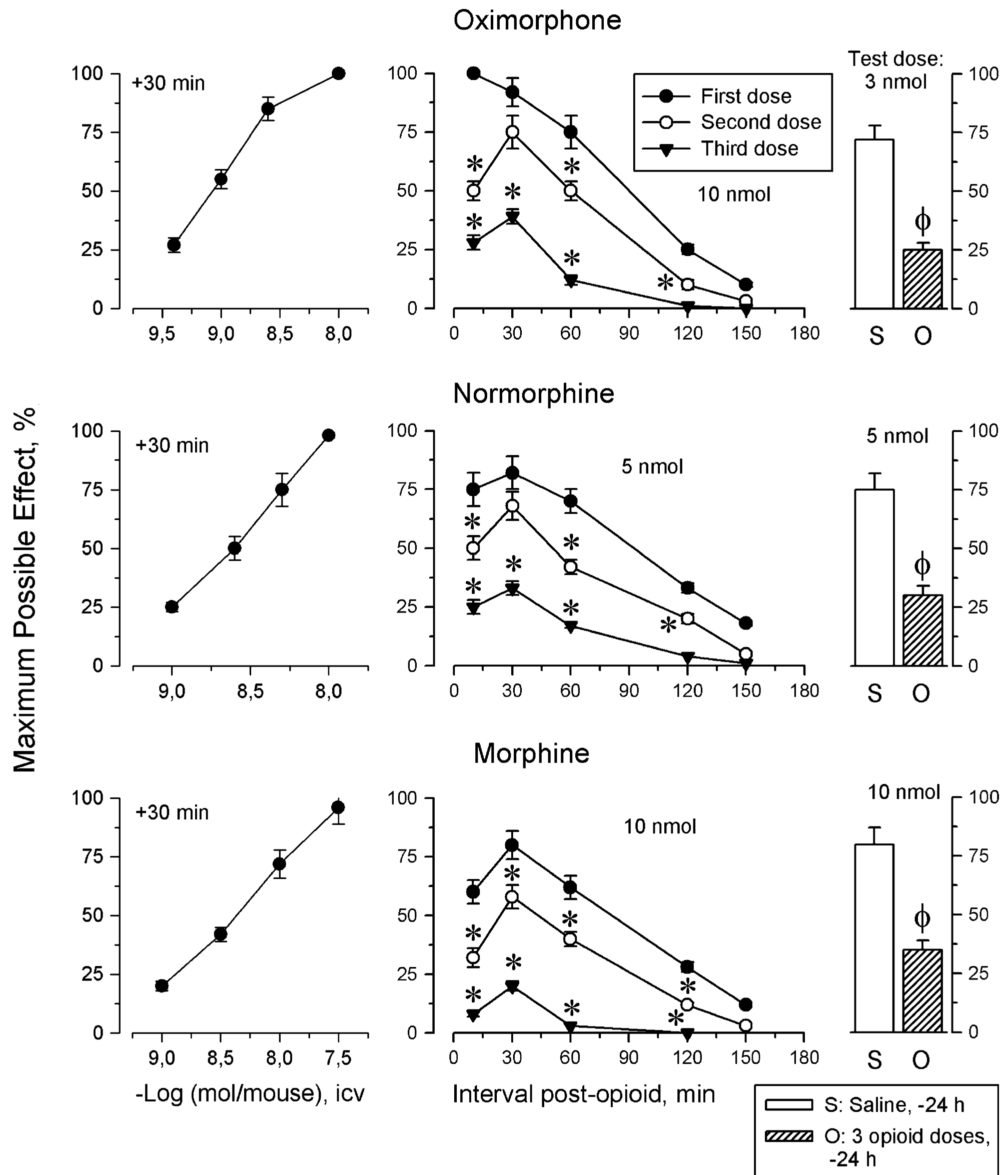

Fig. 2. Desensitizing capacity of oximorphone, normorphine and morphine. Experimental details are given in Section 2 and Fig. 1.

3.3. RGS-Rz and RGS9-2 proteins in the regulation of morphine-induced desensitisation

The expression of RGSZ1 and RGSZ2 in mouse PAG was impaired by the corresponding active ODN. The reductions achieved were: upper glycosylated band, 67 ± 4* %RGSZ1, 58 ± 3* %RGSZ2; lower band, 60 ± 2* %RGSZ1, 83 ± 4* %RGSZ2 (mean ± SEM from five independent experiments). * Significantly different from the ODN-RD control group, ANOVA, Student–Newman–Keuls test, $P < 0.05$) (Fig. 4). The downregulation of RGSZ1 or RGSZ2 greatly increased the potency and the duration of the analgesic effects of an initial dose of 3 nmol morphine, but did not preclude desensitisation to the doses that followed. When compared to the initial dose, the analgesic effects offered by a second, and notably by a third consecutive dose, were significantly less than those seen with normal RGSZ1 or RGSZ2 activity. Impairing the activity of RGS-Rz proteins also brought about an increase in the tolerance to morphine when analysed 24 h after the third dose (Fig. 4).

The ODN directed to RGS9-2 mRNA shows selectivity and efficacy in reducing the coded protein (Garzón et al., 2001, 2003). The percentage decrease in RGS9-2 immunoreactivity was for PAG 55 ± 3* (mean ± SEM from five independent experiments). * Significantly different from the ODN-RD control group, ANOVA, Student–Newman–Keuls test, $P < 0.05$) (Fig. 5). The downregulation of RGS9-2, a member of RGS-R7 subfamily, produced a substantial increase in the effectiveness of the first 3 and 10 nmol

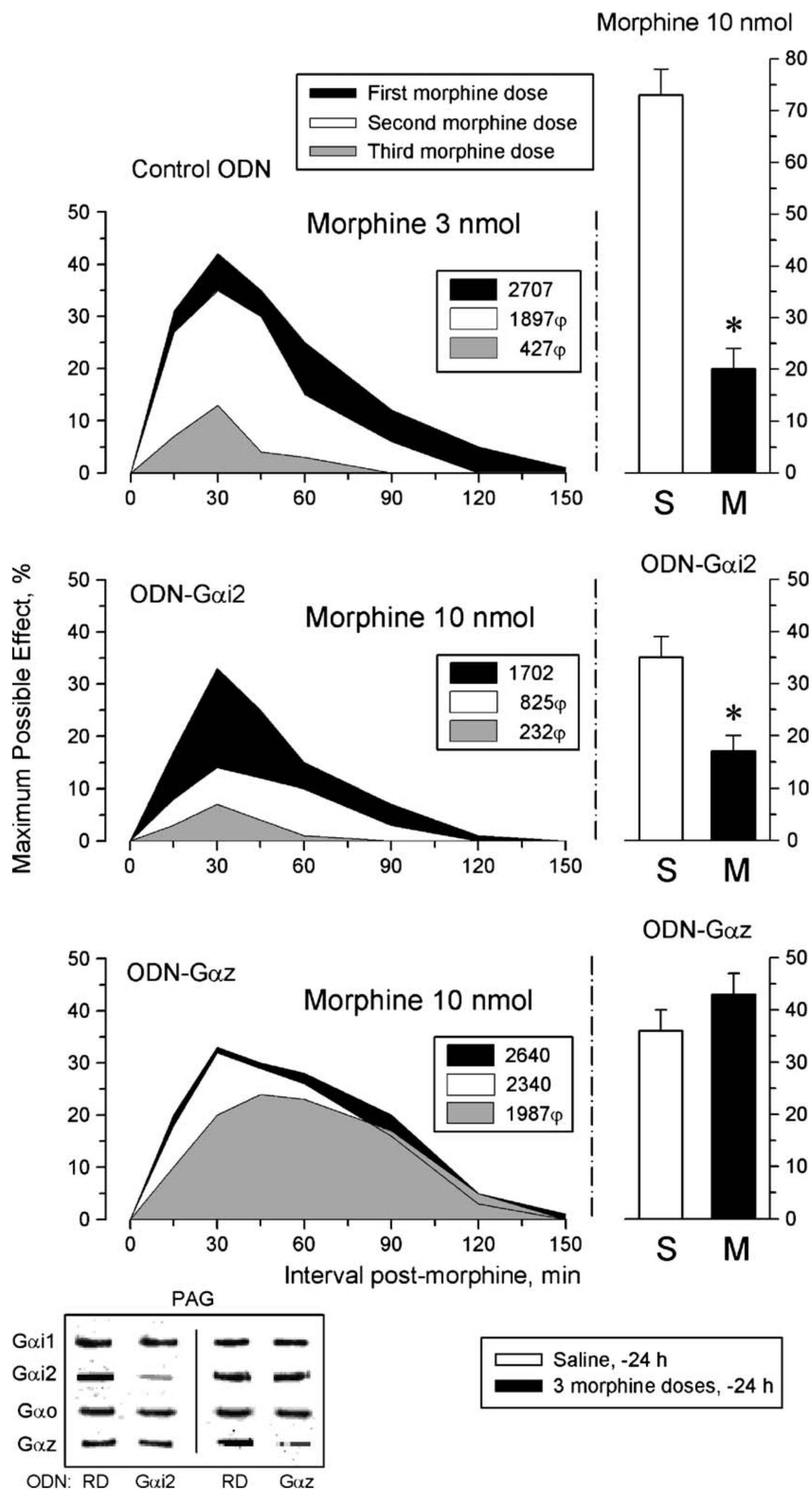

Fig. 3. Role of Gαz and Gαi2 subunits on morphine-induced desensitisation. The time–course of antinociception produced by three consecutive icv doses of 3 nmol morphine, given 180 min apart, was analysed in mice that had received the control ODN-RD. The doses of morphine were raised to 10 nmol in mice that received treatment with the active ODNs against Gαi2 or Gαz subunits for 5 days. Data are presented as the area below the curves and the values are indicated in the corresponding boxes (Sigmaplot v8); φ indicates that at various intervals of the time–course the analgesic, effects were statistically different to those of the first opioid dose; ANOVA, Student–Newman–Keuls test; $P < 0.05$. The columns show the effects of 10 nmol morphine in mice that, 24 h before, received either saline (S) or the three consecutive doses of morphine (M). * Significantly different to the control group that received saline instead of the three doses of the opioid, ANOVA, Student–Newman–Keuls test; $P < 0.05$. Bottom: efficacy of ODNs directed against Gαi2 and Gαz subunits. The control and the active ODNs were icv-injected over 5 days, and on day 6 the mice were sacrificed and the PAG obtained. About 60 μg of SDS-solubilised protein from PAG membranes were loaded per gel lane and resolved by SDS-PAGE and electroblotted. Immunodetection was carried out as described in Section 2.

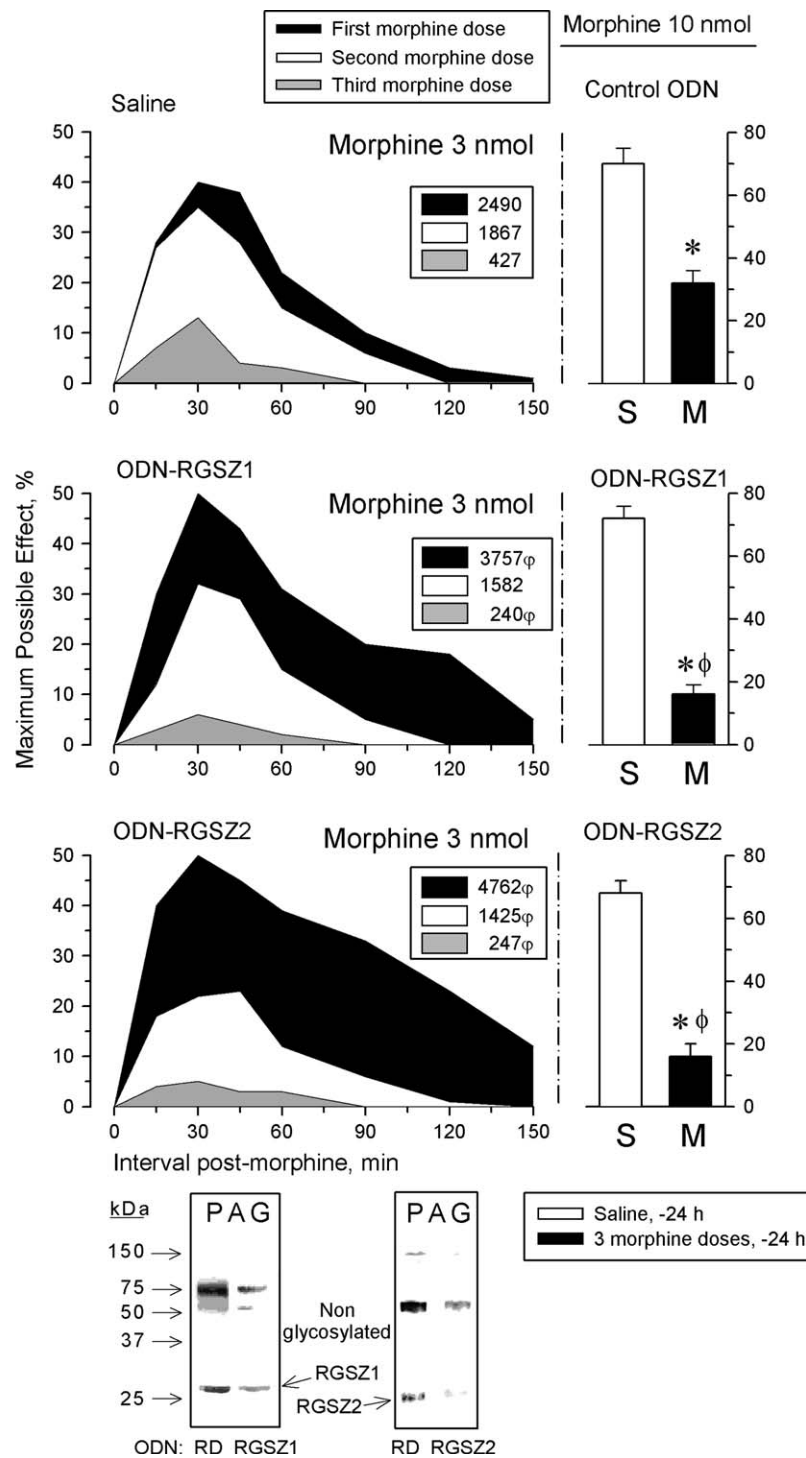

Fig. 4. Effect of RGSZ1 and RGSZ2 knockdown on desensitisation to morphine. The activity of three doses of 3 nmol morphine was studied in mice that received a 5-day treatment with mismatched ODNs or active ODNs against RGSZ1 or RGSZ2 proteins. Since the control ODN produced no changes in the response of the mice to morphine, the group that received saline is shown as a common control. φ Indicates that at various intervals of the time–course, the analgesic effects were statistically different to those of the corresponding dose of the common control (saline group); ANOVA, Student–Newman–Keuls test; $P < 0.05$. * Significantly different to the control group that received saline instead of the three doses of the opioid; φ significantly different to the group that had received the control ODN and the three doses of the opioid, ANOVA, Student–Newman–Keuls test; $P < 0.05$. The bottom figure describes the efficacy of ODNs directed against RGSZ1 and RGSZ2 proteins. Details as described in Section 2 and Fig. 3.

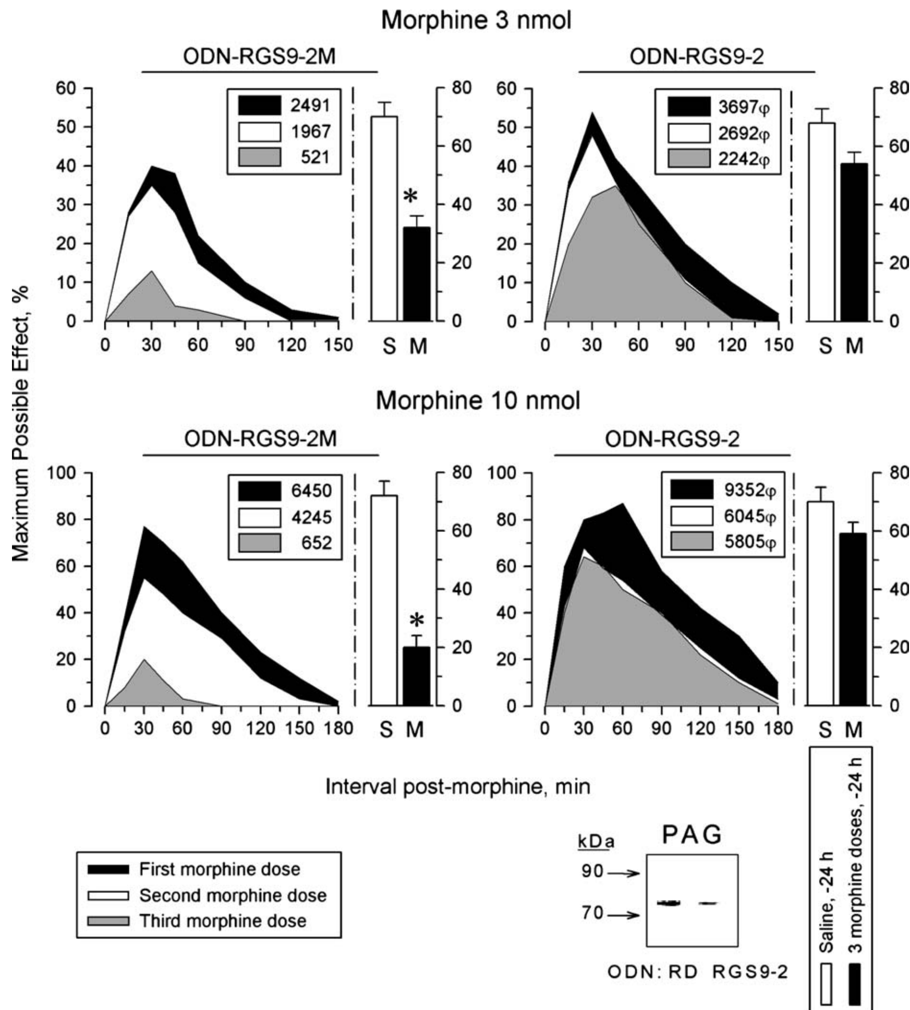

Fig. 5. The absence of morphine-induced desensitisation in mice with reduced levels of RGS9-2 protein. The activity of three successive doses of 3 and 10 nmol morphine was studied in mice that had received the active or mismatched ODN against RGS9-2 proteins for 5 days. φ Indicates that at various intervals of the time–course, the analgesic effects were statistically different to those of the corresponding dose of the mismatched control antisense oligo (ODN-RGS9-2M); ANOVA, Student–Newman–Keuls test; $P < 0.05$. Bottom figure shows the diminishing effects of the active ODN directed against RGS9-2 mRNA. Details as described in Section 2 and Fig. 4.

doses of morphine. More importantly, it virtually abolished the desensitisation to a second and third dose of the opioid, the full analgesic effects of morphine being observed 24 h after the third administration (Fig. 5).

3.4. Co-precipitation of Gαi/o/z subunits with mu-opioid receptors and the effect of exogenous Gαi2 and Gαz subunit administration

In synaptosomal preparations from the mouse PAG, antibodies against mu-opioid receptors precipitated glycosylated proteins of 55–65, 70–80 and 100–110 kDa. Antibodies directed against two epitopes of the mu receptors confirmed that these corresponded to the mu-opioid receptor (Fig. 6 insert). The action of *N*-glycosidase F increased the electrophoretic mobility of these mu receptor-related bands to that of the size deduced from their amino acid sequence (Garzón et al., 1995). Twenty-four hours after receiving an acute icv dose of 10 nmol morphine, tolerance to further morphine administration was observed (Garzón and Sánchez-Blázquez, 2001; Garzón et al., 2004). At this time, there was a 40–60% reduction in the population of Gαi2, Gαz and Gαo subunits that co-precipitated with the mu receptors (Fig. 6). It has been reported that icv administration of Gαi/o subunits to mice in which the function of G proteins is impaired restores the efficiency of opioids in promoting analgesia (Garzón et al., 1999). Moreover, under these conditions the capacity of opioids and agonists of other receptors to activate Gi/o protein-linked GTPase is also restored (Garzón et al., 1999). Interestingly, icv administration of Gαo/i subunits also prevents the onset of morphine acute tolerance (Garzón and Sánchez-Blázquez, 2001). Here we show that icv-injection of Gαi2 and Gαz subunits selectively increased the association of the

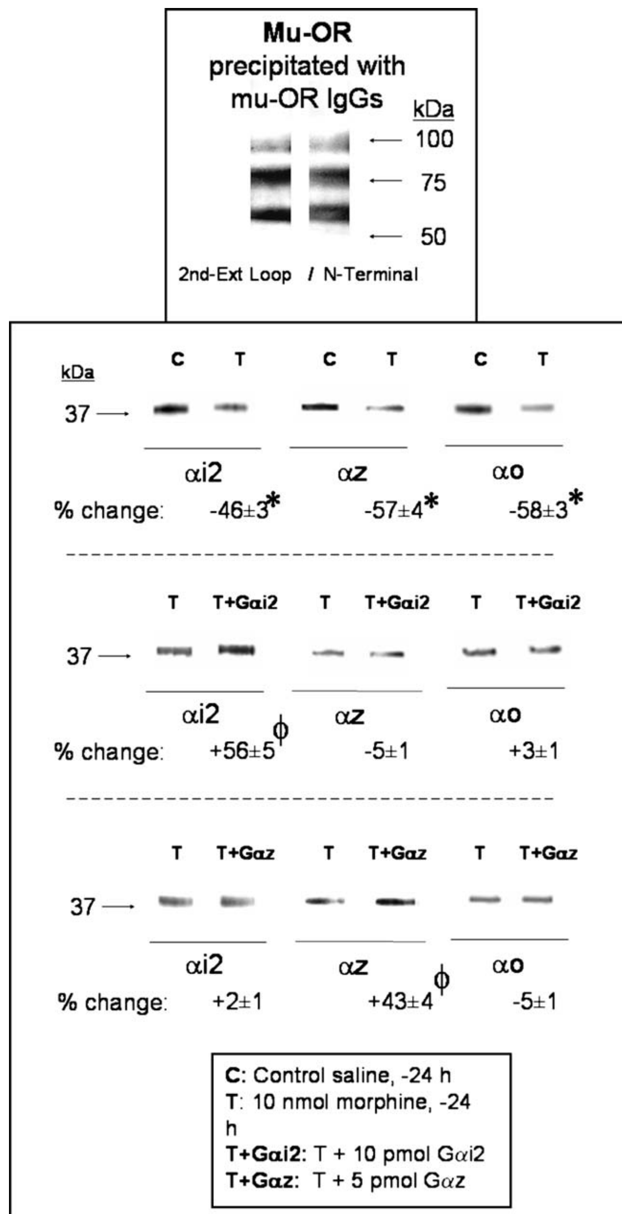

Fig. 6. Co-precipitation of Gα subunits with mu-opioid receptors. Groups of 6–10 mice received a single icv injection of saline or 10 nmol morphine 24 h before being sacrificed. The Gαi2 (10 pmol) or Gαz (5 pmol) subunits were icv injected to mice 23 h after receiving the morphine dose; 1 h later they were killed and the PAG obtained. PAG membranes were solubilised with 1% Nonidet P-40 and incubated overnight at 4 °C with affinity-purified biotinylated IgGs raised against the second external loop of the mu-opioid receptors. Immunocomplexes were precipitated with streptavidin agarose, resolved by SDS-PAGE and visualized in Western blots probed with anti-Gαo, Gαi2 and Gαz antibodies. C: control PAG membranes from mice that had received saline 24 h before sacrifice instead of the single dose of 10 nmol morphine. T: mice that received 10 nmol morphine. The experiment was repeated three times on different groups of mice. Densitometry data are expressed as mean ± SEM. * Significantly different to the control group that received saline instead of the opioid; ϕ significantly different to the group treated with morphine but given saline instead of the exogenous Gα subunits, ANOVA, Student–Newman–Keuls test; $P < 0.05$.

corresponding Gα subunit with the mu-opioid receptors. However, this was seen in PAG from mice that showed acute tolerance to 10 nmol morphine (Fig. 6), but not in the PAG of control mice not exposed to the opioid (not shown).

As expected, microinjection of Gαi2 subunits (0.05–10 pmol/mouse) into the lateral ventricle 1 h before 10 nmol morphine had minimal effects on the analgesic efficacy of morphine within 30 min (Garzón and Sánchez-Blázquez, 2001). In contrast, the injection of Gαz subunits (0.05–5 pmol/mouse) decreased the analgesic capacity of morphine in this interval (Fig. 7A). The analgesic time–course following the administration of 10 nmol morphine indicates that the icv injection of Gαi2 subunits delayed the decline of the opioid effects (Fig. 7B; Garzón and Sánchez-Blázquez, 2001). However, the administration of exogenous Gαz subunits had a biphasic effect on the analgesic time–course provoked by morphine. While a potent increase in morphine antinociception was observed over the first 15 min, this declined more rapidly than in control mice. The action of morphine favours a progressive draining of the pool of mu receptor-regulated G proteins, thus its effects disappear at a faster rate than the pharmacokinetic withdrawal of the opioid from the receptor environment (Garzón and Sánchez-Blázquez, 2001; Garzón et al., 2004). After the effect of morphine peaks, and during its decline, the exogenous Gαi2 subunits available to the mu receptors serve to increase the analgesic activity of this opioid. However, the increased analgesia of morphine promoted by Gαz subunits rapidly triggered the mechanisms that lead to desensitisation of the mu receptors (Figs. 3 and 7B; Garzón et al., 2004).

3.5. Role of Gαz, Gαi2, RGSZ1 and RGSZ2 on morphine-induced acute tolerance

The threshold dose necessary to produce acute tolerance to morphine is about three to four times greater than that required for producing detectable analgesia (Huidobro et al., 1976; Garzón et al., 2002). At its peak (30 min after injection), icv injection of 10 nmol morphine produced an analgesic effect of about 70–80% of the MPE (cut-off time of 10 s). Some 2 h after the injection, a remnant effect of about 20% MPE was observed (Figs. 2 and 7B); however, 24 h later, morphine no longer influenced the baseline response of these animals. To determine the role of Gz and Gi2 proteins in the development of acute morphine tolerance, mice were icv-injected with the corresponding α subunits either 1 h before the priming dose of 10 nmol morphine (24 h before the test dose), or 1 h before the morphine test dose. In control mice that had received the priming dose of morphine, the effect of the opioid test dose was reduced after 30 min (the time of its maximal effect). However, no tolerance to morphine was

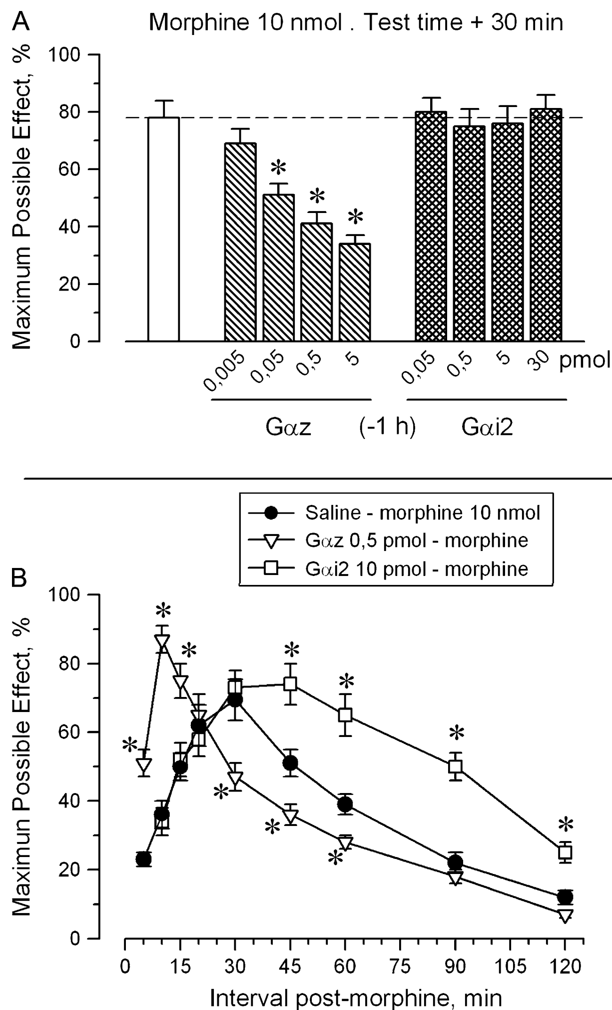

Fig. 7. Differential effect of icv injection of Gαi2 and Gαz subunits on the analgesic activity of morphine. (A) The analgesia evoked by 10 nmol morphine was studied in different groups of 6–10 mice icv-injected with different amounts of Gαi2 or Gαz subunits. The Gα subunits were given 1 h before the opioid and analgesia was evaluated after 30 min post-morphine by the tail-flick test. * Significantly different to the group that received saline instead of the Gαz subunits, ANOVA, Student–Newman–Keuls test; $P < 0.05$. (B) Antinociceptive dose–response curves for 10 nmol morphine in groups of 10 mice treated with saline, Gαz or Gαi2 subunits 1 h before the opioid. * Significantly different to the effect of morphine in the saline-treated group at the corresponding time point, ANOVA, Student–Newman–Keuls test; $P < 0.05$.

seen in mice that had received the Gαi2 subunits 1 or 24 h before the test dose. In contrast, the administration of Gαz subunits 24 h before the test dose brought about an increase in the analgesic effect of morphine after 5 min, but only in mice that had received saline rather than a 10 nmol morphine priming dose. After an interval of 30 min, desensitisation to the test dose of morphine was observed. This profile of the effects of Gαz on the morphine time–course and desensitisation is also seen in Fig. 7B. Indeed, mu receptors were desensitised during the time–course of the priming dose, and this tolerance persisted for 24 h when a test dose was studied both 5 and 30 min post-morphine administration. The injection of Gαz subunits 1 h before the administration of a test dose reduced the effect of the opioid seen in naïve control animals, but slightly increased its potency in mice with acute tolerance that had received a priming dose of morphine (Fig. 8).

The influence of RGSZ1 and RGSZ2 proteins in the development of acute tolerance to morphine was also investigated. After ODN-treatment for 5 days, the mice were challenged on day 6 with saline or 10 nmol morphine and their levels of tolerance tested. The knockdown of RGSZ1 or RGSZ2 did not prevent the onset of acute tolerance to morphine now evident even 5 min after the morphine test dose. The administration of Gαi2 subunits 1 h before the test dose partially recovered the response to morphine of RGSZ1 knockdown mice, an effect not observed when RGSZ2 was impaired (Fig. 8). The knockdown of RGSZ2 reproduced the influence that exogenous Gαz subunits had on morphine activity. The analgesic effects of morphine were greatly increased at early intervals, but later fell below control levels (Figs. 7 and 8).

3.6. *Knockdown of RGSZ1 and RGSZ2 facilitates the development of morphine acute tolerance*

To investigate whether an increase in signalling through the endogenous Gz proteins might affect the desensitisation of mu receptors, mice in which RGSZ1 or RGSZ2 function was impaired were given a priming dose of 3 nmol morphine. The icv injection of 3 nmol morphine did not produce acute tolerance 24 h later in control mice (Fig. 9; Garzón et al., 2004). However, in the RGSZ1 and RGSZ2 knockdown it provoked acute tolerance (Fig. 9). Moreover, the knockdown of RGSZ1 and RGSZ2 brought about an increase in the effects of 3 nmol morphine when evaluated 30 min after its injection. No increase in the effects of a higher 10 nmol dose of morphine could be detected after 30 min in these RGSZ1 knockdown mice. However, this dose did promote desensitisation in RGSZ2 knockdown mice and lack of response to exogenous Gαi2 subunits (Fig. 8; Garzón et al., 2004). These observations indicate that RGSZ2 is more directly involved in the regulation of Gz-mediated mu signalling than the RGSZ1 proteins. An increased presence of GαzGTP at their effectors could reduce their sensitivity to Gα regulation, thereby accelerating the desensitisation of the mu-opioid receptors.

4. Discussion

The administration of opioids in pain management or in the treatment of heroin addiction produces a profound desensitisation of their receptors. Considerable

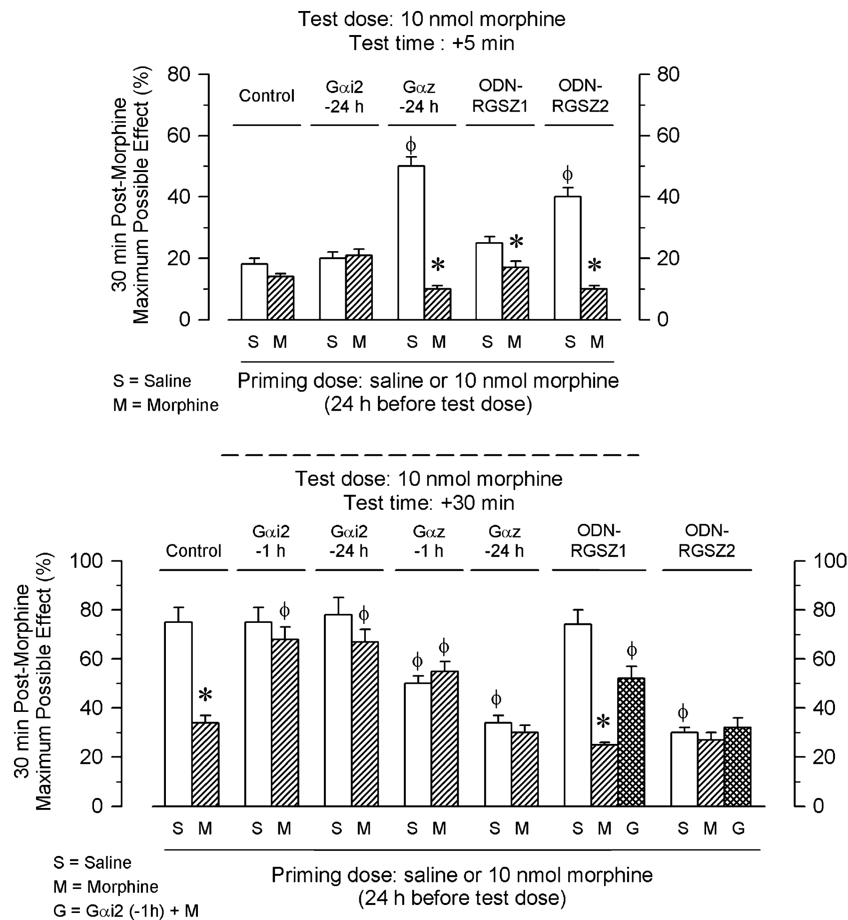

Fig. 8. Influence of icv administration of Gαi2 and Gαz subunits and of RGSZ1 and RGSZ2 knockdown on morphine-induced acute tolerance. A priming dose of 10 nmol morphine was icv-administered to groups of 10–14 mice, 24 h later these animals received a test dose of the same dose of the opioid. Antinociception was evaluated by the tail-flick test 5 and 30 min after injection of the morphine test dose. The Gαi2 (10 pmol) and Gαz (5 pmol) subunits were icv injected 1 h before the priming dose or 1 h before the test dose of morphine. The capacity of morphine to produce acute tolerance was also studied in mice that received a 5-day treatment with ODNs against RGSZ1 or RGSZ2 proteins. * For every treatment indicates a significant difference from the parallel group that received saline instead of the opioid priming dose; ϕ significantly different to the control group that had received no Gα subunits or underwent no RGS-Rz protein depletion, ANOVA, Student–Newman–Keuls test; $P < 0.05$.

effort has been directed toward developing opioid agonists with potent analgesic activity but which are devoid of desensitising effects or a likelihood to lead to abuse. However, limited success has been achieved, and the clinical use of these compounds in the treatment of severe pain relies on their rotation or combination with other drugs. For all the agonist studied here, three consecutive administrations of the same dose produced a long-lasting impairment of their effects. Therefore, this is a common characteristic of opioids that act via mu receptors, and is a serious drawback with respect to their long-term use in the treatment of chronic pain. In recent years, new concepts have come to light regarding the molecular mechanisms responsible for opioid desensitisation that exclude the loss of the agonist-activated mu receptors. This is particularly important with respect to opioids such as morphine, for which high levels of tolerance are provoked without a substantial loss of receptors in the CNS (Keith et al., 1996; Finn and Whistler, 2001). Since the changes caused by morphine in the association of G-proteins with mu-opioid receptors are not a consequence of decreased receptor number but of changes in receptor signalling regulation, this opioid is appropriate for exploring the influence of Gi2/z availability and RGS function on mu receptor desensitisation.

This rapidly evolving field has provided convincing evidence that mu- and delta-opioid receptors form heterodimers in mouse spinal cord (Gomes et al., 2004) and other areas of the CNS (unpublished data). Moreover, when mu and delta receptors form heterodimers they share a common G-protein pool (Alt et al., 2002). This association contributes to their synergy since ligand binding to delta receptors increases the binding of morphine and [D-Ala^2,*N*-MePhe^4,Gly-ol^5] encephalin (DAMGO) to mu receptors, as well as their capacity to activate G proteins and regulate adenylyl cyclase (see Gomes et al., 2004 and references therein). The

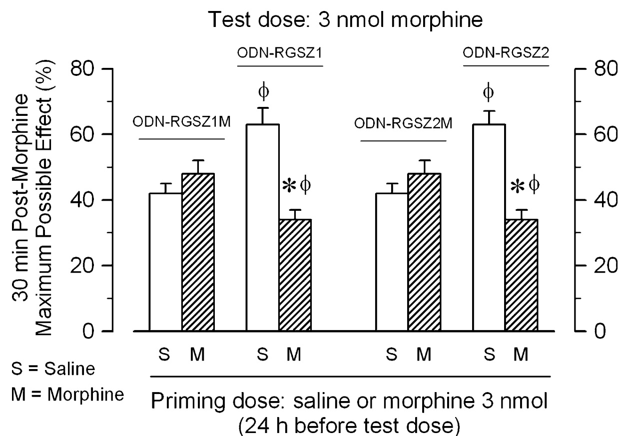

Fig. 9. RGSZ1 and RGSZ2 knockdown facilitates morphine acute tolerance. Either saline or a priming dose of 3 nmol morphine was icv-injected into the mice that had received either the active ODNs against RGSZ1 and RGSZ2 proteins, or control mismatched ODNs. After 24 h, all groups received an icv test injection of 3 nmol morphine, and analgesia was evaluated 30 min later. Values are mean ± SEM from groups of 10–15 mice. * Significantly different to the corresponding group injected with saline before the morphine test dose; φ significantly different to the corresponding group treated with the mismatched ODN, ANOVA, Student–Newman–Keuls test; $P < 0.05$.

delta-opioid receptors have been implicated in mu receptor desensitisation. In fact, selective antagonists of delta receptors (Abdelhamid et al., 1991; Fundytus et al., 1995), knockdown and knockout of delta receptors (Kest et al., 1996; Zhu et al., 1999), and the knockout of the gene coding for delta receptor endogenous ligands (Nitsche et al., 2002), all reduce or abolish the tolerance that develops after morphine administration. Interestingly, the knockdown/knockout of RGS9-2 proteins also prevents agonist-induced desensitisation of mu receptors (Garzón et al., 2001, 2003; Sánchez-Blázquez et al., 2003; Zachariou et al., 2003; present work). These observations suggest that delta receptors influence the signalling of mu receptors by allosteric interactions within the heterodimers, and probably by the activation of G proteins and certain RGS proteins as well.

Although the great majority of mu opioid agonists studied so far propagate their effects through Gi and Gz proteins (Sánchez-Blázquez and Garzón, 1988; Sánchez-Blázquez et al., 2001), they show differences in their preference for activating either one or the other. After binding to mu receptors, the synthetic opioid peptides [D-Ala^2,D-Leu^5] encephalin (DADLE) and [D-Ser^2,Leu^5] encephalin-Thr^6 (DSLET) activate Gi2 proteins much better than Gz proteins (Garzón et al., 1994, 1998). Differences are also observed for the endomorphins, e.g., endomorphin-2 activates Gi2 proteins but endomorphin-1 does not. Moreover, endomorphin-1 activates Gz proteins much better than endomorphin-2 (Sánchez-Blázquez et al., 1999; Garzón et al., 2004). In the production of supraspinal analgesia, morphine mainly triggers the activation of Gi2 and Gz proteins (see Garzón et al., 2000). While the Gi2 proteins contribute little to the extinction of mu receptor-mediated signals, the Gz proteins appear to be involved in the desensitisation of mu receptors (Garzón et al., 2004; present work). Accordingly, knockdown of Gαz subunits abolished desensitisation of mu receptors and the three consecutive doses of morphine produced almost identical effects. Notably, 24 h later these mice showed a response similar to that of controls when an additional dose of the opioid was given. The impairment of the Gαi2 subunits favoured the regulation of Gz proteins by mu receptors, and tolerance to morphine developed more rapidly. The knockdown of RGSZ1 and RGSZ2, selective deactivators of GαzGTP subunits, increased the potency and the duration of the effects evoked by the first dose of morphine, but greatly diminished the analgesic effects of a second and third administration. In fact, 24 h later, tolerance to the analgesic effects of morphine was pronounced. In RGS-Rz knockdown mice, acute tolerance was observed in response to doses of morphine that did not produce such effects in naïve mice (Garzón et al., 2004; present work). It is noteworthy that impairing RGS-Z2 activity facilitated desensitisation during the effective time–course of the first dose of 10 nmol morphine, but not when 3 nmol was used.

Biologically active exogenous Gα subunits injected in the CSF are transported across neural membranes by protein kinase Cβ1-dependent fluid-phase endocytotic process, and incorporated in the inner side of cell membranes where they contribute to receptor-initiated signalling cascades (Garzón et al., 1999; Garzón and Sánchez-Blázquez, 2001). As observed when RGSZ2 activity was impaired, icv injection of exogenous Gαz subunits increased the analgesic response to 10 nmol morphine at early time intervals, followed by a rapid desensitisation. These results suggest that in the regulation of morphine-activated GαzGTP subunits, RGSZ2 is more efficient than RGSZ1. In mice in which the transduction system is impaired, administration of Gαi2/o subunits restores the efficacy with which opioids produce antinociception (Garzón et al., 1999). Notably, Gαi2 subunits increase the morphine-induced analgesia beyond its peak and prevent acute tolerance (Garzón et al., 2001; present work). These effects suggest that morphine decreases the number of mu receptor-regulated G proteins, leading to the development of tachyphylaxis and acute tolerance. The availability of exogenous Gαi2 subunits facilitates the propagation of morphine signalling and restores the transduction regulated by mu receptors such that acute tolerance does not develop. These positive effects are not produced by the Gαz subunits, increasing the availability of this subunit to mu receptors promotes more rapid

induction of tolerance. In these circumstances Gαi2 subunits failed to restore the potency of morphine, suggesting that the activated Gαz subunits caused desensitisation at the effector level. Thus, to minimise the desensitising capacity of Gz proteins on agonist signalling, their regulation by RGS-Rz proteins is essential.

The inhibition of members of the RGS-R7 subfamily promotes an improved response to morphine and abolishes acute tolerance (Garzón et al., 2001, 2003; Sánchez-Blázquez et al., 2003). The RGS9-2 shows a particularly prominent role in these effects (Garzón et al., 2001, 2003; Zachariou et al., 2003). In the present study, impairing RGS9-2 proteins resulted in a significant enhancement of the antinociceptive effects of morphine, paralleled with poor desensitisation to successive doses of the opioid. More importantly, the opioid was still fully effective 24 h later. The RGS-R7 proteins bind Gαi/o/z proteins in their transition state when the GαGTP subunit initiates its spontaneous change into the effector-inactive GαGDP form (Posner et al., 1999). However, they effectively activate only the GTPase at Gαo subunits (Lan et al., 2000). Therefore, upon receptor activation, the binding of RGS-R7 to Gαi/z subunits serves to control the intensity of agonist signalling by reducing the pool of receptor-regulated G proteins (Sánchez-Blázquez et al., 2003). In this scenario, a fraction of the morphine-activated Gαi2/z subunits would be retained by RGS-R7 proteins and, hence, tachyphylaxis and acute tolerance would develop. The RGS-Rz subfamily regulates the action of GαzGTP subunits at their effectors, preventing the rapid desensitisation of mu receptors (Garzón et al., 2004) (Fig. 10). Therefore, tachyphylaxis and acute tolerance triggered by an acute dose of morphine is, for the most part, the consequence of a loss of receptor-regulated G proteins, and not due to a reduction in available receptor molecules. This is further supported by the absence of acute tolerance and decreased tachyphylaxis after increasing the availability of Gα subunits by impairing RGS-R7 function, or by administering exogenous Gαi2 subunits (Garzón et al., 1999, 2001, 2003; Zachariou et al., 2003). In Gαz knockdown mice, the repeated administration of morphine produced no mu receptor desensitisation. Activated GαzGTP subunits might therefore be required in the sequestering activity of RGS-R7 proteins on Gαi subunits.

Delta-opioid receptor shows a limited capacity to regulate Gz proteins in the CNS (Garzón et al., 1998, 2000). In agreement with that, the RGS-Rz proteins are not linked to regulation of this receptor (Garzón et al., 2004). It is therefore unlikely that Gz, and RGZ-Rz proteins are acted upon by delta receptor agonists to attenuate tolerance at mu receptors. In contrast, Gi/o proteins can be regulated by delta receptors (see Garzón et al., 2000 and references therein) and RGS-R7 proteins do affect the function of these opioid receptors (Garzón et al., 2003). Thus, the participation of these signalling elements in the delta-mediated regulation of mu receptor desensitisation merits consideration.

RGS gene expression show rapid changes following a range of experimental and physiological stimuli. Indeed, the RGS-R4 subfamily shows remarkable plasticity. Acute morphine and cocaine reduces the levels of RGS4 in the nucleus accumbens, whereas chronic administration increases its levels (Bishop et al., 2002). Moreover, chronic opioid administration increases the expression of RGS4 mRNA in PC12 cells expressing opioid receptors (Nakagawa et al., 2001). In the rat locus coeruleus, RGS-R4 and RGS-R7 transcripts do not vary during chronic morphine treatment, but RGS2 and RGS4 mRNA levels increase after precipitating opioid withdrawal (Gold et al., 2003). Acute morphine administration increases the levels of RGS9-2 expression in the nucleus accumbens and dorsal horn of the spinal cord, whereas in morphine tolerant-dependent mice levels of this protein are reduced in these structures (Zachariou et al., 2003). Also, acute morphine increases RGS7 mRNA levels in the striatum as well as those of RGS9-2 in the striatum and thalamus, whereas RGS9-2 and RGS11 mRNA levels are reduced in the cortex (Lopéz-Fando et al., 2004). In contrast to that observed for RGS4 and RGS-R7 proteins, RGS-Rz/Gαz genes do not respond rapidly to morphine triggered intracellular changes. In the CNS, the mRNA levels of RGS-Z1, RGS-Z2 and of their target, the Gαz subunits, show no significant variations in response to acute doses of morphine that are sufficient to produce mu receptor desensitisation (unpublished data).

In summary, this paper suggests a key role for activated GαzGTP subunits in the development of mu-opioid receptor desensitisation. However, activated Gαi2GTP subunits do not exhibit this characteristic and restore the effects of acutely tolerant mu receptors. By controlling the activity of GαzGTP subunits, the RGS-Rz proteins reduce their desensitising capacity. In contrast, the RGS9-2 proteins contribute to the development of tachyphylaxis and acute tolerance to morphine, probably by reducing the availability of Gi/z proteins to the receptor. This was achieved without the loss of mu receptor molecules. This knowledge could help in the development of therapies to treat chronic pain where repeated administration of opioids is required. As agonists determine the classes of G proteins that are activated after binding to mu-opioid receptors (Sánchez-Blázquez et al., 2001), the use of mu receptor activating agonists with a high capacity to activate Gi/o proteins but little or no capacity to activate Gz are recommended. This would take advantage of the development of agents directed at reducing the activity of RGS9-2 proteins.

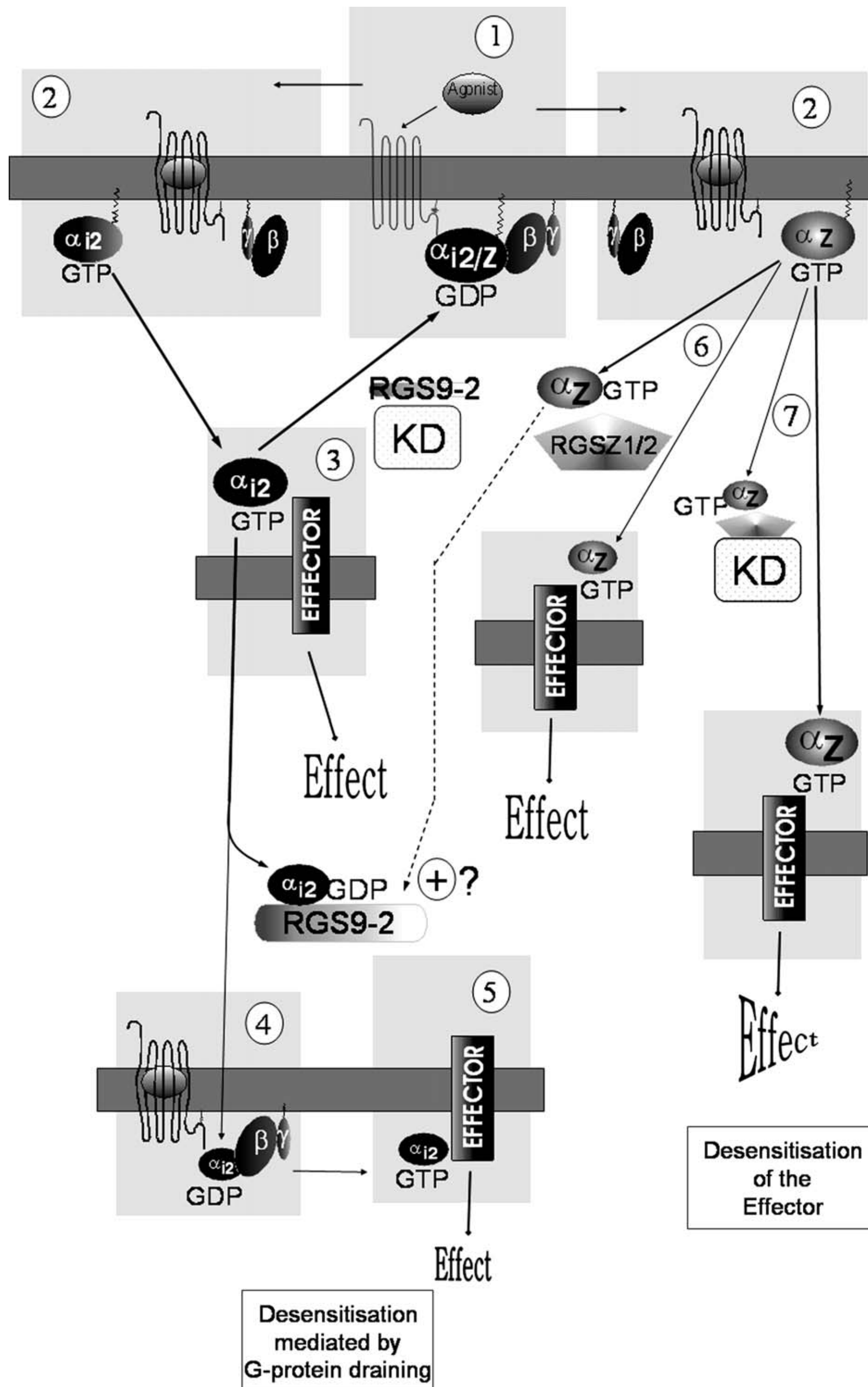

Fig. 10. Proposed role for RGS9-2 and RGS-Rz proteins in regulating mu-opioid receptor activated Gαi2 and Gαz subunits. (1) Mu agonists bind to receptors coupled to Gi2 and Gz proteins. (2) The subsequent exchange of GTP for GDP at the Gαi/z subunits causes their segregation from the Gβγ dimmers. (3) The activated Gαi2GTP subunits regulate the corresponding effector. (4) A fraction of the deactivated Gαi2GDP subunits is now retained by the RGS9-2 proteins (Sánchez-Blázquez et al., 2003), thus reducing the pool of mu-opioid receptor-regulated Gi2 proteins. (5) Consequently, the effect promoted by the mu agonist is of a lower magnitude than that expected from its dosage – G protein-mediated desensitisation. The selective knockdown (KD) of RGS9-2 proteins preserves the availability of Gi2 proteins to mu receptor regulation, and the effect triggered by the agonist is now maintained. (6) Since RGS-Rz proteins are able to bind Gαz subunits in activated GTP form (Glick et al., 1998; Wang et al., 1998) they can act as a trap for mu receptor-activated GαzGTP subunits before they reach the effector. Those that succeed in regulating the effector are also deactivated by the RGSZ1/Z2 proteins to re-form the pool of mu receptor-regulated Gz proteins. (7) The knockdown of either RGSZ1 or RGSZ2 proteins permits a larger fraction of activated GαzGTP subunits to reach and regulate the effector. The inefficient control of Gz signalling – trapping and deactivation of GαzGTP subunits – brings about an initial increase of the effect of the agonist followed by a profound desensitisation, probably due to a reduction of the sensitivity of the effector to G protein regulation (Garzón et al., 2004, present work). This pattern is particularly evident after knockdown of RGSZ2 proteins, where desensitisation occurs even during the time–course of the agonist effect. Since in Gαz KD mice desensitisation to morphine does not develop, a link between activated GαzGTP and the sequestering activity of RGS9-2 proteins on Gαi2 subunits is suggested (dotted line).

Acknowledgments

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