Physiology and Pharmacology

Physiol Pharmacol 19 (2015) 38-45 **www.phypha.ir/ppj**

Original Article

Changes in regulator of G protein signaling-4 gene expression in the spinal cord of adrenalectomized rats in response to intrathecal morphine

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Abstract

Introduction: Regulators of G-protein signaling protein negatively control G protein -coupled receptor signaling duration by accelerating Gα subunit guanosine triphosphate hydrolysis. Since regulator of G-protein signaling4 has an important role in modulating morphine effects at the cellular level and the exact mechanism(s) of adrenalectomy-induced morphine sensitization have not been fully clarified, the present study was designed to determine the changes in the levels of RGS4 mRNA and protein in intact and adrenalectomized (ADX) morphine-treated rats.

Materials and Methods: All experiments were carried out on male Wistar rats. The tail-flick test was used to assess the nociceptive threshold and corticosterone levels were measured by radioimmunoassay. The dorsal half of the lumbar spinal cord was assayed for the expression of RGS4 using semi-quantitative RT-PCR and immunoblotting.

Results: Results showed that the anti-nociceptive effect of intrathecal morphine (5 µg) was significantly increased in ADX rats. The levels of RGS4 mRNA and protein in ADX rats were similar to those in intact animals. However, morphine could elicit a significant increase in both mRNA and protein levels of RGS4 following adrenalectomy. In contrast, the pattern of RGS4 gene expression did not show significant changes in the lumbar spinal cord of intact animals after morphine injection.

Conclusion: Our results demonstrate that in the absence of corticosterone, morphine increases RGS4 through promoting its gene expression.

Keywords:
Morphine;
Analgesia;
RGS4;
Gene expression;
Corticosterone;

Received:
14 Jan 2015
Accepted:
14 Feb 2015

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Introduction

It has been reported that removal of corticosterone through adrenalectomy significantly potentiates morphine analgestic effects (Miyamoto *et al.*, 1990, Esmaeili *et al.*, 2005). Isolated tissue extracted from adrenalectomized (ADX) animals shows an increase in sensitivity to opioid effects (Ozaki *et al.*, 2001). We had previously reported changes in the expression level of specific G-protein subunits involved in adrenalectomy-induced morphine sensitization and that phenomenon is due, at least in part, to lack of adrenal hormone corticosterone (Esmaeili-Mahani *et al.*, 2008).

All three opioid receptor subtypes (μ, δ and κ) belong to the superfamily of G-protein coupled receptors (GPCRs). Opioid receptors induce responses in the nervous system via coupling to $G_{\alpha i/o}$ proteins (Williams *et al.*, 2001), and their activation results in adenylyl cyclase inhibition and/or the regulation of various ion channels and other effectors, leading to hyperpolarization of cells, thereby exerting an inhibitory effect (Law *et al.*, 2000, Nestler, 2004). However, the GPCRs do not act in isolation and their activities are modulated by several accessory proteins such as arrestins, kinases, and regulators of G protein signaling (RGS) proteins (Lamberts and R Traynor, 2013).

RGS proteins are a large family of highly diverse, multifunctional signaling proteins, which share a conserved signature domain (RGS domain) that binds directly to the activated Gα subunits and modulate G protein signaling. These proteins are responsible for Gα binding and markedly regulate the GTPase activity of Gα subunits leading to their deactivation and termination of downstream signals (Baltoumas *et al.*, 2013).

RGS proteins, the negative regulators of G proteins, can also modulate μ-opioid receptor (MOR) signaling and play a controlling role in the effectiveness of opioid receptor ligands both *in vitro* and *in vivo* (Clark *et al.*, 2003, Wang *et al.*, 2009, Lamberts and R Traynor, 2013) Several members of RGSs, in particular RGS4 and RGS9-2 have been demonstrated to affect MOR signaling and morphine-induced behaviors, including analgesia and reward. In addition, morphine modulates the expression levels of RGS proteins, especially RGS4 in a tissue and time dependent manner (Traynor, 2012).

It has been demonstrated that G-protein subunits are under the coordinated control of glucocorticoids in the rat brain and are the physiological targets of glucocorticoids (Saito *et al.*, 1989). Furthermore, glucocorticoids can regulate RGS4 expression levels in nerve tissue (Ni *et al.*, 1999).

Since RGS4 proteins have an important role in the termination of G-protein coupled receptors such as opioid receptors and glucocorticoids have regulating role on RGS4 expression levels the present study was designed to determine the impact of the changes in the levels of RGS4 mRNA and protein in adrenalectomy-induced potentiation of morphine analgesia.

Materials and methods

Animals

All experiments were carried out on male Wistar rats, weighing 200-250 g, that were housed four per cage under a 12 h light/dark cycle in a room with controlled temperature (22 ± 1°C). Food and water were available *ad libitum* except in adrenalectomized rats, which were maintained on 0.9% NaCl drinking solution. Animals were handled daily (between 9:00 and 10:00 A.M) 5 days before the experiment in order to condition them to manipulation and minimize nonspecific stress-related responses. Rats were randomly allocated into several experimental groups, each comprising 6-8 animals. All experiments followed the guidelines on ethical standard for investigation of experimental pain in animals (Zimmermann, 1983).

Drugs

Morphine hydrochloride was dissolved in physiological saline and given intrathecally (i.t.) in the volume of 10 μl.

Antinociceptive test

Anti-nociception effect was assessed by tail-flick test (D'Amour and Smith, 1941). The baseline tail-flick latency for each rat was established as the mean of three values measured at 5 min intervals. The intensity of the beam was adjusted to produce mean reaction times between 2 and 4 s in control animals (only rats with a control latency time from 2 to 4 s were used). The cut-off time was fixed at 10 s in order to avoid any damage to the tail. After determination of baseline latencies, rats received intrathecal injection of morphine (5 μg), and the reaction latency was determined at 15, 30, 60, 120, 180 and 240 min after injection. The tail-flick latencies were converted to the percentage of antinociception according to the following formula: %Antinociception (%MPE) = (Reaction time of test - basal reaction time)/(cutoff time - basal reaction time).

Intrathecal catheter implantation and drug delivery

Animals were anesthetized with ketamine (50 mg/kg) and xylazine (5mg/kg, i.p.). An intrathecal catheter (PE-10) was implanted in each rat according to a previously published method (Yaksh and Rudy, 1976). Animals that exhibited neurological deficits (e.g. paralysis) after the catheter implantation or during drug delivery were excluded from the experiments.

Adrenalectomy

Animals were anesthetized with ketamine (50 mg/kg) and xylazine (5mg/kg) i.p. Both adrenal glands were removed through two dorsal incisions. The sham operation consisted of bilateral dorsal incision, plus locating and exposing the adrenals. All adrenalectomized rats were maintained on 0.9% NaCl drinking solution, whereas the sham-operated rats were kept on tap water. The catheterization was performed at the same time. The adrenalectomized animals were monitored throughout the study to ensure that they were healthy, active, showed no noticeable weight loss, and had clean fur. All animals were retained in the study and appeared active and healthy. The animals were tested 5 days after the adrenalectomy or sham procedure. Plasma level of corticosterone and also postmortem examination of the ADX animals confirmed that the adrenal glands had been completely removed.

Corticosterone assay

On experimental days, rats were killed with decapitation between 9:00-10:00 A.M and trunk blood was collected into tubes containing 5% EDTA. Plasma was obtained by centrifugation of blood at 2500 r.p.m (10 min). Samples were frozen immediately and stored until the time of corticosterone assay at -20°C. Plasma level of corticosterone was measured by radioimmunoassay using a commercial kit for rats ([^{125}I] corticosterone, DRG International, Inc. USA). The sensitivity of assay was 0.25ng/ml and the antibody cross-reacted 100% with corticosterone, 0.34% with desoxycorticosterone, and less than 0.10% with other steroids.

Tissue extraction and preparation

Rats were anesthetized (exposed to a CO_2 atmosphere) and decapitated. The spinal column was cut through the pelvic girdle. Hydraulic extrusion was performed by inserting a 16 gauge needle into the sacral vertebral canal and expelling with ice-cold saline. The spinal cord was immediately placed on ice in a glass petri dish, and the dorsal half of the lumbar cord was dissected. Tissue samples were weighed and immediately frozen in liquid nitrogen and stored at -70°C until assay.

Gene expression study

mRNA analysis

Total cellular RNAs were isolated from the dorsal half of the lumbar spinal cord by a modification of the guanidine isothiocyanate-phenol-chloroform method using RNX$^+$ reagent (Ausubel FM, 2002). A semi-quantitative PCR method was used (Marone *et al.*, 2001). Briefly, the RT-PCR reaction was performed using Oligo-dT primer and M-MuLV reverse transcriptase, based on the manufacturer's protocol (Fermentas GMBH, Germany). The reactions were incubated at 42 °C for 60 min and then inactivated at 70 °C for 10 min. Three separate PCR reactions were used for studying gene expression in the samples obtained from each rat. Each PCR reaction was carried out using selective forward and reverse primers for β-actin (as an internal standard) and RGS4 protein. Based on the results obtained from these experiments, 25 cycles of PCR amplification were used for analyzing all samples. PCR products were subsequently analyzed on 1.5% agarose LMMP (Roche, Germany) gel and bands were quantified by densitometry using Lab Works analyzing software (UVP, UK). The possibility of the presence of contaminating genomic DNA was ruled out by using the yield of reverse transcriptase-minus (RT$^-$) reaction, instead of cDNA template which caused no DNA amplification.

Protein analysis

The dissected spinal tissues were homogenized in ice-cold buffer containing 10mM Tris–HCl (pH 7.4), 1mM

EDTA, 0.1% SDS, 0.1% Na-deoxycholate, 1% NP-40 with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml of leupeptin, 10 µg/ml of aprotinin) and 1 mM sodium orthovanadate. The homogenate was centrifuged at 14000 rpm for 15 min at 4˚C. Protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Muenchen, Germany). Equal amounts of protein (40 µg) were resolved electrophoretically on a 9% SDS-PAGE gel and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare Bio-Sciences Corp. NJ, USA). After blocking (overnight at 4˚C) with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (blocking buffer, TBS-T, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20), the membranes were probed with primary antibody [RGS4 (H-40): sc-98933, Santa Cruz, USA, 1:1000] for three hours at room temperature. After washing, the blots were incubated for 60 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (sc-2004, Santa Cruz, USA, 1:10000). All antibodies were diluted in blocking buffer. The antibody-antigen complexes were detected using the ECL system and exposed to Lumi-Film chemiluminescent detection film (Roch, Germany). Lab Work analyzing software (UVP, UK) was used to analyze the intensity of the expression. β-actin immunoblotting (antibody from Cell Signaling Technology, INC. Beverly, MA, USA; 1:1000) was used to control for loading.

Statistical analysis

The results are expressed as mean ± SEM. The difference in %MPE (antinociception) between groups over the time course of study was determined by one- or two-way analysis of variance (ANOVA) followed by the Newman-Keuls test The averages of RGS4/β-actin ratio for different groups were compared using ANOVA followed by the Newman-Keuls test. $P < 0.05$ was considered significant.

Results

The effect of adrenalectomy and sham operation on the levels of plasma corticosterone

The level of plasma corticosterone were significantly reduced (to undetectable levels) in adrenalectomized animals compared with sham- operated animals (263.4±35.6 ng/ml). It means that the adrenal glands had been completely removed and there were no ectopic sources of corticosterone secretion.

The effect of morphine on nociceptive threshold in the presence or absence of adrenal glands

Fig. 1, depicts the analgesic effects of 5 µg (i.t.) morphine on the tail-flick test in sham-operated and adrenalectomized animals. Administration of saline had no anti-nociceptive activity in experimental groups. Morphine produced an analgesic response in sham-operated animals at 15 and 30 min after injection [$F_{(6,42)}=19.882, P=0.0001$].

In adrenalectomized rats, administration of morphine (5 µg i.t.) could affect nociceptive threshold and induce analgesic response peaked 30 min after injection and persisting for up to 120 min [$F_{(6,41)}=32.523, P=0.0001$]. The analgesic property of morphine in adrenalectomized animals was greater than sham-operated animals at 15, 30 and 60 min after injection ($P<0.01$, $P<0.05$ and $P<0.001$ respectively).

The effect of adrenalectomy and morphine on the levels of RGS4 mRNA

Removal of adrenals had no effect on the mRNA levels of RGS4 in the dorsal portion of lumbar spinal cord as compared to sham-operated and control animals. Adrenalectomized animals that received morphine showed a significant increase ($P<0.05$) in RGS4 mRNA levels (Fig. 2). There were no changes in the mRNA levels of RGS4 following the injection of 5 µg morphine in sham-operated rats ($P>0.05$).

The effect of adrenalectomy and morphine on the levels of RGS4 protein

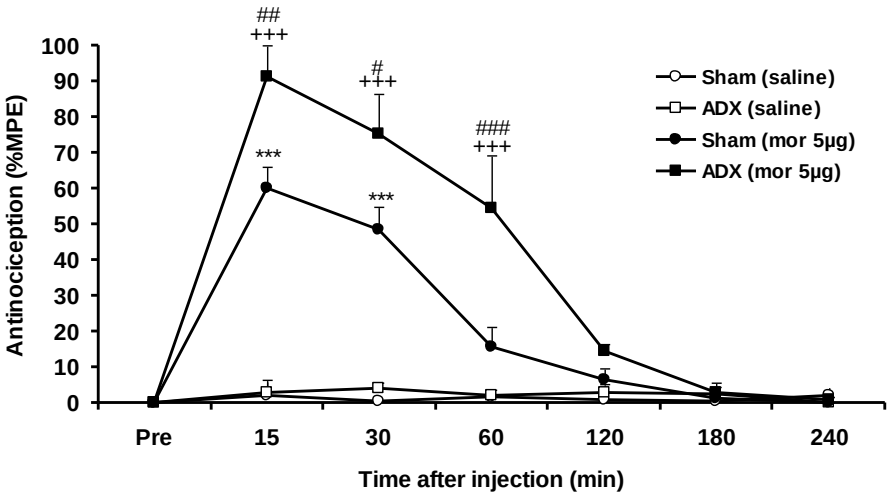

Fig. 1: The effect of intrathecal injection of saline or 5 µg morphine on the nociceptive threshold in sham-operated and adrenalectomized (ADX) rats. Values represent mean ± SEM (n = 6–8). +++P < 0.001 significantly different versus saline-treated ADX group at the same time. ***P < 0.001 versus saline-treated sham group at the same time. #P < 0.05, ##P < 0.01 and ###P < 0.001 significantly different versus morphine-treated sham animal at the same time

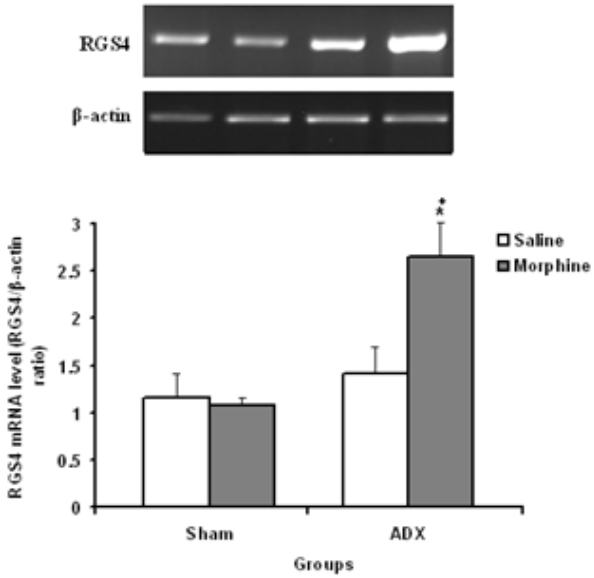

Fig 2: The effect of intrathecal morphine on the levels of RGS4 mRNA in dorsal portion of lumbar spinal cord in sham-operated (Sham) and adrenalectomized (ADX) animals. Each value in the graph represents the mean ± SEM band density ratio for each group (n = 6–7). Beta-actin was used as an internal control. The density of RGS4 band in the dorsal portion of lumbar spinal cord increased significantly in the morphine-treated ADX group.
*P < 0.05 versus saline-treated ADX group. +P < 0.05 versus morphine-treated sham group.

Immunoblot analysis of the dorsal portion of the lumbar spinal cord extract probed with anti-RGS4 revealed bands with predicted sizes. Our results showed a significant increase of RGS4 protein in morphine-treated ADX animalscompared with to saline-treated adrenalectomized rats (P<0.01). Injection of morphine had no effect on RGS4 protein level in sham-operated rats (Fig. 3).

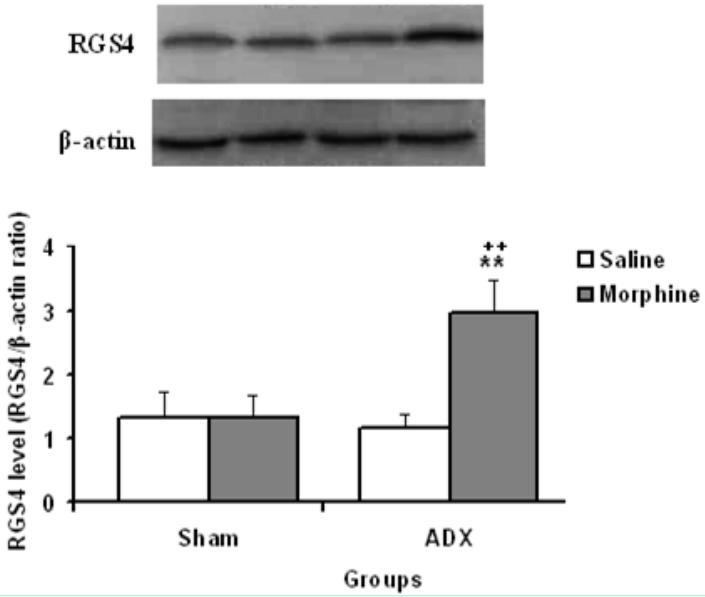

Fig 3: The average band density ratio of RGS4 protein in sham-operated (Sham) and adrenalectomized (ADX) animals that received saline or morphine (5 µg). Each value in the graph represents the mean ± SEM band density ratio for each group (n = 6–7). Beta-actin was used as an internal control. The density of RGS4 protein in the dorsal portion of lumbar spinal cord increased significantly in the morphine-treated ADX group. **P < 0.01 versus saline-treated ADX group. ++P < 0.01 versus morphine-treated sham group.

Discussion

Although it has been shown that adrenalectomy potentiates the analgesic effect of morphine, the exact mechanisms underlying this potentiation/sensitization- especially the role of changes in RGS proteins gene expression- have not yet been identified. Our results show that intrathecal injection 5 µg of morphine elicited greater analgesic effects in ADX than in sham-operated animals.

It is well known that corticosterone can reduce morphine analgesia (Takenaka *et al.*, 2003). Therefore, it follows that in ADX animals (no corticosterone) morphine is able to induce grater analgesic properties.

We previously showed that one of the possible mechanisms for morphine sensitization in adrenalectomized animals is, at least in part, is eliminating the source of corticosterone (Esmaeili-Mahani *et al.*, 2007, Esmaeili-Mahani *et al.*, 2008).

In addition, it is well documented that beta-endorphin plasma levels increase in adrenalectomized rats (Bogdanov and Yarushkina, 2004, Vissers *et al.*, 2004). Therefore, we can speculate that the observed increase in morphine analgesia in adrenalectomized rats is also the result of a synergistic mechanism involving morphine and beta-endorphin.

Our results show that adrenalectomy (after 5 days) had no effect on RGS4 gene expression while, ADX animals that received morphine had increased level of RGS4 gene expression (Figs 2 and 3).

Traditionally, it has been documented that opioids inhibit adenylate / adenylyl cyclase and modulate ion channels in different cell types and that these acute effects are mediated via $G_{\alpha i/o}$ and associated $G_{\beta\gamma}$ proteins (Williams *et al.*, 2001, Al-Hasani and Bruchas, 2011).

RGSs modulate µ-opioid receptor signaling and control the effectiveness of opioids (T Lamberts and R Traynor, 2013). The changes in the expression of RGS4 proteins may reflect altered functioning of the opioid signaling cascade. It seems elevated level of RGS4 in morphine-treated ADX animals terminates the amplified am opioid signaling and analgesia. However, further studies are needed \to clarify its exact mechanisms.

Ni and colleagues reported that corticosterone

decreases RGS4 mRNA levels in the rat's hypothalamus (Ni *et al.*, 1999). The molecular mechanisms of RGS4 mRNA regulation seem to be attributable, at least in part, to opposite changes in mRNA stability. The differential regulation of RGS4 expression in the brain nucleiand in key relays/pathways of the hypothalamic-pituitary-adrenal axis could contribute to the brain's region-specific and long-term adaptations to stress.

In rodents, opioids are produced in response an stressful experience initiating a neuroendocrine responses, which in turn promote sthe release of glucocorticoid hormones. Glucocorticoids send feedback to the brain that prevents excessive opioid responses to subsequent challenges. Here, it seems that the increased in RGS4 gene expression in morphine-treated ADX animals prevent excessive opioid responses and further reduce toxicity.

it seems that the changes in RGS4 gene expression following opioids provide an additional mechanism by which adrenalectomy affects morphine analgesia.

Acknowledgments

This work was supported by funds from Neuroscience Research Center, Shahid Beheshti Medical University.

Conflict of interest

All authors declared that there is no conflict of interest.

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