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The Intracerebroventricular Administration of W-7, A Calmodulin Inhibitor, Attenuate the Development of Morphine Tolerance in Rats

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Abstract: Problem statement: The present study was performed to determine the effect of Intracerebroventricular (ICV) administration of W-7, a specific calmodulin inhibitor, on the analgesic effect and development of tolerance to antinociceptive effect of acute and chronic morphine administration respectively. **Approach:** This study was carried out on male wistar rats, weighing 200-250 g. For acute experimental protocol, Morphine was injected intraperitonealy in a single dose (5 mg kg^{-1}). For chronic experimental protocol, Morphine was administered daily (15 mg kg^{-1} for 8 days). The threshold to thermal nociceptive stimuli was measured by tail-flick test. In acute and chronic experiments, W-7 (0.25, 0.5 and 1 µmol/rat) was injected through ICV at different paradigms. Maximal Possible Effect percentage (MPE%) was considered as analgesia index. **Results:** Our result showed that W-7 (0.25, 0.5 and 1 µmol/rat) injections before acute morphine administration significantly reduced the analgesic effect of morphine compared with morphine treated group (all $p<0.05$). Chronic morphine exposure induced tolerance to its antinociceptive effect and administration of W-7 (0.5 and 1 µmol/rat) decreased the development of tolerance to it. **Conclusion:** In conclusion these data showed that acute administration of W-7 reduced the analgesics effect of morphine and chronic injection of W-7 inhibited the development of morphine tolerance which indicates that calmodulin and its dependent pathways may play a role in the morphine tolerance processes.

Key words: Calmodulin inhibitor (W-7), morphine, tolerance, tail-flick test, pain, rat

INTRODUCTION

Tolerance is indicated by a decreased efficacy of the drug after chronic use, leading to the requirement for a higher dose to get the desired effect (Hamdy *et al*., 2004). Accumulating evidences have revealed that prolong exposure to opiates such as morphine and heroin can significantly alter brain function, leading to the development of tolerance to opiates (Eisch *et al*., 2000; Kelley *et al*., 2000; Nestler and Aghajanian, 1997). However, the underlying central mechanisms for opiate tolerance are not entirely understood.

Calcium ions are thought to play an important role in many cellular processes. Interaction of calcium with several calcium-binding proteins, the main one of which, Calmodulin (Gnegy, 1993), is a critical step for activating or deactivating of different cellular pathways such as enzyme activation, plasma Ca^{2+} pump regulation and protein phosphorylation and dephosphorylation cascades (Cheung, 1982; Dinsmore and Sloboda, 1988; Ye *et al*., 2004). A number of studies indicate that opioid tolerance is associated with alteration in the calcium homeostasis and free intracellular Ca^{2+} concentration is higher in the brain (Diaz *et al*., 1995; Welch and Olson, 1991). Increased in cytosolic calcium concentration activate several intracellular enzymes including protein kinases (Wroblewski and Danysz, 1989). Protein kinases such as Ca^{2+}/Calmodulin-dependent protein Kinases (CaMK) have been reported to phosphorylate opioid receptor, leading to receptor desensitization (Koch *et al*., 1997; Mestek *et al*., 1995); a phenomenon which plays a

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critical role in opioid tolerance (Breivogel *et al*., 1997; Yabaluri and Medzihradsky, 1997). If this were to be the case, an essential process for opioid receptor phosphorylation and desensitization could be the activation of the Ca^{2+}/calmodulin complex. A previous report which described the inhibition of morphine tolerance by intraventricular application of CaMK II inhibitor favors this possibility (Tang *et al*., 2006). However, the effect of specific calmodulin inhibitors such as W-7 on the antinociceptive effect and tolerance to morphine is not determined yet. So the major aim of the present study was to study the effect of supraspinal inhibition of calmodulin by its specific inhibitor, W-7, on the analgesic effects and development of tolerance to morphine in male rats.

MATERIALS AND METHODS

Animals: Male Wistar rats, weighing 200-250 g were used in this study. Subjects were housed four per cage in a temperature-controlled room at 25±1°C on 12:12 h light-dark cycle with lights on at 07:00 am. The experiments were carried out during the light phase of the cycle. The animals had free access to commercial food for rodents (Teklad Rodent Diet, Iran) and drinking water. Rats were divided randomly into several experimental groups, each comprising 7-9 animals. All of the procedures were in accordance with guidelines for caring and using of laboratory animals in Neuroscience Research Center of Kerman University of Medical Sciences and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs: Morphine hydrochloride (Temad Co, Iran) was dissolved in saline. W-7 (N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide) was purchased from Alexis Company; USA and was dissolved in 100% DMSO as vehicle (Dimethyl Sulfoxide, Merck Company, Germany).

Surgical procedure: Each rat was anaesthetized by an intraperitoneal injection (i.p) of 60 mg kg^{-1} ketamine and 5 mg kg^{-1} xylazine. They were then placed in the stereotaxic instrument (Stoelting Company, USA). A single incision was made on the midline of the scalp. Once the area had been prepped, a stainless steel 21 gauge cannula was placed in the lateral ventricle according to the Paxinos and Watson (1986) atlas. The coordinates were 1.0 mm posterior to the bregma and 1.5 mm lateral of the midline and 3.0 mm inferior of the duramater. The cannula was kept in place on the skull by dental cement and bone screws. The rats were allowed a 7days recovery period after the surgery for implantation of the cannula. The injections were done, using a 23 gauge stainless steel cannula attached to polyethylene tubing and 10 µL Hamilton syringe (Hamilton Inc., Reno, NV).

After the completion of experiments, the animals were sacrificed by an overdosage of ketamine and xylazine combination and then dye was injected through the cannulas to mark the ventricular space. The brain sections were visually examined to verify that the tip of the cannula was located in the lateral cerebral ventricle.

Tail-flick test: Antinociception was assessed by tail-flick test. Radiant heat was applied at 5-8 cm from the tip of the tail using a tail-flick apparatus (PANLAB 7160, Spain). Tail Flick Latency (TFL) was measured as the time of the beam exposure to the withdrawal time of the tail. The mean of three consecutive TFL was measured at 1 min intervals before drug or solvent administration (basal latency) and then similar TFL was measured at specific times after drug or solvent administration, (experimental latencies) (Mahani *et al*., 2005a). The intensity of radiant heat was adjusted to establish the basal latency of 3-5 sec. To avoid tissue damage, a cut-off time of 15 sec was set. Trials were automatically terminated if a response did not occur within 15 sec (Doi *et al*., 1988). Maximal Possible Effect percentage (MPE%) was considered as analgesia index which was calculated by the following formula:

$$\text{MPE\%} = \frac{\text{Experimental latency-basal latency}}{\text{15-basal latency}} \times 100$$

Effect of W-7 on morphine-induced analgesia: All rats underwent surgical procedure and a cannula was placed in the lateral ventricle of rats' brain. The animals were tested 7 days after Cannulation. On the experimental day, a single dose of morphine (5 mg kg^{-1}) was administered intraperitonealy. In control group, saline was injected i.p. Tail-Flick Latency (TFL) was measured before and 30, 60, 90 and 120 min after morphine administration (Mahani *et al*., 2005b). In experimental groups, W-7 (0.25, 0.5 and 1 µmol/rat) was injected ICV 10 min before the intraperitoneal administration of morphine. DMSO (vehicle) was administered ICV in the same schedule as control group. All of ICV injections were performed with a micro injector in an amount of 10 µL at a constant rate within 1 min.

Experimental groups:

1. Rats that received a single dose of morphine (5 mg kg^{-1}/i.p) and DMSO Intracerebroventricularly (ICV)
2. Rats that received saline intraperitoneally and DMSO was injected by ICV
3. Rats that received maximum dose of W-7 (1 µmol/rat /ICV) 10 min before intraperitoneal saline injection

4-6. Experimental treated rats that received W-7 (0.25, 0.5 and 1 µmol/rat/ICV) 10 min before morphine (5 mg kg^{-1}/i.p) injection

Morphine tolerance: All rats underwent surgical procedure and were tested 7 days after cannulation. Tolerance to morphine was induced by daily morphine injections (15 mg kg^{-1}/i.p) for 8 days. Tail-Flick Latency (TFL) was measured both before and 30 min after morphine administration in days 1, 3, 5 and 8 (Mahani *et al*., 2005a). W-7 (0.25, 0.5 and 1 µmol/rat) was administered Intracerebroventricularly (ICV) 10 min before morphine administration but in days that tail-flick test was performed (1, 3, 5 and 8), W-7 was injected after doing the tail-flick test. All of ICV injections were performed with a micro injector in an amount of 10 µL at a constant rate within 1 min.

Experimental group:

1. Rats that received morphine (15 mg kg^{-1}/i.p) for 8 days
2. Rats that received saline i.p for 8 days
3. Rats that received saline i.p and DMSO Intracerebroventricularly (ICV) for 8 days
4. Rats that received morphine (15 mg kg^{-1}/i.p) and DMSO (ICV) for 8 days
5. Rats that received saline i.p and maximum dose of W-7 (1 µmol/rat /ICV) for 8 days

6-8. Experimental treated rats that received ICV injection of W-7 (0.25, 0.5 and 1 µmol/rat) and morphine (15 mg kg^{-1}/i.p) for 8 days

Statistical analysis: The results are expressed as mean ± SEM of at least 6 rats in each group. The difference in MPE% (antinociception) between groups over the time course of study was determined by two-way Analysis Of Variance (ANOVA), followed by the Tukey post hoc test with 5% level of significance ($p<0.05$). For accessing tail flick latencies or MPE% in one group for several days we used repeated measure test. One sample t-test was used to determine the cause of difference and Bonferroni adjustment was used for reducing error.

RESULTS

Effect of ICV injection of W-7 on morphine-induced analgesia: We first tested whether supraspinal inhibition of calmodulin activity by its specific inhibitor (W-7), interfere with the analgesic effect of morphine. W-7 (0.25, 0.5 and 1 µmol/rat) or its vehicle (DMSO) was injected ICV 10 min before the intraperitoneal administration of a single dose of morphine (5 mg kg^{-1}). Tail-flick test was performed before and 30, 60, 90 and 120 min after morphine administration. Following morphine administration, an analgesic response (about 70%MPE) produced since 30 min and lasted for about 120 min. Administration of W-7 (1 µmol/rat) by itself produced slight antinociceptive effect (18.5%MPE). However, the injection of W-7 at 0.25, 0.5 or 1 µmol/rat prior to morphine administration caused a decrease in the peak response to morphine to nearly 40%MPE which is significantly lower than morphine treated group ($p<0.05$). The analgesic effect of W-7+ morphine also last shorter (about 90 min) than that of DMSO + morphine-treated group (about 120 min) (Fig. 1).

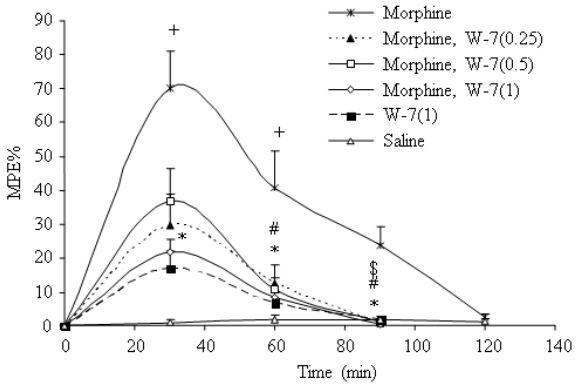

Fig. 1: The effect of ICV injection of W-7 (0.25, 0.5 and 1 µmol/rat) on the antinociceptive effect of morphine (5 mg kg^{-1}/i.p) in rats. W-7 was injected 10 min prior to morphine injection and tail flick response was measured before and 30, 60, 90 and 120 min after morphine administration. W-7 and DMSO were injected intracerebroventricularly and morphine and saline were injected intraperitonealy. Values represent mean ± SEM of at least 6 rats in each group. (+) $p<0.05$ compared to Saline and W-7 treated groups; (*) $p<0.05$ Morphine+W-7 (1 µmol/rat) with Morphine; (#) $p<0.05$ Morphine+W-7 (0.5) with Morphine at 60 and 90 min; ($) $p<0.05$ Morphine compared to W-7 treated groups

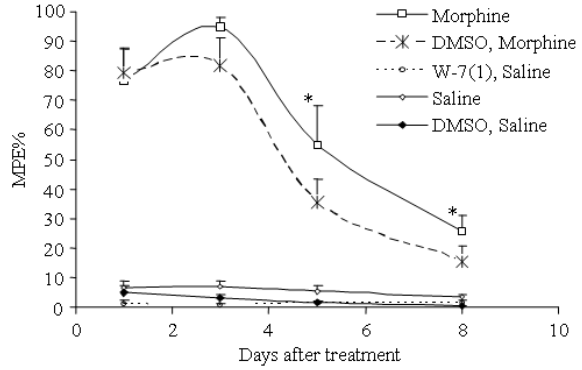

Fig. 2: Tolerance to analgesic effects of morphine induced by chronic morphine administration. Morphine (15 mg kg^{-1}/i.p) administered for 8 days. W-7 and DMSO were injected intracerebroventricularly, 10 min prior to saline or morphine injection except on days which tail flick test performed. Tail flick response was measured before and 30 min after morphine administration on days 1, 3, 5 and 8. Values represent mean ± SEM of at least 6 rats in each group. Chronic morphine administration (15 mg kg^{-1} 8 $days^{-1}$/i.p) induced tolerance to its antinociceptive effect; MPE% on the fifth and eighth days were significantly reduced compared with MPE% on the first day, in the morphine group and DMSO+ morphine group ($p<0.05$). ICV injection of W-7 (1 µmol/rat) with i.p administration of saline did not affect MPE % compared with saline- treated group ($p>0.05$)

Effect of ICV injection of W-7 on the development of tolerance to analgesic effect of morphine: In the second part of the experiment, we examined whether chronic supraspinal inhibition of calmodulin by W-7 could affect the development of morphine tolerance. As it is shown in Fig. 2, chronic administration of morphine alone for 8 days induced tolerance to its antinociceptive effect; the MPE% on day 5(45%) and day 8 (30%) was significantly reduced compared with MPE% on the first day (80%) in this group (all $p<0.05$). The tolerance to the analgesic effect of morphine treatment alone was not significantly different from that of DMSO + morphine - treated group. ICV injection of W-7 (1 µmol/rat) with i.p administration of saline did not affect the Maximal Possible Effect percentage (MPE%) compared with saline- treated group ($p>0.05$) (Fig. 2).

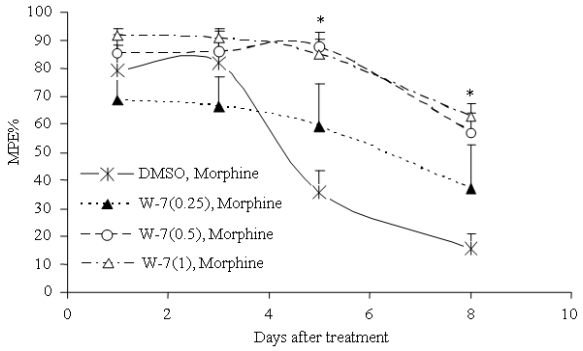

Fig. 3: The effect of ICV administration of W-7 (0.25, 0.5 and 1 µmol/rat) on the development of tolerance to analgesic effect of morphine (15 mg kg^{-1} 8 $days^{-1}$/ i.p) in rats. Other notations are the same as in Fig. 2. (*) $p<0.05$ W-7 (1 µmol/rat) + Morphine and W-7 (0.5 µmol/rat) + morphine as compared to morphine + DMSO-treated rats

The MPE on day 8 in morphine + W-7 (0.25 micromol/rat) group was about 35% which slightly increased in comparison with morphine + W-7 Vehicle-treated rats. However, this increase was not statistically significant ($p>0.05$). However, MPE% on day 5 and 8 in rats receiving morphine + W-7 (0.5 micromol/rat) was about 90 and 62% respectively which showed a significant increase in morphine analgesic response compared with that of morphine + DMSO-treated rats (both $p<0.05$). MPE% on 5th and 8th day in group treated with morphine + W-7 (1 µmol/rat) was 85 and 65% which significantly increased in comparison with rats treated with morphine + DMSO ($p<0.05$). In fact, MPE% on day 5 and 8 in rats receiving W-7 (0.5 and 1 µmol/rat) did not show any significant difference as compared with MPE% on first day in their own groups. It means that chronic ICV administration of W-7 in dose 0.5 and 1 µmol/rat could effectively inhibited the development of morphine tolerance (Fig. 3).

DISCUSSION

The present study was designed to evaluate the effect of acute and chronic calmodulin inhibition by microinjection of its specific inhibitor "W-7" into the lateral ventricle of the rat's brain on the analgesic effect and development of morphine tolerance. Our results showed that acute ICV administration of W-7 before morphine injection inhibited analgesic effects of morphine. Our results are consistent with recent studies indicating that calmodulin is involved in G protein signal pathway initiated by G protein-coupled receptors

such as opioid receptors (Ye *et al*., 2004; Wang *et al*., 1999). Wang *et al*. (1999) has been shown that calmodulin interact directly with the mu opioid receptor at the third intracellular loop and may play as an independent second messenger molecule that is released upon receptor stimulation. Also, it is reported that morphine increases CaM contents in mouse brain (Niu *et al*., 2000). Therefore, it seems logic that inhibition of calmodulin activity could attenuate the analgesic effects of morphine. Our result showed that *in vivo* administration of calmodulin inhibitor could inhibit the morphine analgesic effects and support our hypnosis.

In addition, we found that chronic ICV administration of W-7 strongly attenuated the development of tolerance to chronic morphine exposure, as evidenced by a significant increased in the Maximal Possible Effect (MPE %) on day 5 and 8 in comparison with morphine + W-7 Vehicle-treated group.

Since on days that we measured the antinociceptive effect of morphine, W-7 was injected after performing tail-flick test, it is unlikely that the inhibition of morphine tolerance by W-7 was due to the acute interaction between W-7 and morphine.

It has been shown that calmodulin content and location changed during chronic morphine administration. It is reported that chronic morphine treatment increased calmodulin contents in the rat's brain (Nehmad *et al*., 1982). Moreover, the intracellular calcium elevation by opioid receptor stimulation (Diaz, *et al*., 1995; Welch and Olson, 1991) leads to calmodulin dissociation from cell membrane (Wang *et al*., 1999). The increase in cytosolic calmodulin prompts the translocation of calmodulin into the nucleus (Wang *et al*., 2000) which regulates the gene expression by morphine (Niu *et al*., 2000; Deisseroth *et al*., 1998). However, it is unclear whether these finding may play a role in the development of morphine tolerance or not.

In addition, several lines of evidence indicated that calmodulin dependent pathways such as Ca^{2+}/Calmodulin Kinase II (CaMKII) activation may involve in the morphine tolerance (Liang *et al*., 2004). It has been reported that intraventricular application of CaMK II inhibitor attenuated morphine tolerance (Tang *et al*., 2006). However, an important characteristic of CaMK II is its autophosphorylation which enables this kinase to phosphorylate substrates in a Ca^{2+}/calmodulin-independent manner and thus prolongs the duration of its effect (Lou *et al*., 1999). Our results showed that in rat, administration of calmodulin inhibitor, W-7 inhibited the development of morphine tolerance. So, it could be possible that calmodulin activity is important in the mechanisms underlying morphine tolerance.

CONCLUSION

In summary, our results indicated that chronic inhibition of calmodulin activity by ICV injection of its specific inhibitor, W-7, attenuated morphine tolerance but acute W-7 administration decreased the antinociceptive effects of morphine. Further studies need to be carried out to better understand the underlying mechanism (s) of these findings and determine the relevance of them in human.

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