Original Article

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The Role of BK Potassium Channels in Analgesia Produced by Alpha-2 Adrenergic Receptors

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ABSTRACT

BACKGROUND AND OBJECTIVE: Millions of people suffer from pain worldwide, and annually, great economic costs are imposed on societies for pain relief. Analgesics such as alpha-2 adrenergic receptor agonists, which have low risk of complications, can be effective in assuaging pain and reducing costs. According to former studies, potassium channels play an important role in the analgesic mechanism of these receptors. This study aimed to determine the role of BK potassium channels in analgesia induced by alpha-2 adrenergic receptors.

METHODS: This study was performed on 56 male Wistar rats weighing 250-300 g that were divided into seven groups of eight rats. We administered 0.7 mg/kg intraperitoneal (IP) injection of clonidine, 1 mg/kg IP injection of yohimbine, and 5 mg/kg intracerebroventricular (ICV) injection of yohimbine. Iberiotoxin at a dose of 100 nm was also injected ICV. Normal saline and DMSO were applied as solvents. Pain severity was evaluated using formalin test at a concentration of 2%.

FINDINGS: The chronic pain induced by formalin injection was relieved by IP injection of 0.7 mg/kg clonidine. Moreover, 5 µg/kg and 1 µg/kg ICV administration of yohimbine with mean chronic pain scores of 2.29±0.13 and 2.09±0.07, respectively, could significantly inhibit analgesic effect of clonidine with mean chronic pain score of 1.55±0.14 ($p<0.001$). ICV injection of iberiotoxin with mean chronic pain score of 2.33±0.16 at a dose of 100 nm significantly diminished analgesic effects of clonidine.

CONCLUSION: Alpha-2 adrenergic receptor agonists could induce analgesia in the animals, and the antagonist of this receptor inhibited the analgesic effect of agonists of these receptors. BK channel inhibition prevented analgesic effect of adrenergic receptor agonists, as well.

KEY WORDS: *Alpha-2 adrenergic receptor, BK potassium channel, Formalin test, Pain.*

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Introduction

Alpha-2 adrenergic receptors are a member of the large family of adrenergic receptors, which act via binding to G inhibitory (Gi) proteins. Alpha-2 adrenergic receptors have three subtypes namely, α_{2A}, α_{2B}، and α_{2C} (1). The subtypes of alpha-2 adrenergic receptor are widely distributed in the central and peripheral nervous systems (2-4). In general, alpha-2 adrenergic receptor agonists are used for general anesthesia, critical care, treatment of hypertension and glaucoma, and reduction of morphine withdrawal symptoms (5, 6).

Agonists of alpha-2 adrenergic receptors block nerve fibers, especially type C. In addition, they exert their analgesic effect through opioid and adenosine receptors (7). These receptors influence ionic conductivity by Gi stimulation or reduction of intracellular cAMP level (6, 7). According to various studies, clonidine, as an alpha-2-adrenergic receptor agonist, can alleviate pain (8-10).

Potassium channel plays a major role in the analgesic mechanism of the alpha-2-adrenergic receptors (11), one of the most important of which is calcium-activated potassium channel (KCa). Based on conductance level, these channels are categorized as big (BK), intermediate (IK), and small (SK) conductance calcium-activated potassium channels (12). According to previous studies, BK potassium channels are associated with pain (13-15). It was also suggested that blockage of these channels in the spinal cord can cause neuropathic pain in rats (16). In a former study, it was reported that activation of potassium channels could promote analgesic effect of clonidine (17).

Opening of potassium channels leads to outward potassium currents and in turn, hyperpolarization of cell membrane potential, which can also happen by alpha-2 adrenergic receptors on the potassium channels (18, 19). Understanding the role of potassium channels in the etiology of pain can provide deeper insight into pain treatment. Therefore, the present study aimed to determine the role of BK potassium channels in the analgesia caused by alpha-2 adrenergic receptor agonists using formalin test.

Method

This experimental study was performed on 56 adult, male Wistar rats weighing 250-300 g, purchased from North Branch of Pasteur Institute, Iran. The animals were kept in separate cages at 20-22°C on a 12:12-h light:dark cycle. The animals had free access to food and water with minimum stress. Moral considerations for minimizing pain in the animals were taken into account based on the Medical Ethics Committee principles and Laboratory Animal Management of Golestan University of Medical Sciences, Gorgan, Iran.

In this study, the animals were randomly divided into seven groups of eight as follows: A) control group: the rats receiving 1 m/kg intraperitoneal (IP) injection of normal saline; B) clonidine group (alpha-2 adrenergic receptor agonist): the rats receiving IP injection of clonidine (Sigma) at a dose of 0.7 mg/kg (volume: 1 mg/kg) (20); C) yohimbine group (alpha-2 adrenergic receptor antagonist): IP+clonidine, IP injection of yohimbine (Tocris) at a dose of 1 mg/kg (21) and IP injection of clonidine five minutes later; D) DMSO group: intracerebroventricular (ICV) injections of 5 µl DMSO; E) ICV injection of yohimbine+ clonidine group: ICV injection of yohimbine at a dose of 5 µg/kg (21) and IP injection of clonidine five minutes later; F) iberiotoxin group (specific inhibitor of BK potassium channels): ICV injection of iberiotoxin (Tocris) at a dose of 100 nm (22); G) iberiotoxin+clonidine group: ICV injection of iberiotoxin and IP injection of clonidine five minutes later.

The surgical procedure and cannulation of the lateral ventricle of the brain: The rats were anesthetized by IP injection of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) combination. After anesthetization, the rats were placed in a stereotaxic apparatus with the head fixed. A flat-plate heater was placed under the animal's body, and their head was shaved. The animals' eyes were kept wet during the surgery. The surgical field was disinfected by betadine. With respect to sterile principles, a 2-cm incision was made in the scalp using surgical blade number 22. Bleeding was prevented using cotton

swabs, and the protective layer of skull was slowly pushed aside so that the skull, bregma, and lambda regions as well as the brain fissures were obvious. Based on Rat Brain Atlas of Paxinos, the exact cannulation point was marked (at coordinates: AP=-0.8, ML=1.5, and DV=-4.2) on the skin using stereotaxic apparetus. Using a dental drill, a hole was drilled through the skull (in the frontal bone) in the proximity of the guide cannula, and another hole was drilled in the occipital bone, and a dental screw was implanted in each of them. Thereafter, a hole was drilled in the marked area to insert the guide. A guide cannula (G 22) was unilaterally entered into the right lateral ventricle of the brain with a depth of 3 mm and was linked to the screws by dental cement; then, the rats were placed in separate cages.

The animals were expected to recover seven days after the surgery. ICV injection was performed by Hamilton syringe (volume 100 µl). In all the groups, chronic pain was induced by formalin test. Formalin test is a valuable model for the measurement of chronic pain induced by a chemical stimulus (23). Formalin test can be divided into two phases, each of which demonstrating a different aspect of pain. The first phase or acute phase of pain occurs immediately after drug injection (0-7 minutes). This pain arises from stimulation of peripheral receptors and tissue destruction caused by the activation of C-type nerve fibers against the environmental stimuli.

The interphase stage begins at minutes 8-14 and the pain behaviors diminish or disappear at this stage. The second or delayed phase occurs at minutes 15-60. In this phase, chronic pain, which is caused by stimulation of peripheral nerves, increased sensitivity of central neurons, as well as functional and histological alterations in the posterior grey column, is mild (23). To perform formalin test, 50 µl formalin with 2% concentration was subcutaneously injected to the left front paw of the rats, using needle number 31. The rats were placed in a formalin chamber (a transparent plastic container with dimensions of 40 × 40 × 40 cm) immediately after the injection. A mirror was embedded on the floor of the chamber at an angle of 45 degrees. Observable behaviors associated with pain were evaluated during 0-60 minutes, and scores were recorded every fifteen seconds.

Under normal conditions, the animal puts its paws on the ground; however, behaviors associated with pain were observed immediately after formalin injection. Score 0 was given to the animals putting their paws completely on the floor. Score 1 was assigned when the animals putting their paw lightly on the floor. Score 2 was given to the animals that completely elevated their paw from the floor. Finally, score 3 was given to animals that began to lick and bite the injected paw. Afterwards, the mean scores at one and three minutes were recorded. All the scores were given solely by one person (24).

Formalin test was conducted on a daily basis, from 9 am to 12 pm. The test was performed for two weeks in the first, second, and third groups, while it was carried out for one week in the fourth and fifth groups. In addition, formalin test was performed for one week in the sixth and seventh groups with respect to timing of surgery and recovery in both groups. The week was chosen based on recovery from cannulation by stereotactic apparatus. This study focused on the second phase of pain (chronic pain); therefore, the mean pain scores (at 15 to 60 minutes) were compared in different groups. All the rats were sacrificed by deep anesthesia with chloroform two hours following the completion of the test. Then, methylene blue 1% (50 µl) was injected via guide cannula and the rats' brain was removed from the skull and was placed in 4% paraformaldehyde solution. The insertion site of cannula to the right lateral ventricle of the brain was observed by loupe microscope after one week. If the cannula location was incorrect, the animal was excluded from the study (24). Finally, data were recorded in Graph-Pad PRISM 6.01 software. One-way ANOVA and Fisher's LSD post-hoc test were run using SPSS, version and $p<0.05$ was considered statistically significant

Results

Pain behavior induced by formalin injection: Severe pain behaviors secondary to formalin injection were

observed in all the pain phases (phases one and two as well as interphase). Figure 1 demonstrates pain behaviors induced by formalin injection in the control group, 0-60 minutes after the injection. Based on the recorded three-minute mean scores of the formalin test, 20 sections were obtained. Sections one and two represent the first phase, sections three, four, and five represent interphase, and sections 6-20 exhibit the second phase or chronic pain (fig 2).

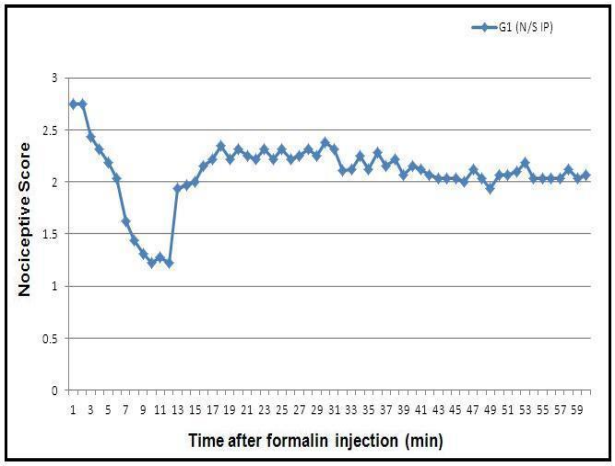

Figure 1. Pain behaviors 60 minutes after formalin injection in the control group (normal saline) based on one-minute mean scores (from minutes 0-7 in the first phase, minutes 8-14 in the interphase in which pain behaviors reduced, from minutes 15-60 in the second or delayed phase)

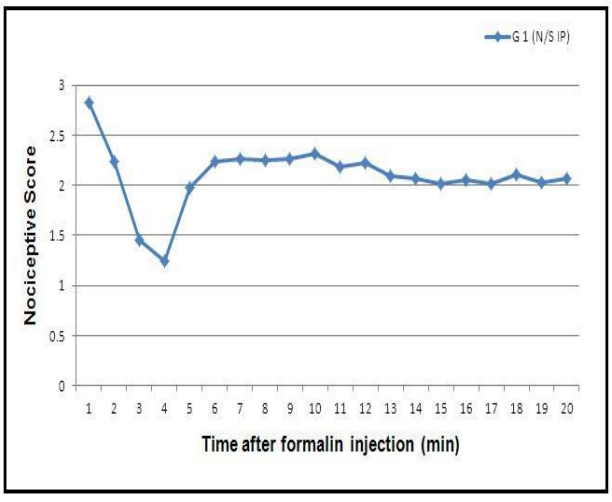

Figure 2. Pain behaviors 60 minutes after after formalin injection in the control group (normal saline) based on three-minute mean scores; 1 and 2 phase one, 3, 4, and 5 interphase, and from minute 6 to 20 the second or delayed phase

The agonist and antagonist effects of alpha-2 adrenergic receptors on the chronic pain caused by formalin injection: clonidine injection alone at a dose of 0.7 mg/kg led to significant reduction of pain behavior mean score in the chronic phase compared to the control group (1.56±0.14 vs. 2.15±0.10; $p<0.001$). Normal saline injection could not significantly affect pain. However, clonidine, as an alpha-2 adrenergic receptor agonist, had analgesic effect on the rats; hence, the rats felt less pain. ICV injection of yohimbine as an alpha-2 adrenergic receptor antagonist at a dose of 1 mg/kg, before clonidine injection, significantly reduced the analgesic effect of clonidine ($p<0.001$; fig 3). The use of DMSO (volume 5 µl) did not affect pain. DMSO group was considered as the control group for ICV injection of yohimbine group, since DMSO is yohimbine solvent. The comparison of DMSO and normal saline groups showed that there was not a significant difference between the two groups. In addition, the comparison of ICV injection of yohimbine group with DMSO group showed that there was not a significant difference between the two groups ($p<0.05$). In contrast, the comparison of ICV injection of yohimbine and clonidine groups demonstrated a significant difference ($p<0.001$). In addition, the obtained results of this study showed a significant difference between the IP and ICV yohimbine injection groups ($p<0.001$). This finding suggests that pain in the IP yohimbine group was more severe than the ICV yohimbine group (fig 3).

The inhibitory effects of BK channels on chronic pain and the analgesia caused by alpha-2 adrenergic receptor agonist based on formalin test: compared to the control group, a significant increase in the second phase (chronic pain) was observed in the ICV injection of iberiotoxin group ($p<0.001$). Severe pain behaviors were observed in the ICV injection of iberiotoxin+clonidine group, which was significantly different from the control group ($p<0.001$). The severity of pain was more in ICV injection of iberiotoxin+clonidine group compared to the control group. The comparison of the groups receiving iberiotoxin and iberiotoxin+clonidine with the clonidine group showed a significant difference

between them, signifying analgesic effect of clonidine. However, the rats in the iberiotoxin and iberiotoxin+clonidine groups showed severe pain behaviors (fig 4).

Also, there was a significant difference between iberiotoxin and iberiotoxin+clonidine groups, that is, the rats in iberiotoxin group had severe pain, while in the iberiotoxin+clonidine group the pain was decreased ($p<0.01$) (fig 4).

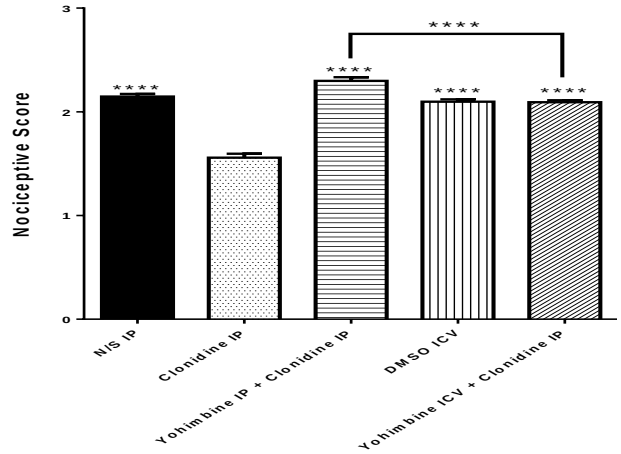

Figure 3. The effect of alpha-2 adrenergic receptors on pain: group 1: 1 ml/kg IP normal saline, group 2: 0.7 mg/kg IP clonidine, group 3: 1 mg/kg IP yohimbine+0.7 mg/kg IP clonidine, group 4: 5 µl ICV DMSO, group 5: ICV injection of 5 µg/kg yohimbine+0.7 mg/kg IP clonidine (n=15). ****Significantly different from the clonidine group ($p<0.001$)

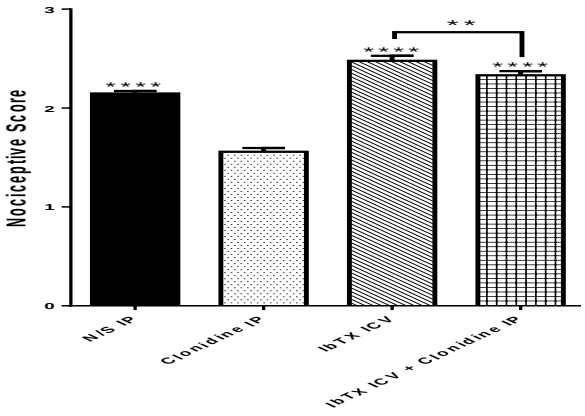

Figure 4. Analgesic mechanisms of alpha-2 adrenergic receptor

The comparison of clonidine (IP at a dose of 0.7 mg/kg), iberiotoxin (ICV at a dose of 100 nm), iberiotoxin (ICV at a dose of 100 nm)+clonidine (IP at a dose of 70 mg/kg), and normal saline (IP injection) groups (n=15) Mean±SEM. ** $p<0.01$, **** $p<0.001$

Discussion

According to our results, BK potassium channels play an important role in the analgesic effects of alpha-2 adrenergic receptor agonists. It was found that clonidine, as an alpha-2 adrenergic receptor agonist, induced analgesic effect in rats, which was in accordance with the findings of similar studies (25). Yohimbine as an alpha-2 adrenergic receptor antagonist inhibited the analgesic effect of clonidine. In agreement with our results, former studies demonstrated that normal saline and DMSO did not have a significant effect on rats' pain after formalin test. The analgesic effect of clonidine alone or in combination with other drugs was demonstrated in previous studies, as well (26-32). Various routes of administration (e.g., intrathecal, epidural, and topical ointment) were employed for clonidine, and its analgesic effect was confirmed in several studies (33-35). Our results indicated that yohimbine, as an alpha-2 adrenergic receptor antagonist, did not have any analgesic effects. After administering yohimbine, alpha-2 adrenergic receptors were inhibited; thus, injection of clonidine following IP and ICV injection of yohimbine failed to show any analgesic effects. These results are consistent with those of other studies (36, 37).

This study showed that the rats in the group receiving IP injection of yohimbine sustained a higher level of pain, compared to the group receiving ICV injection of yohimbine. This finding suggests that alpha-2 adrenergic receptors were inhibited in the group receiving IP injection of yohimbine; therefore, the analgesic effect of clonidine was completely controlled. However, in the group receiving ICV injection of yohimbine, only central alpha-2 adrenergic receptors were antagonized and the analgesic effect of clonidine was partially observed. Thus, less pain was observed in the group receiving ICV injection of yohimbine. According to the present study, BK channel inhibitor iberiotoxin (38) could promote pain in the rats.

In addition, the inhibition of BK potassium channels controlled analgesic effect of clonidine as an alpha-2 adrenergic receptor agonist. The current study

exhibited that the inhibition of BK channels by iberiotoxin impeded analgesic effect in the rats receiving ICV injection of clonidine following iberiotoxin injection. Therefore, the rats showed pain-related behaviors. Given the extensive distribution of BK potassium channels in the central nervous system (39, 40), Bk channels are associated with pain, which was confirmed by other studies (16, 18). Stimulation of these channels increases outward potassium current and hyperpolarization of cellular membrane potential (16, 18).

Moreover, the severity of pain in the group receiving IP injection of clonidine after ICV injection of iberiotoxin was lower than that of the group receiving ICV injection of iberiotoxin alone. This result indicates that pain was partially controlled by clonidine despite inhibiting its analgesic effect by iberiotoxin. These findings suggest that the analgesic effect of clonidine can affect pain through other pathways, except for BK potassium channels. The role of BK potassium channels is recognized in the analgesic effect of alpha-2 adrenoceptor agonists, as was confirmed by previous studies and the current findings (11, 18). Clonidine and other alpha-2 adrenoceptor agonists lead to hyperpolarization of cellular membrane through potassium channels (41). According to our findings and those of previous studies, evaluation of the potassium channels can be helpful in understanding pain mechanism and might provide a deeper insight into pain treatment in clinical practice (42-44). Alpha-2 adrenergic receptor agonists have analgesic effect on animals and its antagonists inhibit the analgesic effect of the receptor. Moreover, inhibition of BK potassium channels impedes analgesic effect of the receptors.

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