International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 8 (5): 344-354, 2012
ISSN 1811-7775 / DOI: 10.3923/ijp.2012.344.354

Interaction between Anxiolytic Effects of Testosterone and β-1 Adrenoceptors of Basolateral Amygdala

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Abstract: Many studies have shown that anxiety-like behaviors are influenced by peripheral and central parameters including hormones and neurotransmitters in the different areas of Central Nervous System (CNS). Many investigations have proven the anxiolytic effect of androgens in different methods. Also, there are many reports about the modulating role of the amygdala complex, especially Basolateral Amygdala (BLA), through adrenergic system on anxiety. Among various types and subtypes of adrenergic receptors, β-1 adrenoceptors (β-1 ARs) of BLA account for the anxiety. The purpose of this study was to examine the relationship between testosterone and β-1 ARs in the BLA as an anxiety regulating key of CNS. Using Elevated plus Maze (EPM), the anxiety-like behaviors of four groups of intact adult male Wistar rat were assessed in the presence of different doses of testosterone (0, 20, 30 and 40 mg kg^{-1}, Intraperitoneal (i.p.). Then, the effects of intra-BLA microinjection of different doses of betaxolol, a selective β-1 ARs antagonist, (0, 0.025, 0.1 and 0.4 μg rat^{-1}), were evaluated in the other four groups. Finally, the interaction between the ineffective dose of testosterone via i.p. and betaxolol via intra-BLA was investigated. The results obtained revealed that testosterone (i.p.) and betaxolol (intra-BLA) alone had anxiolytic effects on the male Wistar rats in a dose-dependent manner. Our findings also showed that the anxiolytic effects of testosterone (i.p.) were reinforced by the intra-BLA injection of betaxolol. Co-administration betaxolol and testosterone also showed the synergistic actions on anxiolytic effects in the adult male Wistar rats. Our results interestingly proposed that the interaction between testosterone and β-1 ARs is in part related to common mechanism and other neurotransmitters include gamma-aminobutyric acid (GABA) and serotonin.

Key words: Testosterone, BLA, beta-1 adrenoceptor, betaxolol, anxiety-like behaviors, elevated plus maze

INTRODUCTION

Various humoral and neurochemical substances are involved in anxiety-like behaviors (Samad *et al*., 2005; Raj *et al*., 2006; Boyle *et al*., 2006; Velisek, 2006; Edinger and Frye, 2006; Sadeghi *et al*., 2007; Blum *et al*., 2007; Taiwe *et al*., 2010; Naghibi *et al*., 2011; Mahbub-E-Sobhani *et al*., 2011). Anxiety are reduced by anxiolytic drugs and controlled by two large limbic system structures, the amygdala and hippocampus (David, 1999; Wang *et al*., 2011). The amygdala complex seems to be in charge of mediating anxiety in both humans and animals (Killcross *et al*., 1997). This complex has a crucial role in regulating anxiety and emotional responses (Wang *et al*., 2011). The lateral and Basolateral Amygdala (BLA) are known to be of great importance for integrative processes and are the primary input nuclei of amygdala (Heldt and Ressler, 2006; Shibata *et al*., 1989). Adrenergic system and other neurotransmitters play an important role in the BLA on modulating the anxiety-like behaviors (Quirarte *et al*., 1997; Roozendaal *et al*., 2006; Shibata *et al*., 1989). Among various types and subtypes of adrenergic receptors, β-Adrenoceptors (β-ARs) of BLA are involved in the anxiety-like behaviors (Rudoy and Van Bockstaele, 2007; Fu *et al*., 2008). However, some studies suggest that various subtypes of β-ARs including β-1, β-2 and β-3 may exert different effects on anxiety (Ordway *et al*., 1991; Pandey *et al*., 1995). Some studies have shown that selective β-2 ARs antagonist appears to be ineffective in treating acute anxiety while it is effective in treating chronic anxiety. Other investigations have shown that β-1 ARs expression in rats' amygdala increased in cocaine withdrawal-induced anxiety (Rudoy and Van Bockstaele, 2007; Fu *et al*., 2008).

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On the other hand, the anxiolytic effect of testosterone has been found in many studies on rodents. Aikey *et al*. (2002) indicated that testosterone reduces anxiety-like behaviors rapidly in male house mice. Clark and Henderson (2003) showed that testosterone propionate decreased anxiety in adult male rat in Elevated Plus-maze (EPM).

There is a suggestion showing that the mechanism of testosterone in producing anxiolytic effects may be related, in part, to its metabolism. Testosterone is metabolized into Dihydrotestosterone (DHT) by 5α-reductase which activates the androgen responses. Then, the DHT metabolized into 3α-androstenediol (3α-diol) by 3α-hydroxysteroid dehydrogenase. Testosterone and DHT enjoy a high affinity for intracellular androgen receptors (Verhoeven *et al*., 1975; Cunningham *et al*., 1979; Christiansen and Knussmann, 1987; Frye *et al*., 2002, 2007) but 3α-Diol does not (Roselli, 1991). Androgen regimes, that elevate 3α-Diol concentrations significantly, increase the duration of open arm time in the EPM (Gee, 1988; Frye *et al*., 1996; Frye and Reed, 1998; Monjo *et al*., 2003; Fernandez-Guasti and Martinez-Mota, 2005). GABAA/benzodiazepine Receptors (GBRs) complexes have a key role in modulation of anxiety. 3α-Diol is a potent modulator at GABAA/benzodiazepine receptors (GBRs), whereas both testosterone and DHT have a weak activity in this regard (Gee, 1988; Frye *et al*., 1996; Frye and Reed, 1998; Fernandez-Guasti and Martinez-Mota, 2005). Some studies suggest that testosterone reduces serotonin level in the brain area and anti-anxiety effects of testosterone are possibly done by reducing serotonin level (Birger *et al*., 2003). Up-regulation of serotonin in the brain area such as BLA causes anxiety (Van der Wee *et al*., 2008; Akimova *et al*., 2009).

Until now, the relationship between the anxiolytic effects of testosterone and BLA β-1 ARs is not clearly understood. Accumulating evidence suggests that ARs subtypes display a differential regulation by testosterone (i.e. up-regulation of β-1 ARs and down-regulation of β-2 and β-3 ARs) (Monjo *et al*., 2003). It has been found that β-1 ARs mRNA levels in different tissue are evaluated when all the sexual hormones are injected (Monjo *et al*., 2003). We therefore hypothesized that existed interaction between anxiolytic effects of testosterone and β-1 ARs of BLA. This study has been designed to examine the effect of testosterone in the presence and absence of BLA β-1 ARs on anxiety-like behaviors in the EPM and to reveal the interaction between them in intact adult male rats.

MATERIALS AND METHODS

All procedures were carried out in accordance with the Institutional Guidelines for Animal Care and Use of Laboratory Animals and approved by the Biology Department of Shahid Chamran University (Ahvaz, Khuzestan Province, Iran).

Animals: Intact male Wistar rats (purchased from Jondi Shapour University of Ahwaz, Khuzestan province, Iran), aged 13±2 weeks and weighing 200±20 g, at the time of surgery were used. The animals were housed four/cage in a colony room with a constant 12 h reverse-light/dark cycle (7:00 AM-19:00 PM light off) at 22±1°C and relative humidity of 30-50%. The animals had free access to food and water except during the time of behavioral test session. All animals were allowed to adapt with the laboratory conditions for at least 1 week before the surgery. The rats were handled about 5 min each day prior to behavioral testing. In our study, rats are always tested in their dark phase (between 9:00 AM and 14:00 PM), when rodents are most active and have consistent differences in their endogenous concentrations of androgens. Each animal was used once only. Eight animals were used in each group of the experiments and all animals had stereotaxic surgery stress.

Stereotaxic surgery: The rats were anesthetized intraperitoneally (i.p.) using ketamine hydrochloride (50 mg kg^{-1}) and xylazine (4 mg kg^{-1}) and placed in a stoelting stereotaxic instrument (Stoelting Co, Illinois, USA). The animals head was restrained in a stereotaxic apparatus. Two Guide cannulas (14 mm stainless steel, 21 gauge) were implanted bilaterally into BLA (from bregma 2.8 mm caudally, 5 mm laterally and from skull surface 6.8 mm ventrally). A stylus (15 mm stainless steel, 27 gauge) was placed in the guide cannulae to prevent coagulation of clogging. After recovery from anesthesia, the rats were returned to their home cage for one week before behavioral test session.

Drugs: Testosterone [testosterone enanteat 250 mg mL^{-1}, Abureihan Co., Tehran, Iran] was dissolved in sesame oil (testosterone vehicle manufactured by Barvich Co., Tehran, Iran). The final testosterone concentrations were 0, 20, 30 and 40 mg/animal kg. The selective β-1 ARs antagonist, betaxolol (betaxolol hydrochloride, Tocris Bioscience, IO Center Moorend Farm Avenue, Bristol BS 11, OL, UK) was dissolved in 0.9% sodium chloride saline (as betaxolol vehicle) and the final concentrations were 0, 0.025, 0.1 and 0.4 μg rat^{-1} (Cecchi *et al*., 2007).

Drug injection: For intra-BLA betaxolol or betaxolol vehicle injections, the stylus was withdrawn into the guide cannula and replaced by the injection unit (15 mm stainless steel 27 gauge tubing), terminating at 1.5 mm below the tip of the guide cannula (Cecchi *et al*., 2007). Each injection unit has been connected to a 2 μL Hamilton syringe by polyethylene tubing. The animals received an injection of 1 μL rat^{-1} intra-BLA (0.5 μL/per cannula) at a 60 sec period. The inner cannula was left in place for an additional 60 sec to allow diffusion of the drug or vehicle and to reduce the possibility of reflux. 15 min after intra-BLA microinjection, testosterone or vehicle was injected via i.p. (ElAttar *et al*., 1964). Then, the behavioral testing was done 45 min post i.p. injection.

Behavioral testing: The EPM paradigm was used effectively to assess anxiety and exploratory behaviors (Walf and Frye, 2007; Matuszewich *et al*., 2007). The EPM consisted of four arms (50 cm long and 10 cm wide) elevated 73 cm off the ground. Two arms were enclosed by 30 cm high walls and the other two arms were exposed. As described by Pellow and File (1986) and Dunn *et al*. (1989), the rats were placed at the junction of the open and closed arms of the EPM and observed for 5 min. The number of entries and the amount of time spent on the open and closed arms were assessed using a video device (Sony Handycam HDR-CX110 Camcorder-1080i). The rats were considered to be in either the closed or the open arms of the EPM and the open arm time was recorded only when the rats had all four paws on the open arms of the EPM. Numbers of total arm entries reflect the motor component of the exploratory activity (locomotors activity). The apparatus was cleaned with alcohol after each rat was tested.

Experimental design:

- **Experiment 1: Effects of testosterone alone on the anxiety-like behaviors in adult male rats:** In this stage, four groups of animals (control or 0, T20, T30 and T40) received betaxolol vehicle (normal saline 1 μL rat^{-1} intra-BLA) and then 15 min post-intra-BLA injections, each group received testosterone 0, 20, 30 and 40 mg kg^{-1}, i.p. ,respectively. The behavioral test session was performed 45 min after the i.p. injection and the percent of open arm time (OAT%), the percent of open arm entries (OAE%) and locomotors activity were assessed (Fig. 2)

- **Experiment 2: Effects of betaxolol alone on the anxiety-like behaviors in adult male rats:** In this stage, four groups of animals (control or 0, B0.025, B0.1 and B4) received betaxolol 0, 0.025, 0.1 and 0.4 μg rat^{-1} intra-BLA, respectively. Then, 15 min later, the all these groups received testosterone vehicle (1 mL kg^{-1} sesame oil via i.p.). The behavioral test session was performed 45 min after the i.p., injection and OAT%, OAE% and locomotors activity were assessed (Fig. 3)

- **Experiment 3: Interaction of betaxolol and testosterone on the anxiety-like behaviors:** In this stage, using the one-way ANOVA follow up by Turkeys' test of prior experiments, one group of rats (BT group) received an ineffective dose of betaxolol (0.025 μg rat^{-1} via intra-BLA) and, 15 min later, an ineffective dose of testosterone (20 mg kg^{-1}, via i.p.). The behavioral test session was performed 45 min post i.p. injection and OAT%, OAE% and locomotors activity were assessed. This group was compared with control, T20 and B0.025 groups (Fig. 4)

Verification of cannula placement: After completion of all experimental sessions, the animals were killed with an overdose of chloroform. Subsequently, 0.5 μL per cannula of ink (0.1% aquatic methylene blue solution) was injected intra-BLA by a 15 mm stainless steel 27 gauge which was projected a further 1.5 mm ventral to the tip of the guide cannula to aid in histological verification. The animals' brain was removed and fixed in 10% formalin 10 days before sectioning. All sections were examined to determine the location of the cannula aimed for BLA. The cannula placement was verified using the Atlas of Paxinos and Watson (1998). The data from rats with cannula placement outside the BLA were excluded from the analyses.

Statistical analyses: Statistical analyses were performed using the Statistical Package for the Social Science (SPSS-PC, version 15. SPSS, Inc., Chicago, IL). The data were expressed as Mean±SEM. The data analysis for the experiments 1, 2 and 3 was performed by one-way analysis of variance (ANOVA). Then a post-hoc analysis (Tukey) was performed for assessing specific group comparisons. The differences between the experimental groups at each point were considered as statistically significant ($p<0.05$).

RESULTS

Histology: Figure 1 shows the approximate point of drug injection intra-BLA. The histological results were plotted on the representative section taken from the rat brain (2.80 mm posterior to bregma; Paxinos and Watson (1998). The data from the animals with the injection sites located outside the BLA were not used in the analysis.

Effect of testosterone alone on the anxiety-like parameters: Figure 2 shows the effects of i.p. injection of different doses of testosterone (0, 20, 30 and 40 mg kg^{-1}) on the anxiety-related parameters in the EPM. A one-way

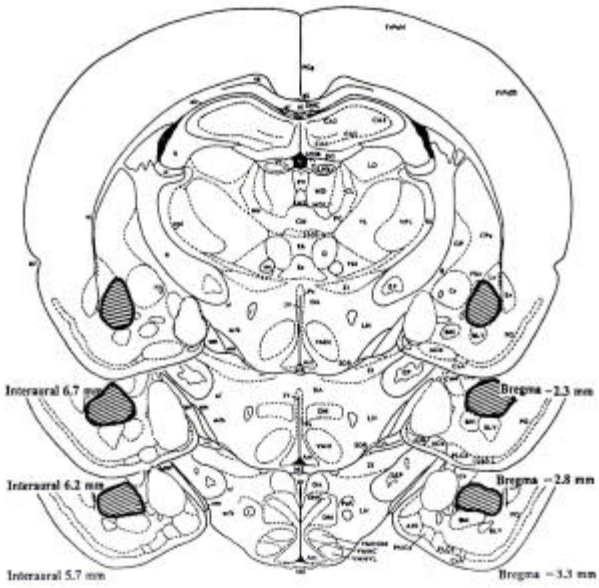

Fig. 1: Schematic illustration of coronal section of the rat brain (2.80 mm posterior to bregma; Paxinos and Watson (1998) showing the approximate location of the BLA sites in the study

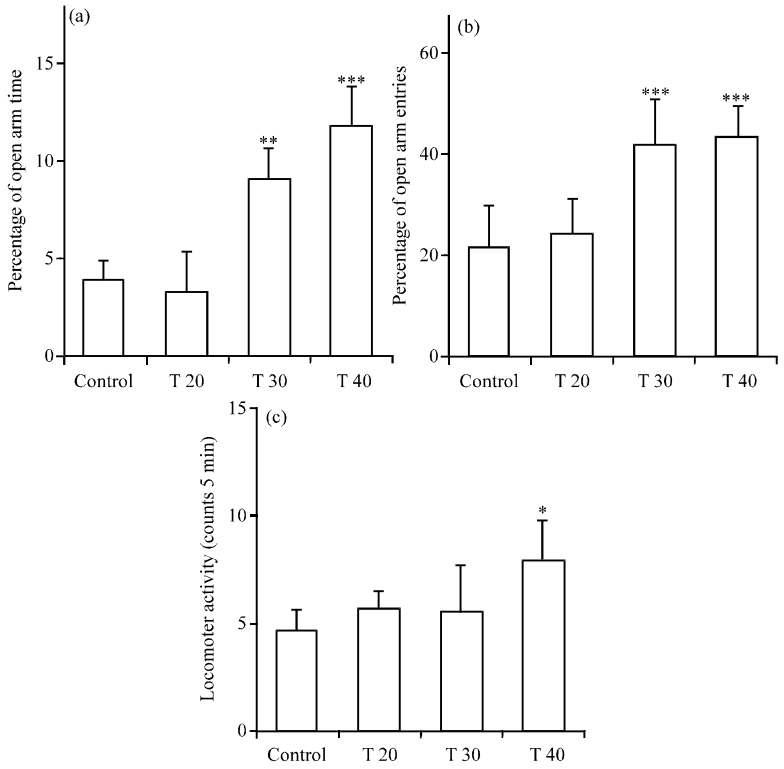

Fig. 2(a-c): Anxiolytic effects of testosterone on the performance of rats in the EPM. (a) OAT%, (b) OAE% and (c) Locomotor activity (number of total arm entries). Betaxolol vehicle (1 μL rat^{-1} Saline via intra-BLA) was microinjected 15 min before the i.p., infusion of different doses of testosterone (0, 20, 30 and 40 mg kg^{-1}). The behavioral test session was performed 45 min after i.p., injection. The data were expressed as Mean±SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with control group. n = 8 for all groups

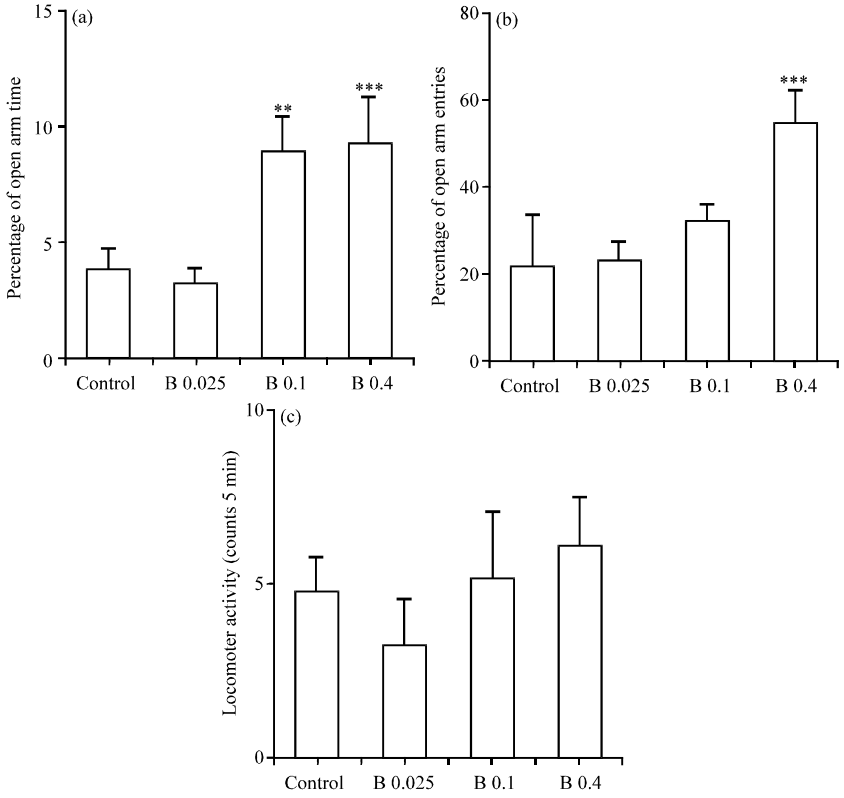

Fig. 3(a-c): Anxiolytic effects of betaxolol on the performance of rats in the EPM. (a) OAT%, (b) OAE% and (c) Locomotor activity (No. of total arm entries). Different doses of betaxolol (0, 0.025, 0.1 and 0.4 μg rat^{-1}) were infused 15 min before the i.p. injection of testosterone vehicle (1 mL kg^{-1} sesame oil via i.p.). The behavioral test session was performed 45 min after i.p. injection. The data were expressed as Mean±SEM, ** $p<0.01$ and ***$p<0.001$ compared with control group. n = 8 for all groups

ANOVA follow up by Turkeys' test revealed that anxiety-related parameters were altered by testosterone in comparison with the control group: OAT% [T20 = 3.38±2, $p<0.05$; T30 = 9.14±1.6, $p<0.01$; T40 = 11.85±2, $p<0.001$] (Fig. 2a), OAE% [T20 = 24.4±7, $p>0.05$; T 30 = 42±9, $p<0.001$; T40 = 43.42±6, $p<0.001$] (Fig. 2b) and locomotor activity [T20 = 4.57±1.95, $p>0.05$; T30 = 5.57±2.2, $p>0.05$; T40 = 8.00±1.7, $p<0.05$] (Fig. 2c). These results revealed that testosterone has an anxiolytic effect in a dose-dependent manner. Control group received betaxolol vehicle (1 μL rat^{-1} saline via intra-BLA) and testosterone vehicle (1 mL kg^{-1} sesame oil via i.p.). The anxiety-related parameters in control group: OAT% = 4±0.91; OAE% = 22±6; locomotor activity = 4.71±0.95. The data were expressed as Mean±SEM n = 8 for all groups.

Effects of intra-BLA injection of betaxolol on the anxiety-like parameters: Figure 3 shows the intra-BLA injection effects of betaxolol (0, 0.025, 0.1 and 0.4 μg rat^{-1} via intra-BLA) on the anxiety-related parameters. One-way ANOVA follow up by Turkey's test revealed that betaxolol also alters anxiety-related parameters in the EPM in a dose dependent manner and reduces the animals' anxiety. In comparison with the control group, betaxolol significantly altered the OAT% [B0.025 = 3.14±0.75, $p>0.05$; B0.1 = 8.86±1.50, $p<0.01$; B0.4 = 9.16±2.00, $p<0.001$] (Fig. 3a) and the OAE% [B0.025 = 23.25±4.00, $p>0.05$; B0.1 = 31.14±3.50, $p>0.05$; B0.4 = 55.71±8.00, $p<0.001$] (Fig. 3b) but it had no effects on the animals' locomotor activity [B0.025=3.28±1.26, $P>0.05$; B0.1 = 5.14±1.86, $p>0.05$; B0.4 $(7,56) = 6.00$±1.47, $p>0.05$] (Fig. 3c). As mentioned earlier, control group received betaxolol vehicle (1 μL rat^{-1} saline via intra-BLA) and testosterone vehicle (1 mL kg^{-1} sesame oil via i.p.). The data were expressed as Mean±SEM n = 8 for all groups.

Interaction of testosterone i.p. injection and betaxolol intra-BLA injection on the anxiety-like parameters: Figure 4 shows the effects of betaxolol (0.025 μg rat^{-1} via intra-BLA) on the anxiolytic effects of testosterone

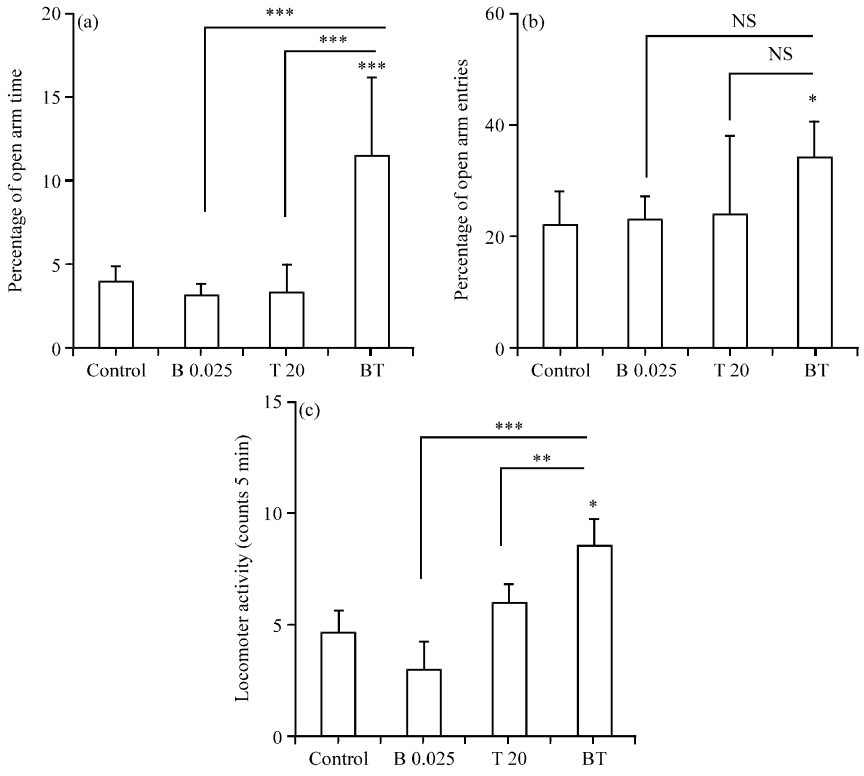

Fig. 4(a-c): Interaction of testosterone and betaxolol on the performance of rats in the EPM. (a) OAT%, (b) OAE% and (c) Locomotor activity (No. of total arm entries). BT group received an ineffective dose of betaxolol (0.025 μg rat^{-1} intra-BLA) and an ineffective dose of testosterone (20 mg kg^{-1}, 15 min later intra-BLA injection via i.p.). The behavioral test session was performed 45 min after i.p. injection. The data were expressed as Mean±SEM *p<0.05, **p<0.01 and ***p<0.001 and BT group compared with the control, T20 and B0.025. The anxiety-related parameters of T20 and B0.025 groups were expressed in previous sections. n = 8 for all groups

(20 mg kg^{-1} via i.p.) in the anxiety-related parameters. A one-way ANOVA follow up by Turkey's test revealed that the BT group in comparison with the control, T20 and B0.025 groups has a significant difference on the anxiety-related parameters in the EPM. In the previous experiments, betaxolol (0.025 μg rat^{-1} via intra-BLA) and testosterone (20 mg kg^{-1} via i.p.) that had no effect alone on the anxiety-like behaviors, when used together, have an interactive and significant anxiolytic effect on the OAT%, OAE% and locomotor activity. Co-administration of ineffective dose of betaxolol and testosterone (BT group) in comparison with control group significantly altered the OAT% [BT = 12.14±4.68, $p<0.001$] (Fig. 4a) and OAE% [BT = 34.00±6.80, $p<0.05$] (Fig. 4b) and locomotion activity [BT = 8.28±1.97, $p<0.01$] (Fig. 4c). The data were expressed as Mean±SEM.

DISCUSSION

The EPM was employed as an animal model for studying the anxiety-like behaviors (Sullivan *et al*., 2009; Woode *et al*., 2009, 2010; Zuloaga *et al*., 2011; Skurlova *et al*., 2011). Our findings showed that testosterone has an anxiolytic effect with dose dependent manner in the intact adult male rats. Also the inhibition of BLA β-1 ARs by betaxolol significantly decreased the anxiety-like behaviors in the EPM.

The results of this study also demonstrated that there is an interaction and synergic actions between testosterone and BLA β-1 ARs antagonist. We suggest that the anxiolytic effect of testosterone might have occurred in BLA, because microinjection of betaxolol into BLA reinforces the anxiolytic effects of testosterone.

In support of our findings, the anxiolytic effects of testosterone have been shown in many investigations on rodents. Aikey *et al*. (2002) indicated that testosterone rapidly reduces anxiety but not locomotor activity in male house mice, Clark and Henderson (2003) showed that testosterone propionate decreases anxiety in adult male rats in the EPM. Some studies suggest that androgen administration can have anxiolytic effects in both male and female rats (Forman *et al*., 1989; Bitran *et al*., 1993; Molina *et al*., 1994).

Testosterones mechanism to produce analgesia and to enhance cognitive performance may be in part due to its metabolism. Testosterone is metabolized into DHT by 5α-reductase which is, in turn metabolized by 3α-hydroxysteroid dehydrogenase into 3α-Diol, as described in more detail in the introduction part. Testosterone and DHT have a high affinity for intracellular androgen receptors (Verhoeven *et al*., 1975; Cunningham *et al*., 1979; Christiansen and Knussmann, 1987; Frye *et al*., 2002, 2007) but 3α-Diol does not (Roselli, 1991). 3α-Diol is a potent modulator at GBRs, whereas testosterone and DHT have only weak activity at GBRs (Gee, 1988; Frye *et al*., 1996; Frye and Reed, 1998; Fernandez-Guasti and Martinez-Mota, 2005). Androgens can change GBR pharmacodynamics, as gonadectomy decreases sensitivity to diazepam-induced sedation (Svensson *et al*., 2000). The similar effects of testosterone, DHT and 3α-Diol to produce analgesia and anxiolytic effects suggest that their actions may stem from their metabolism or production of 3α-Diol and subsequent actions at GBRs (Frye and Lacey, 2001). For example, administration of 3α-Diol to Ovariectomized (OVX) rats produced analgesia in the tail-flick model (Frye *et al*., 1996). Androgen regimes that increase 3α-Diol concentrations significantly increase the OAT% in the EPM (Monjo *et al*., 2003). Our findings also confirmed the anxiolytic effects of testosterone in the intact adult male rats.

In addition, the results of this study indicated that administration of antagonist β-1 ARs in the BLA has anxiolytic effects and does not have any influence upon locomotor activity. It has been also confirmed that the amygdala receives sever noradrenergic innervations (Clayton and Williams, 2000) and down regulation of adrenergic system in long or short term may occur after stressor cassation. For example, stress can severely and lastingly impair the function of α-1 ARs in the BLA (Braga *et al*., 2004). β-1 ARs mainly exist in postsynaptic membrane and known to be coupled to Gs (Stimulatory G protein), such that stimulation of these receptors increases cyclic intracellular adenosine monophosphate (cAMP) levels via a direct activation of adenylate cyclase. Immunofluorescence studies have also demonstrated that β-1 ARs were located in the cell membrane and the number of immunoreactive neurons was significantly increased in the amygdala of the fear conditioned rats (Beitner-Johnson and Millhorn, 1998; Rosen and Schulkin, 1998).

Fu *et al*. (2008), using western blotting analysis, showed up-regulation of β-1 ARs in the amygdala after fear training and anxiety condition. Also it was confirmed that microinjection of metoprolol as antagonist β-1 ARs in the rats BLA inhibits up-regulation of β-1 ARs.

Furthermore, our results obviously revealed that there exists an interaction and synergic action between testosterone and BLA β-1 ARs on the anxiety-like behaviors. The administration of either the lowest dose of testosterone (i.p.) or betaxolol (intra-BLA) alone did not have any effects on the anxiety-related parameters. Co-administration of testosterone and betaxolol attenuated the anxiety-like behaviors in the BT group in comparison with control, B0.025 and T20 groups. This proves the synergistic actions of testosterone and β-1 ARs antagonist on the anxiety. There are miscellaneous reports showing the intervention of androgen hormones, especially testosterone in ARs regulation. This interaction between testosterone and BLA β-1 ARs probably depends on the common intracellular signaling pathway or common neurochemical system in the BLA. One mechanism of synergic actions of testosterone and betaxolol on the anxiety-like parameters might be occurring through modulation of serotonergic and GABAergic systems. For example, activity of projection neurons in the BLA is under strong inhibitory control of GABAergic synaptic transmission (Royer *et al*., 1999; Szinyei *et al*., 2000) and intensive anxiety attenuates inhibitory GABAergic control in the BLA (Braga *et al*., 2002; Rodriguez Manzanares *et al*., 2005). Other studies have indicated that the dorsal raphe nucleus serotonergic system is a critical modulator of the GABAergic system in the BLA and dysregulation of this system has been recognized in stress and anxiety disorders (Southwick *et al*., 1999; Van Praag, 2004).

Some studies have shown that up-regulation of serotonin in the brain regions (including the amygdala) caused anxiety and the serotonin levels are strongly enhanced in the BLA during stressful experiences (Amat *et al*., 1998; Minor and Hunter, 2002). Therefore, manipulation of serotonergic system within the BLA has a critical influence on emotional and anxiety-related parameters (Kim *et al*., 2005; Van Nobelen and Kokkinidis, 2006). It seems that both testosterone and betaxolol decreases the concentration of serotonin in various brain nuclei including the BLA.

In support of this finding, other studies have shown that β-AR antagonists inhibit serotonin uptake by pulmonary vascular cells in culture and this condition attenuates serotonin function (Lee and Fanburg, 1991). Further, serotonin level in the pineal gland decreases due to the effect of specific β-1 and β-2 AR antagonists (Brownstein *et al*., 1973). In this study, betaxolol, as a selective β-1 ARs antagonist, probably tends to have affinity for serotonin BLA receptors. Other studies have confirmed that testosterone reduces serotonin level in the brain regions and anxiolytic effects of testosterone are possibly taken place by reducing serotonin level

(Birger *et al*., 2003). According to the findings of this study, testosterone and selective β-1 ARs antagonist likely reduce serotonin function or concentration in the BLA and thus testosterone hormone and betaxolol have an interaction and anxiolytic effects. However, the effect of specific β-1 ARs antagonist occurs in the BLA and betaxolol increases the anxiolytic effects of testosterone in this region.

The second mechanism that it can also be suggested is the role of GABAergic system alone on this synergic effect. Some studies have shown that adrenergic systems, especially α-1A ARs, generally impair facilitation of GABAergic transmission in the BLA (Braga *et al*., 2004). Further β-1 ARs mediate the facilitation of Purkinje cell in response to GABA in the cerebellum of rats (Yeh and Woodward, 1983). Therefore, betaxolol as β-1 ARs antagonist probably attenuates this system and stops the inhibitory effect of GABAergic system in the BLA. But testosterone and particularly its metabolites have a high affinity to GBRs and reinforce the GABAergic system. Thus it is likely that in contrast to testosterone, selective β-1 ARs antagonists naturally inhibit GABAergic system. It is in contrast to our finding. However, β-1 ARs have a high heterogeneity in different brain regions and the precise mechanism between the all subtypes of β-1 ARs in the BLA and GABAergic system is not clearly understood. Further study is needed to investigate the modulatory effects of GABAergic system in this synergic action.

Finally, we conclude the findings of this study confirmed the synergistic actions of testosterone and betaxolol, β1-AR antagonist, on the anxiolytic effects in the BLA. Also, our results interestingly proposed that the interaction between testosterone and β-1 ARs is probably in part related to common neurotransmitter systems include serotonergic or GABAergic systems. Further study is needed to investigate the precise mechanism of synergistic action of betaxolol and testosterone in the BLA.

ACKNOWLEDGMENT

The financial and technical support of Shahid Chamran University of Ahvaz, Iran, is acknowledged.

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