Pergamon

Pharmacology Biochemistry and Behavior, Vol. 52, No. 4, pp. 701–706, 1995

0091-3057/95 $9.50 + .00

0091-3057(95)00153-0

Regulation of Anxiety by $GABA_A$ Receptors in the Rat Amygdala

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Received 9 June 1994

SANDERS, S. K. AND A. SHEKHAR. *Regulation of anxiety by $GABA_A$ receptors in the rat amygdala.* PHARMACOL BIOCHEM BEHAV **52**(4) 701–706, 1995.—Blockade of GABAergic inhibition in the region of the anterior basolateral amygdala (BLA) of rats elicits physiologic changes associated with a defense reaction. The present study was undertaken to determine whether GABA receptors in the BLA might be involved in regulating experimental anxiety using the social interaction (SI) and conflict test. Guide cannulae were stereotaxically implanted bilaterally in the BLA of rats for intracerebral microinjections. In the BLA, injection of the $GABA_A$ receptor antagonists bicuculline methiodide (BMI) and picrotoxin (PIC) produced anxiogenic-like effects in the SI paradigm, as did BMI injection using the conflict paradigm. Injection of the $GABA_A$ agonist muscimol (MUS) into the central nucleus of the amygdala (Ce) produced anxiolytic-like effects in the SI test. Microinjection of MUS, baclofen ($GABA_B$ agonist), 2OH-saclofen ($GABA_B$ antagonist) or strychnine (glycine antagonist) into the BLA or BMI into the Ce elicited no change in experimental anxiety as measured by the SI test. These results suggest that endogenous GABA acts tonically at $GABA_A$ receptors in the BLA to inhibit anxiety responses.

Social interaction Bicuculline Picrotoxin Muscimol Anxiety conflict

THE AMYGDALA is thought to be an important structure in the mediation of emotions such as anxiety. Bilateral ablation of the amygdala in primates has been shown to produce emotional blunting (19). In man, lesions of the amygdala produce a calming effect (9), whereas electrical stimulation of the amygdala in patients under local anesthesia elicits feelings of fear and confusion (1). Likewise, electrical stimulation of the amygdala in several mammalian species elicits a cardiovascular and behavioral arousal associated with a fight-or-flight response (3–5). Chemical stimulation by microinjection of D,L-homocysteic acid into the amygdala has also been shown to elicit these cardiovascular and motor effects (7). Several studies employing intracerebral (IC) microinjection techniques have determined that injection of BDZs or the $GABA_A$ agonist muscimol directly into the amygdala produces anxiolytic-like effects (6,8,11,14,17,18). These studies were all performed using a conflict (16) paradigm, which is an operant test involving punishment-induced suppression of responses. Because of its time-consuming nature, the potential effects on other motivations besides anxiety, and the involvement of electric shock, the conflict test has come under increasing criticism. Other paradigms, especially ethologically based tests such as the social interaction test (2), have been used more frequently (15).

We have previously shown that microinjection of drugs that block $GABA_A$ receptor function into the region of the anterior basolateral amygdala (BLA) produces increases in heart rate (HR), blood pressure (BP), and locomotor activity (13); this suggests that a tonic GABAergic mechanism exists in the BLA that, when disinhibited, brings about the cardiovascular and behavioral responses. The present study was conducted to test the hypothesis that a tonic GABAergic mechanism in the BLA, possibly acting through the $GABA_A$ receptor, may be involved in regulating experimental anxiety in rats. The contribution of the BLA in mediating experimental anxiety was measured using both a conflict (16) and social interaction test (2,15) following IC microinjection of various drugs acting at the $GABA_A$ receptors. Because of the ease of administration and reliability, the social interaction test was used to test the effects of microinjecting GABAergic and other drugs into the amygdala on experimental anxiety. The conflict

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test, which requires prolonged training of the animals, was used as an additional study to verify the role of $GABA_A$ receptor function in the BLA. To test for pharmacologic specificity, we also injected the $GABA_B$ agonist and antagonist baclofen (BAC) and 2OH-saclofen (SAC), respectively, or the glycine antagonist strychnine (STR), and determined social interaction time. Anatomic specificity of these responses was ascertained by repeating a parallel series of experiments in rats with guide cannulae implanted in the central nucleus of the amygdala (Ce).

METHODS

Animals

Male Wistar rats, obtained from Harlan (Indianapolis, IN) and maintained at 80% free-feeding weight on a 12 L : 12 D schedule (light off at 1900 h) at 72°F, were used in the following experiments. All rats were housed separately in plastic boxes. Surgeries were carried out under pentobarbital (50 mg/kg, IP) anesthesia. Animals were allowed at least 4 days for recovery from surgery before the beginning of any experiment.

Arterial Catheter Placement and Measurement of HR and BP

We constructed arterial catheters using two sizes of Tygon tubing (Fisher Scientific, Pittsburgh, PA). We fused 5 cm of 0.01-in. (254 μm) tubing with 30 cm of 0.02-in. (508 μm) tubing using cyclohexanone. The smaller tubing was inserted into the artery and the larger tubing was routed subdermally to the dorsal aspect of the neck, where it was stabilized with a leather jacket. HR and BP were measured from the arterial catheters originating in the femoral artery. The direct blood pressure signals from the arterial catheters were recorded on a Beckman R511 Dynograph (Fullerton, CA) through a Sensormedics pressure transducer (Anaheim, CA). The BP signal was fed into a cardiotachometer that produced the HR output.

Implantation of Microinjection Cannulae

Cannulae were placed using a stereotaxic apparatus (David Kopf, Tujunga, CA) with the incisor bar at -3.3. Guide cannulae (26 ga) were fixed onto the stereotaxic arms, with coordinates used for the BLA at: A, -2.0; L, $+5.1$; V, -7.5; those for the Ce were: A, -2.4; L, $+4.1$; V, -7.6 (10). Small openings were drilled in the skull at these coordinates and the guide cannulae were inserted. The guide cannulae were held in place using three 2.4-mm stainless-steel screws anchored to the skull with cranioplastic cement. The guide cannulae were sealed with obturators.

Drug Injections

Bilateral drug injections of 250 nl were accomplished using a Sage (Cambridge, MA) pump fitted with two 10-μl Hamilton (Bonaduz, Switzerland) syringes connected to the injection cannulae (33 ga) with PE-50 polyethylene tubing. Injection cannulae were inserted through and extended 1 mm beyond the tip of the guide cannulae. The entire system was filled with the drug solution and the pump was set to deliver 250 nl/30 s. The pump was turned on for 30 s and the injectors were left in place an additional minute before being removed.

Social Interaction Test

The social interaction (SI) apparatus consisted of a large wooden open field 91.44 × 36 × 91.44 cm, with walls 30.48 cm high. A videocamera was mounted above the SI field to record each session on VHS videotape. During a test session, the experimental rat was placed in the SI field with an unfamiliar partner rat. During each 5-min session, the total time the experimental rat spent interacting (grooming, crawling over and under, sniffing, etc.) with the partner rat was recorded. A decrease in interaction indicated an increase in anxiety and vice-versa. Each session was scored separately by two observers unaware of treatment conditions (intraobserver reliability, $r = 0.97$, $n = 6$).

Conflict Test

The conflict apparatus (Coulbourn Instruments, Lehigh Valley, PA) consisted of a large modular test cage 25.4 × 11 × 27.94 × 30.48 cm, fitted with a shock floor, house light, response lever, liquid dipper with a 0.02 cc dipper cup and liquid reservoir, and tone module. A cable connecting the grid floor shocker with the dipper provided a shock to the animal only when it tried to drink the sweetened milk, thereby completing the circuit. The entire apparatus was placed inside a sound-attenuated isolation cubicle equipped with a ventilation fan. Rats were trained to press the lever for a reinforcement of sweetened milk and eventually put on a variable interval schedule with an average reinforcement rate of once per 30 s (VI 30). The conflict interval consisted of a VI 30 schedule plus a constant low volume tone and low amplitude shock (0.3–0.4 mA) delivered through the dipper. The shock level was adjusted for each animal to obtain a slight decrease in responding during the conflict interval (CON) as compared to the nonpunished VI 30 interval (VI). The testing protocol began with a 5-min warmup period, followed by alternating 5-min VI and CON intervals for a total of 30 min. Total bar presses for VI and CON were obtained when each of the 5-min periods (three VI and two CON) was averaged. This was compared to average baseline (no drug injection) VI and CON responding. A selective decrease in responding during the CON period with no change in the VI period indicated an increase in anxiety. If both VI and CON responding were decreased, we interpreted it to mean that the drug concentration was too high and that there was a generalization of suppression to all periods. All IC drug injections were made 5 min before placing the rat in the testing apparatus.

Drugs

BMI, PIC, MUS, and STR were obtained from Sigma (St. Louis, MO) and were dissolved in 0.9% sodium chloride (saline). Baclofen and SAC were obtained from RBI (Natick, MA). Baclofen was dissolved in saline and SAC was dissolved in 0.1 N HCl and diluted with saline with pH adjusted to 7.4 using 1 N NaOH. All drugs were administered IC and bilaterally in a volume of 250 nl saline as described earlier, in Drug Injections.

Experimental Protocol

Effects of injecting the drugs acting at $GABA_A$, $GABA_B$, and glycine receptors into the BLA were tested in the social interaction test. The effects of blocking $GABA_A$ receptors in the BLA on anxiety were further verified in the conflict test.

Experimental procedures commenced following at least a 4-day recovery period from surgery. In the first set of experiments, microinjection cannulae were inserted into the guide cannulae to administer a 250-nl IC injection of either saline (vehicle) or a drug solution into awake and freely moving

animals. The injection cannulae were removed 1 min after completion of the injection and replaced with obturators. For the SI test, the animal was removed from its home cage 10 min following the injection and placed in the social interaction field with an unfamiliar partner rat for 5 min. This 5-min session was videotaped to be scored by an observer unaware of treatment conditions. All drug injections were given randomly using a counterbalancing method to eliminate any day effect. The amount of time each rat spent interacting with a partner following the drug injection was compared with the amount of time the same rat spent interacting with a different partner following vehicle injection. At least 48 h elapsed between injections and no animal received more than six injections. For the conflict test, the animals were placed in the test box 5 min following the injection and began the 5-min warmup period. Bar presses during the VI and CON intervals were scored by a computer.

Because blockade of GABAergic inhibition in the region of the BLA is associated with emotional arousal (13), including increases in HR, BP, and locomotor stimulation, one may argue that the behavioral effects are a direct result or effect of the cardiovascular changes and not a cognitive anxiety. To determine whether the HR and BP changes lead to emotional arousal or whether the emotional component is intrinsic to the BLA, the cardiovascular component was attenuated using peripheral administration of atenolol, a selective β_1 receptor antagonist, before GABAergic inhibition in the BLA. The femoral arterial catheter of an awake and freely moving rat was connected to a Beckman R511 Dynograph to measure HR and BP. The animals then randomly received either a saline or atenolol (10 mg/kg) injection, IP. We administered BMI 20 pmol/250 nl, IC, 10 min following the IP injection, and monitored HR and BP for 10 min. At the end of the 10-min period, the animal was placed in the SI field with an unfamiliar partner rat and videotaped to be scored later. Experimental runs occurred on consecutive days, and no rat received more than three IC drug injections.

Histology

Injection sites were marked by injecting 250 nl of a 50% solution of india ink (250 nl). Animals were sacrificed under pentobarbital anesthesia using a Harvard Apparatus (Cambridge, MA) guillotine. Their brains were then removed and stored in 10% neutral buffered formalin for at least 48 h. Microtome sections (40 μm) were placed on slides and stained with Neutral red to determine the precise anatomic location of the injection areas.

Statistical Analysis

The results of the conflict test were converted to the percent change in bar presses/5-min period from baseline (vehicle injection) and expressed as mean ± SEM. For the SI test, the results were tabulated as the mean of the percent change in social interaction time from baseline (vehicle injection) ± SEM. Statistical analysis employed a paired t-test when two different drug injections were being compared in one group of animals or a repeated measures analyis of variance (ANOVA) when more than two different drug injections were being compared in a group of animals. Statistical significance was set at $p < 0.05$.

RESULTS

The sites of the bilateral injection areas for 29 of the 31 animals used in these experiments as determined by histologic observation are represented in Fig. 1. All 26 rats with implants at the BLA coordinates were found to respond to microinjections of 50 pmol BMI, IC, with increases in HR and BP. Of the 29 animals represented in Fig. 1, 24 had cannulae in the BLA and five had cannulae implanted in the Ce according to the atlas of Paxinos and Watson (10).

Effect of Microinjecting $GABA_A$-Selective Drugs into the BLA and Ce

Figure 2 shows the effects of injecting GABAergic drugs into the BLA on the social interaction time as a percent of baseline (saline injection). Anxiogenic-like effects were produced by injection of 20 pmol BMI [paired t-test, $t(5) = 6.28$, $p = 0.008$] or 10 pmol PIC [paired t-test, $t(3) = 6.88$, $p = 0.006$] into the BLA. However, when BMI 20 pmol was injected into the Ce, no change in social interaction was observed [paired t-test, $t(4) = 0.32$, $p = 0.767$].

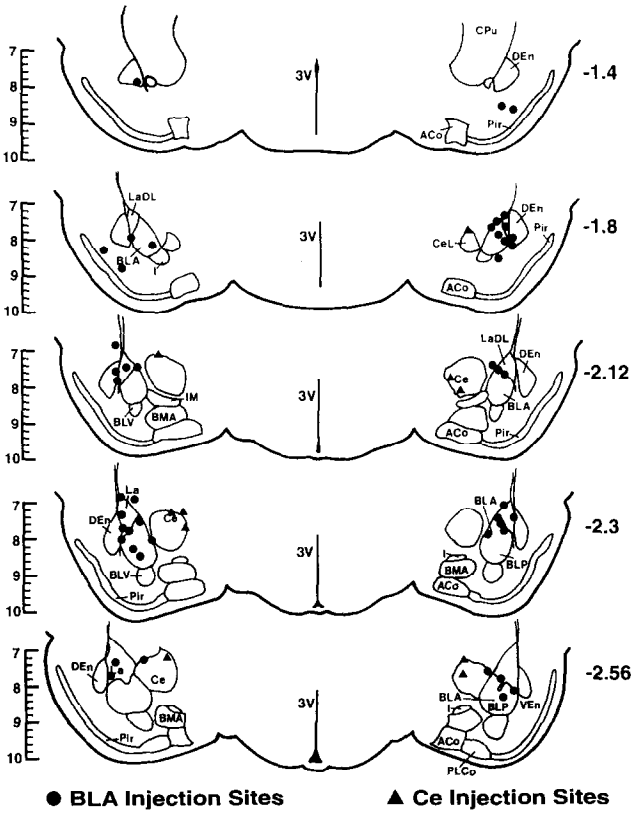

FIG. 1. Schematic representation of the bilateral injection sites as determined by histology. Brain sections are represented according to the atlas of Paxinos and Watson (10). Numbers on the right indicate distance (mm) posterior from bregma. The scale on the left represents distance (mm) ventral to bregma. ●, Implantation of guide cannulae in the region of the BLA; ▲, implantation of guide cannulae in the region of the Ce. ACo, anterior cortical amygdaloid nucleus; BLA, anterior basolateral amygdaloid nucleus; BLP, posterior basolateral amygdaloid nucleus; BLV, ventral basolateral amygdaloid nucleus; BMA, anterior basomedial amygdaloid nucleus; Ce, central amygdaloid nucleus; CeL, lateral central amygdaloid nucleus; CPu, striatum; DEn, dorsal endopiriform nucleus; I, intercalated amygdaloid nuclei; IM, main intercalated amygdaloid nucleus; La, lateral amygdaloid nucleus; LaDL, dorsolateral lateral amygdaloid nucleus; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; VEn, ventral endopiriform nucleus; 3V, third ventricle.

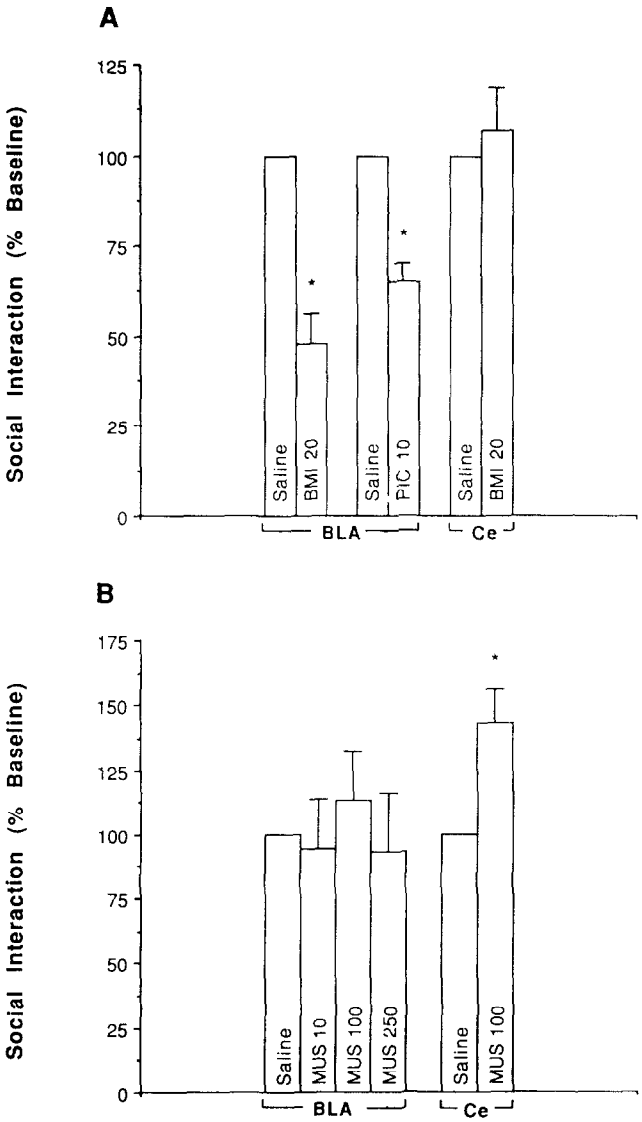

FIG. 2. The effects on SI of modifying $GABA_A$-receptor function in the BLA and Ce. (A) Blockade of $GABA_A$ receptors in the BLA using BMI 20 pmol (n = 6) or PIC 10 pmol (n = 4) as well as 20 pmol BMI injected into the Ce (n = 5). (B) Stimulation of $GABA_A$ receptors in the BLA using MUS 10 (n = 5), 100 (n = 5), or 250 (n = 5) pmol/250 nl or MUS 100 (n = 5) pmol/250 nl injected into the Ce. The total time each experimental rat spent interacting with its partner is represented on the abscissa as a percent of baseline (saline) interaction ±SEM. *$p < 0.05$ change in SI significantly different from baseline (saline) by repeated measures ANOVA coupled with the least mean squares or paired t-test.

Microinjection of three doses of muscimol (10, 100, and 250 pmol) into the BLA did not alter baseline anxiety [repeated measures ANOVA for the 10- and 100-pmol doses, $F(2, 8) = 0.35$, $p = 0.713$, and paired t-test for the 250-pmol dose, $t(4) = 0.32$, $p = 0.767$]; injections of 100 pmol MUS into the Ce produced a significant anxiolytic-like effect [paired t-test, $t(4) = 3.3$, $p = 0.03$].

The response of rats in the conflict paradigm following microinjection of several doses of BMI is presented in Fig. 3. A dose-dependent decrease in bar pressing was observed as

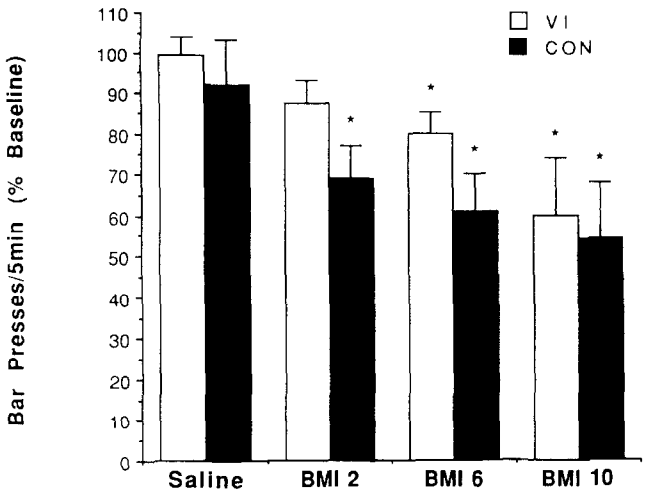

FIG. 3. The effects on the response in the conflict test of blockade of $GABA_A$ receptors in the BLA: average number (percent baseline) of bar presses/5-min period (VI or CON) ±SEM following BMI (0, 2, 6, 10 pmol; n = 5) injection into the BLA of rats. *$p < 0.05$ change in either VI or CON compared to baseline (no injection) using repeated measures ANOVA coupled with least mean squares.

the dose of BMI increased in both the VI [repeated measures ANOVA, $F(4, 16) = 6.63$, $p = 0.002$] and the conflict [repeated measures ANOVA, $F(4, 16) = 5.79$, $p = 0.004$] intervals. Injection of 2 pmol BMI into the BLA elicited an anxiogenic-like effect in that responding during the conflict period ($p = 0.02$ by least mean squares) was suppressed, whereas responding during the VI period ($p = 0.15$ by least mean squares) is unchanged. However, the larger doses of BMI (6 and 10 pmol), when injected into the BLA, suppressed responding in both periods.

Effect of Microinjecting $GABA_B$-Selective and Glycinergic Drugs Into the BLA

Table 1 illustrates the effects on SI of injections of $GABA_B$-selective drugs, BAC (50, 250, and 1000 pmol) and SAC (200, 400, and 800 pmol), as well as the glycine antagonist STR (50 and 250 pmol) into the BLA. Neither the $GABA_B$-selective

TABLE 1

EFFECTS ON SOCIAL INTERACTION OF MICROINJECTING BACLOFEN, 20H-SACLOFEN, OR STRYCHNINE INTO THE BLA OF RATS

Drug	Dose (pmol)	n	SI*
Baclofen	50	5	97 ± 19
	250	4	111 ± 38
	1000	5	96 ± 37
20H-Saclofen	200	6	119 ± 20
	400	4	109 ± 24
	800	4	87 ± 10
Strychnine	50	4	100 ± 10
	250	4	79 ± 11

*Values represent the mean SI ± SEM as a percent of baseline (saline) SI.

drugs [BAC: repeated measures ANOVA for the 250- and 1000-pmol dose, $F(2, 4) = 0.45$, $p = 0.669$, and paired t-test for the 50-pmol dose, $t(4) = 0.18$, $p = 0.866$; or SAC: repeated measures ANOVA, $F(3, 17) = 0.75$, $p = 0.539$] nor the glycine antagonist STR [50 pmol; paired t-test, $t(3) = 0.00$, $p =$ 1000 or 250 pmol; paired t-test, $t(3) = 1.92$, $p = 0.151$] had an effect on SI responses of rats.

Effect of Peripheral Atenolol Administration Before Microinjecting BMI into the BLA

The increase in HR elicited by microinjecting BMI into the BLA was significantly inhibited [paired t-test, $t(3) = 13.752$, $p = 0.001$], whereas BP was not significantly affected [paired t-test, $t(3) = 1.5$, $p = 0.231$] by prior injection of atenolol IP (Fig. 4A). In the SI test, prior administration of atenolol did not attenuate the anxiogenic effects of BMI even though HR responses were blocked [Fig. 4B; repeated measures ANOVA, $F(2, 6) = 34.33$, $p = 0.001$].

DISCUSSION

The data presented here show that a significant anxiogenic-like effect may be produced following microinjection of $GABA_A$-receptor antagonists into the BLA. This suggests that a tonic GABAergic inhibition exists in the BLA, which when blocked, produces anxiety. That injections of the $GABA_A$ agonist MUS into the BLA elicited no changes in experimental anxiety suggests that the GABA tone in the BLA may be maximal. Taken together with our previous study (13), these data indicate that a tonic GABAergic system exists in the BLA, and blocking this $GABA_A$ receptor-mediated inhibition results in a syndrome resembling a fight-or-flight response consisting of cardiovascular and locomotor stimulation as well as an increase in anxiety. In addition to postsynaptic $GABA_A$ antagonists, we have also shown that injection of the GABA synthesis inhibitor L-allylglycine also produces increases in HR, BP, and anxiety similar to that produced by $GABA_A$-receptor blockade (12). $GABA_A$ receptors also seem to have a role in modulating anxiety in the Ce in that injection of MUS produced an anxiolytic-like effect. The anxiogenic-like effect observed as a result of $GABA_A$ receptor blockade in the BLA was not observed following injection of the same dose of BMI into the Ce, suggesting that the GABAergic system in the Ce is not a tonic system.

Microinjection of various benzodiazepines (8,11,14,17) or the $GABA_A$ agonist muscimol (6,14) into the amygdala has been shown to increase punished responding in a conflict paradigm. However, there is much disagreement as to which area of the amygdala mediates this response. Microinjection of 25 ng/0.5 μl MUS into the lateral portion of the amygdala increased punished responding in some studies (14), whereas microinjection of 10-30 ng/1 μl MUS into the central but not the basolateral area of the amygdala increased punished responding in others (6). One possible explanation is that whereas both nuclei contain intrinsic GABAergic mechanisms involved in mediating anxiety, the level of inhibition in two systems might differ. In the BLA, GABA tone may be maximal, and muscimol has no anxiolytic properties in this region at the dose range tested. On the other hand, GABA tone in the Ce might not be maximal and therefore is subject to further action by muscimol in decreasing anxiety. For the same reason, blockade of GABAergic inhibition by injections of BMI and PIC into the BLA elicited an anxiogenic-like effect, whereas no effect on anxiety was observed following BMI injection into the Ce at the same dose of BMI.

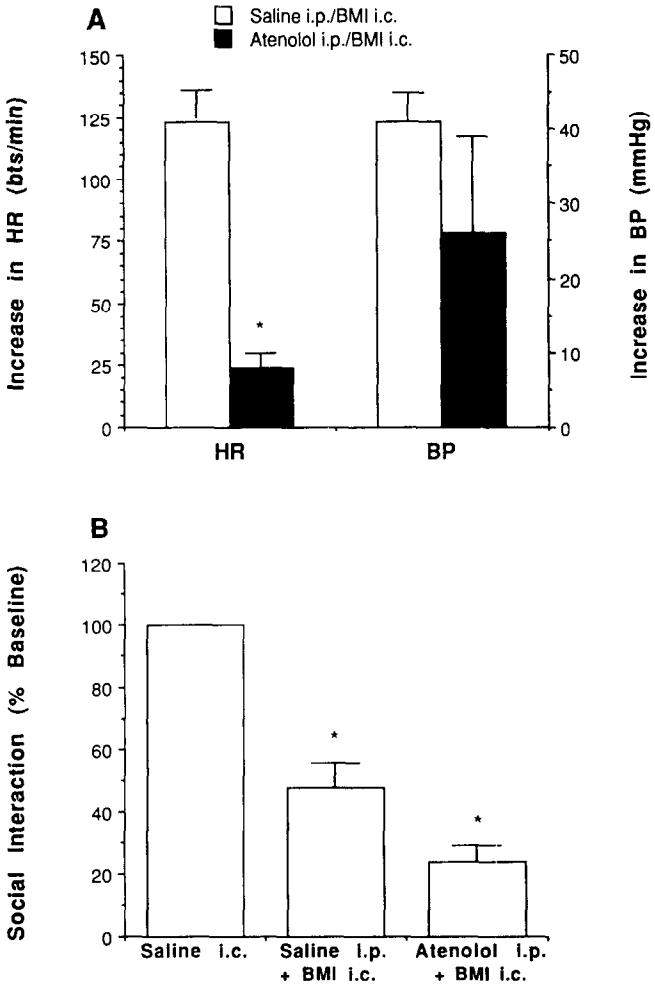

FIG. 4. Attenuation of cardiovascular effects of GABA blockade in the BLA by the peripherally acting β_1 antagonist atenolol, without decreasing anxiety. (A) Changes in HR and BP elicited by injections of BMI 20 pmol into the BLA preceded by either an IP injection of saline (□) or atenolol 10 mg/kg (■). Data are represented as an increase in HR or BP (mean ± SEM) over preinjection baseline values 10 min following the IC injection. *$p < 0.05$ change in HR or BP significantly different from saline IP/BMI IC injection by paired t-test ($n = 4$). (B) Changes in experimental anxiety as measured by the social interaction test. Data are represented as percent change in SI (mean ± SEM) from a previous SI trial in which the same rats received an IC injection of saline (250 nl). *$p < 0.05$ change in SI significantly different from baseline (saline, IC) by repeated measures ANOVA coupled with the least mean squares ($n = 4$).

Although injections of $GABA_A$ receptor antagonists into the BLA produce an anxiogenic-like effect, other explanations are possible. Because injections of both BMI and PIC into the BLA produce cardiovascular changes (13) associated with anxiety (increase in HR and BP), the peripheral cardiovascular effects might induce an anxiety-like state. However, we have found that before GABA blockade in the BLA, peripheral administration of atenolol, a selective β_1-receptor antagonist that inhibits sympathetic stimulation of the heart with negligible central effects, can attenuate the cardiovascular symptoms without increasing SI time (Fig. 4). This finding suggests that

the anxiogenic-like effect may develop independently of the cardiovascular responses.

In summary, microinjection of $GABA_A$ antagonists BMI and PIC into the BLA and the $GABA_A$ agonist MUS into the Ce, elicited anxiogenic- and anxiolytic-like effects, respectively. Injecting BMI into the Ce or MUS into the BLA had no effects on experimental anxiety. No changes in anxiety-related behavior were obtained upon injection of $GABA_B$-receptor antagonist and agonists SAC and BAC, respectively, or the glycine antagonist STR into the BLA. The increase in HR produced by BMI injection into the BLA was attenuated by prior IP administration of the β_1 antagonist atenolol without attenuating the anxiogenic-like response. These results suggest that a population of tonically active GABAergic neurons in the BLA act to regulate cardiovascular and behavioral effects associated with anxiety by their inhibitory action at $GABA_A$ receptors, and that the experimental anxiety induced by blocking the $GABA_A$ receptors in the BLA is not secondary to increases in HR and BP.

ACKNOWLEDGEMENTS

This work was supported in part by PHS Grant MH 45362 and by the IUPUI Research Investment Fund. The authors thank Tony Gardner and William Rusche for technical assistance.

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