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Modulation of the pressor response elicited by carbachol and electrical stimulation of the amygdala by muscarinic antagonists in conscious rats

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1 The nature of the muscarinic receptor involved in mediating cardiovascular changes caused by unilateral microinjection of carbachol (5 nmol) into, and electrical stimulation (200–300 μA) of, the amygdaloid complex was investigated in conscious, unrestrained female Sprague-Dawley rats.

2 Unilateral microinjection of carbachol (5 nmol; $n=6$) and electrical stimulation (200–300 μA, 80 Hz, 30 s; $n=4$) caused a significant rise in blood pressure of 21 ± 4 mmHg and 25 ± 5 mmHg, respectively. These changes were associated with no overall effect on heart rate. The effects of electrical stimulation were found to be repeatable.

3 Pretreatment i.c.v. with pirenzepine (5–20 nmol; $n=6-7$ for each dose), dose-dependently inhibited the rise in blood pressure induced by carbachol, whereas AF-DX 116 (100 nmol; $n=6$) failed to have any effect on the carbachol-induced pressure response. Neither antagonist alone had any effect on resting baseline variables.

4 Unilateral microinjections of atropine sulphate (1–100 nmol; $n=4-6$ for each dose), pirenzepine (0.03–10 nmol; $n=4$ for each dose) or AF-DX 116 (10–60 nmol; $n=4-5$ for each dose), into the amygdala, dose-dependently inhibited the rise in blood pressure caused by electrical stimulation (200–300 μA). The ID_{50} values were 1.05, 0.23 and 39.5 nmol, respectively. Although pirenzepine seemed to be more potent than atropine, this difference was not significant.

5 It is concluded that the rise in blood pressure elicited by unilateral microinjection of carbachol into, or electrical stimulation of, the amygdaloid complex is mediated by M_1-muscarinic receptors.

Keywords: Muscarinic receptors; M_1-muscarinic receptors; carbachol; atropine; pirenzepine; AF-DX 116; amygdala; blood pressure; conscious rats

Introduction

Centrally acting cholinoceptor agonists and cholinesterase inhibitors are known to induce pressor (Varagic, 1955; Dirnhuber & Cullumbine, 1955; Krstic & Djurkovic, 1973; Ozawa & Uematsu, 1976; Buccafusco & Brezenoff, 1979; Brezenoff & Giuliano, 1982; Oktay *et al*., 1984; Sundaram *et al*., 1988; Özkutlu *et al*., 1993) and depressor responses (De Wildt & Porsius, 1981; Criscione *et al*., 1983; Murugian *et al*., 1989; Sundaram *et al*., 1989; Hara *et al*., 1992; Ally *et al*., 1993) associated with a tachycardia or a bradycardia depending on species and mode of administration. In rats, activation of central muscarinic receptors results primarily in hypertension (see Buccafusco, 1996). This occurs when cholinomimetics are administered into the cerebral ventricles or microinjected into the following brain areas; posterior hypothalamic nucleus (Brezenoff & Jenden, 1969; Brezenoff & Wirecki, 1970; Brezenoff, 1972; Buccafusco & Brezenoff, 1979; Martin, 1992), AV3V region (Nattie & Li, 1990; Menani *et al*., 1990), hippocampus (Haruta *et al*., 1992), locus ceruleus (De Luca *et al*., 1990), C1 area of the rostral ventrolateral medulla (Sundaram *et al*., 1988; Giuliano *et al*., 1989) and the amygdaloid complex (Ohta *et al*., 1991).

The amygdaloid complex forms dense neuronal connections with the hypothalamus and brainstem, areas that are known to play an important role in cardiovascular regulation (see Dampney, 1994). The complex has been demonstrated to contain acetylcholine and acetylcholinesterase (Hoover *et al*., 1978) and muscarinic binding sites (Rotter *et al*., 1979). Further, the pressor response evoked by the cholinomimetic carbachol (i.c.v.) was suppressed by electrolytic lesions of the central nucleus of the amygdaloid complex (Özkutlu *et al*., 1995). The present study was designed to investigate the type of muscarinic receptor that mediates the carbachol-evoked pressor response when injected into the central nucleus of the amygdaloid complex and whether a similar muscarinic receptor is involved in mediating the pressor response to electrical stimulation of this nucleus. A preliminary account of some of these observations has been presented to the British Pharmacological Society (Aslan *et al*., 1995).

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Methods

Animals

Experiments were performed in female albino Sprague-Dawley rats weighing 250–300 g. All animals were fed a standard diet and water *ad libitum* and kept at room temperature ($20\pm3^{\circ}$C) in an air-conditioned room.

Surgical procedures

Rats were anaesthetized with ketamine (100 mg kg^{-1}, i.p.) and chlorpromazine (1.0 mg kg^{-1}, i.p.). After the onset of surgical anaesthesia the head was fixed in a stereotaxic apparatus (Stoelting Model 51600). In one group of rats (electrical stimulation group), a guide cannula-electrode (0.25 mm in diameter; C313G-MS303, simultaneous bipolar electrode-cannula system, Plastic One Inc.), bared at the tip, was implanted into the right central nucleus of the amygdala (2.3 mm caudal to bregma, 4.2 mm lateral to the midline and 8.0 mm ventral to the surface of the skull) on the basis of the stereotaxic atlas of Paxinos and Watson (1982) (Figure 1). In another group

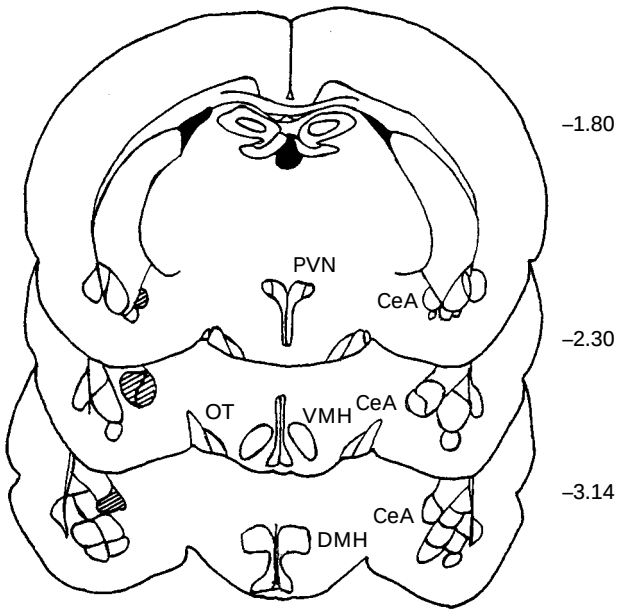

Figure 1 The sites of stimulation (hatched areas) shown on diagrams of coronal section through ventral forebrain (1.80, 2.30 and 3.14 mm posterior to bregma). CeA, central nucleus of the amygdala; DMH, dorsomedial hypothalamic nucleus; OT, optic tract; PVN, paraventricular nucleus; VMH, ventromedial hypothalamic nucleus.

(carbachol-injected group), 26 gauge stainless steel guide cannulae were implanted into the right central nucleus of the amygdala (coordinates given above) and the left lateral cerebral ventricle (1 mm caudal to bregma, 1.5 mm lateral to the midline, and 3.4 mm ventral to the surface of the skull). Injection stylets extending 1 mm below the tips of the guide cannulae were inserted for paranchymal administration of drugs. The cannulae were fixed by dental cement together with three screws driven into the skull, and plugged with a removable stylet except at the time of drug injections.

Three to five days after this surgical intervention, animals were anaesthetized with ether and a polyethylene catheter (PE 10 attached to PE 50) filled with heparin/saline (500 u ml^{-1}) solution, was inserted into the abdominal aorta through the right femoral artery for direct blood pressure recordings. The other end of the tubing was passed beneath the skin and exteriorized for a distance of 3 cm through an incision in the nape of the neck. A stainless wire plug was placed in the exposed end of the catheter until the experiment. Each animal was moved into a plexiglass cage ($25 \times 25 \times 30$ cm). Experiments were conducted at least 2 h after the operation in conscious and freely moving rats.

Microinjections of drugs

Microinjections into the central nucleus of the amygdala were done slowly within 1 min in a final volume of 200 nl via a 1 μl microsyringe (Hamilton) through the cannula connected to an infusion pump (Kd Scientific, model 120, U.S.A.). Intracerebroventricular (i.c.v.) injections were done in a total volume of 10 μl.

At the end of the experiments, 200 nl and 10 μl of Indian ink was injected into the central nucleus of the amygdala and lateral cerebral ventricle, respectively, and the animals were transcardially perfused with 4% buffered formalin solution. The brains were then removed, 40 μm coronal sections were cut through the amygdala region by using a cryostat (Microm, F.R.G) and stained with thionin for light microscopic examination. Only proper cannula and cannula-electrode placements were included in the study.

Electrical stimulation

After the stabilization period, the central nucleus of the amygdala was stimulated by using a stimulator (Grass, model S88, U.S.A.) through the cannula-electrode implanted 3–5 days before the experiment. The electrical stimulation consisted of 1 ms pulses at 80 Hz for 30 s, at an intensity of 200–300 μA. The guide cannula served as anode.

Experimental protocols

The arterial cannula was connected to a pressure transducer (Grass, Model P23ID, U.S.A.) and arterial blood pressure was recorded on a polygraph (Grass, Model 7, U.S.A.). Following a 2 h stabilization period, basal arterial blood pressure and heart rate were established. The heart rate of rats in the carbachol injected group was recorded continuously via a tachograph (Grass, Model 7P44, U.S.A.) whereas basal heart rate was obtained by direct counting of pulsatile pressure wave-forms in the electrical stimulation group. Since preliminary experiments have shown us that heart rate changes in response to the electrical stimulation of the central nucleus of the amygdala were variable (unpublished observations), heart rate was not measured in these experiments.

Carbachol injected group Carbachol (5 nmol) was injected into the central nucleus of the amygdala preceded by intracerebroventricular saline (10 μl; $n=6$), pirenzepine (5–20 nmol; $n=6-7$ for each dose) or AF-DX 116 (100 nmol; $n=6$) injection 15 min earlier. Arterial blood pressure was monitored for 30 min after the carbachol injection.

Electrical stimulation group Following two successive control stimulations, saline (200 nl; $n=4$), atropine (1–100 nmol; $n=4-6$ for each dose), pirenzepine (0.03–10 nmol; $n=4$ for each dose) or AF-DX 116 (10–60 nmol; $n=4-5$ for each dose) was injected into the central nucleus of amygdala 15 min before the third stimulation. Only saline or a single dose of atropine, pirenzepine or AF-DX 116 was injected into each animal.

Drugs

The following drugs were used: ketamine HCl (Ketalar, Parke-Davis, gift from Eczacibasi, Turkey), chlorpromazine HCl (gift from Eczacibasi, Turkey), atropine sulphate (Sigma, St. Louis, MO, U.S.A.), pirenzepine dihydrochloride (Sigma, St. Louis, MO, U.S.A.), AF-DX 116 (11-[[2-[(Diethylamino) methyl] -1-piperidynl]acetyl] -5,11-dihydro - 6H-pyridol[2,3 - b][1,4] benzodiazepin - 6 - one, gift from Dr Karl Thomae GmbH, F.R.G.), heparin sodium (Liquemine, gift from Roche, Turkey). All drugs were dissolved and diluted in saline.

Data analysis

The results are expressed as mean $\pm$ s.e.mean of 4–7 rats in each group. Mean arterial pressure (MAP) was calculated as '1/3 pulse pressure + diastolic blood pressure'. Data were statistically evaluated by one way analysis of variance (ANOVA) and *post-hoc* comparisons were made with Dunnett's test. A P value <0.05 was taken to indicate a significant difference. The ID_{50} values were calculated by probit analysis, the confidence limits were determined and the potencies of the antagonists were compared according to Litchfield and Wilcoxon (1949). In the electrical stimulation group, the mean of two subsequent pressor responses before drug injection was considered as control and % changes from this control response, after atropine, pirenzepine and AF-DX 116 were determined. In the carbachol injected group, the mean pressor response to carbachol given into the central nucleus of the amygdala in saline pretreated rats was accepted as the control response and those obtained in antagonist-treated animals were expressed as %

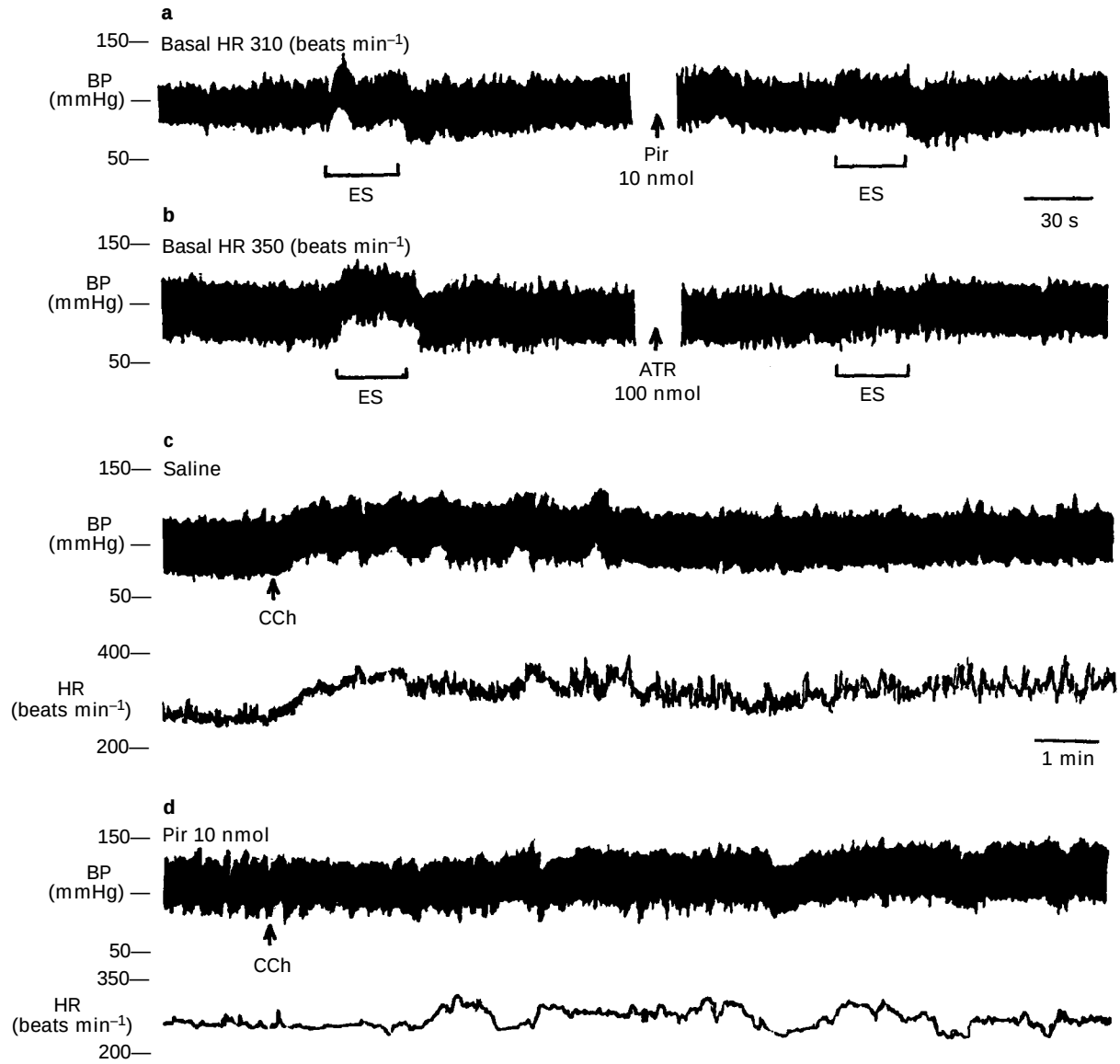

Figure 2 (a and b) Typical traces obtained from two different animals showing the blood pressure (BP) responses to electrical stimulation (ES; 1 ms, 80 Hz, 200 μA for 30 s) of central nucleus of the amygdala before and after (a) 10 nmol pirenzepine (Pir) and (b) 100 nmol atropine (Atr). (c and d) Representative records of the blood pressure (BP) response to carbachol (CCh; 5 nmol) injected into the central nucleus of the amygdala and the accompanying heart rate (HR) changes of (c) an animal pretreated with i.c.v. saline (10 μl) and (d) another pretreated with 10 nmol i.c.v. pirenzepine.

Table 1 Heart rate changes in response to carbachol (5 nmol) injected into the central nucleus of the amygdala of rats pretreated with i.c.v. saline, pirenzepine or AF-DX 166

	Heart rate (beats min^{-1}) *Time after carbachol injection* (min)					
Group	*0*	*1*	*3*	*5*	*15*	*30*
Saline ($n=6$)	362±22	393±12	417±22	398±19	357±29	365±12
Pirenzepine 5 nmol ($n=6$)	357±23	373±20	387±20	400±21	365±23	360±21
Pirenzepine 10 nmol ($n=6$)	363±23	362±23	377±25	375±18	373±18	360±20
Pirenzepine 20 nmol ($n=7$)	357±10	366±18	367±18	370±22	351±13	350±14
AF-DX 116 100 nmol ($n=6$)	308±14	312±17	342±22	360±22	356±39	350±27

Numbers of animals (n) in each group are in parentheses. One-way ANOVA did not show any statistically significant change in any group.

changes from control.

Results

The resting MAP and heart rate in all animals ($n=85$) were 104 ± 1 mmHg and 363 ± 9 beats min^{-1}. There were no significant differences in the pre-drug resting MAP or heart rate between the groups. None of the antagonists affected baseline MAP or heart rate values significantly.

Chemical stimulation with carbachol

Carbachol, when injected into the central nucleus of the amygdala at a dose of 5 nmol, caused a 21 ± 4 mmHg increase in MAP ($n=6$) (Figures 2 and 3). The pressor response started immediately after carbachol injection, reached its maximum at 10 min and MAP returned to the basal values within 30 min. Although carbachol produced tachycardia in five and a bradycardia in one of the six rats, the overall increase was not significant (Table 1). Pirenzepine pretreatment inhibited the carbachol-induced pressor responses dose-dependently ($ID_{50}=7.99$ nmol, confidence limits: 1.63–9.03). The MAP changes were completely abolished by 20 nmol pirenzepine injected into the lateral cerebral ventricle whereas 100 nmol AF-DX 116 had no significant effect on these responses (Figure 3).

Electrical stimulation

Electrical stimulation of the central nucleus of the amygdala caused reproducible increases in MAP. A rapid rise in blood pressure was seen immediately after the initiation of the electrical stimulation and blood pressure returned to basal values as soon as stimulation was stopped (Figure 2). The pressor responses to two subsequent stimulations were not significantly different from each other. The mean control pressor response to electrical stimulation of the central nucleus of the amygdala was 25 ± 5 mmHg ($n=4$). Some behavioural changes such as rotation of the body and jaw movements were also observed. Saline injection into the central nucleus of the amygdala did not alter the behavioural or the pressor responses to electrical stimulation (30 ± 5 mmHg). The mean control responses were not significantly different from each other between groups injected with either saline, atropine, pirenzepine or AF-DX 116.

Atropine blocked the pressor effect of electrical stimulation of the central nucleus of the amygdala in a dose-dependent manner ($P<0.01$; Figure 4). The inhibition was 19.0 ± 6.8, 56.4 ± 5.5, 71.0 ± 5.7 and $101.8\pm3.2\%$ with 0.1, 1, 10 and

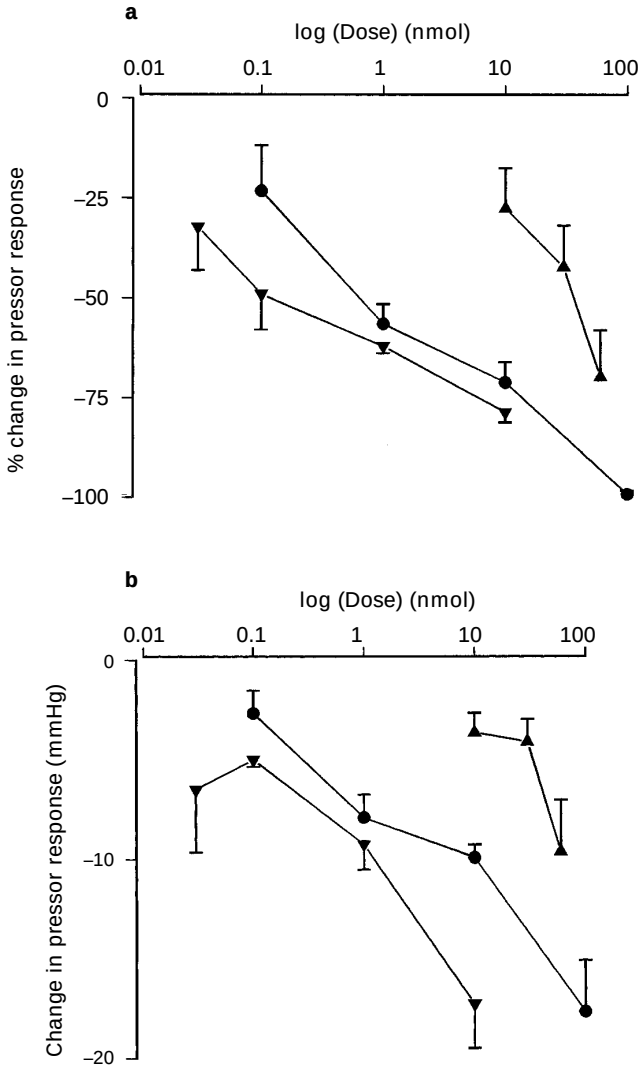

Figure 4 The inhibitory effect of atropine (●), pirenzepine (▼) and AF-DX 116 (▲) on the pressor responses to the electrical stimulation of the central nucleus of the amygdala. Responses expressed as (a) % change and (b) mmHg. Atropine, pirenzepine or AF-DX 116 was injected into the central nucleus of the amygdala. Each point represents the mean of 4–6 independent experiments; vertical lines show s.e.mean. ID_{50} values with 95% confidence limits in parentheses were: pirenzepine 0.23 nmol (0.01–5.42), atropine 1.05 nmol (0.13–8.39), AF-DX 116 39.5 nmol (11.4–137.5).

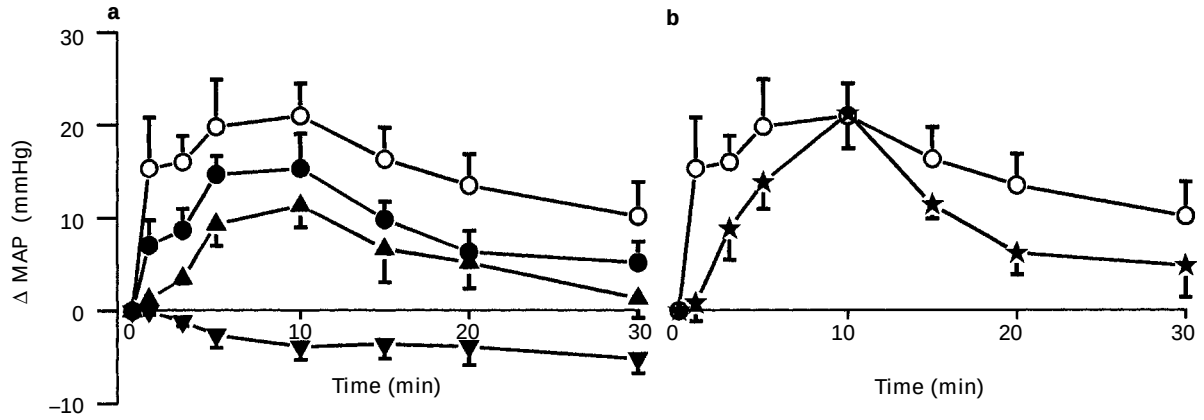

Figure 3 Changes in MAP induced by carbachol (5 nmol) injected into the central nucleus of the amygdala of animals pretreated with either (a) saline (○), pirenzepine (5 (●); 10 (▲) and (▼) 20 nmol) or (b) AF-DX 116 100 nmol (★). Data from the saline group is shown in both (a) and (b). Each point represents the mean of 6–7 independent experiments; vertical lines show s.e.mean.

100 nmol atropine, respectively. The ID_{50} was 1.05 nmol (95% confidence limits: 0.13 – 8.39). Atropine completely abolished the behavioural changes at the highest dose used in this study.

Similarly, pirenzepine prevented the increase in MAP in response to electrical stimulation of the central nucleus of the amygdala dose-dependently ($P<0.01$; Figure 4). The inhibitory effect of pirenzepine was 32.2 ± 12.5, 49.0 ± 10.2, 62.0 ± 2.0 and 78.5 ± 3.0% with 0.03, 0.1, 1 and 10 nmol doses, respectively (Figure 4). The ID_{50} was 0.23 nmol (95% confidence limits: 0.01 – 5.42). The potencies of atropine and pirenzepine in inhibiting electrical central amygdaloid nucleus stimulation-induced pressor responses were not significantly different from each other.

On the other hand, AF-DX 116 ($ID_{50}=39.5$ nmol; 95% confidence limits: 11.4 – 137.5) was 140.3 times less potent than pirenzepine in antagonizing electrical stimulation-induced pressor responses (Figure 4). The inhibitory effect of AF-DX 116 was 27.5 ± 10.0, 42.2 ± 10.4 and 69.6 ± 11.7% with 10, 30 and 60 nmol doses, respectively. Interestingly, both pirenzepine and AF-X 116 did not seem to abolish the behavioural changes at the doses used in this study.

Discussion

The present data demonstrate that the microinjection of carbachol into, and also that electrical stimulation of, the central nucleus of the amygdala in conscious, unrestrained female Sprague-Dawley rats evokes a pressor response associated with no change in heart rate. Similar results have been obtained by Gelsema *et al*. (1987) and Ohta *et al*. (1991). The carbachol-evoked pressor response was found to be attenuated in a dose-dependent manner by i.c.v. pretreatment with the selective M_1-muscarinic antagonist, pirenzepine (Hammer *et al*., 1980) but not by selective M_2-muscarinic receptor antagonist, AF-DX 116 (Giachetti *et al*., 1986). The dose of AF-DX 116 was 5 times larger than the highest dose of pirenzepine used in this study. However, unilateral microinjections of atropine, pirenzepine and AF-DX 116 dose-dependently inhibited the evoked rise in blood pressure caused by electrical stimulation of the central nucleus. The potency of pirenzepine was not significantly different from atropine whereas AF-DX 116 was 140 times less potent in inhibiting the electrically-induced pressor response. As atropine and pirenzepine have similar potencies and AF-DX 116 is 50 times less potent than pirenzepine at M_1-muscarinic receptors (Michel *et al*., 1989; Doods *et al*., 1993), the combined data suggest that these receptors mediated the above evoked pressor response. In addition, as AF-DX 116 is ten times more potent at M_2-muscarinic receptors and also 5 times less potent at M_4-muscarinic receptors than pirenzepine (Doods *et al*., 1993), these data further support the view that it is the M_1-subtype mediating this pressor response.

The amgydala has been implicated in the control of several autonomic functions such as respiration and cardiovascular functions including the regulation of blood pressure (Dampney, 1994; Davis *et al*., 1994). For instance, the ablation of the amygdala has been shown to delay the development of hypertension and attenuate the exaggerated pressor responses to noise stress in spontaneously hypertensive rats (Galeno *et al*., 1982; 1984). Further, ablation of the central nucleus of the amygdala significantly attenuates i.c.v. carbachol-induced pressor responses in both anaesthetized and conscious rats (Özkutlu *et al*., 1995). These combined observations suggest that, at the level of the amygdaloid complex, cholinergic pathways acting via M_1-muscarinic receptors may play an important role in the regulation of blood pressure and in stress-induced hypertension. However, the present data suggest that this pathway is more important in changes in peripheral resistance than in control of changes in cardiac output, as heart rate was unaffected by the above interventions.

In conclusion, the present data support the view that M_1-muscarinic receptors seem to be the major subtype involved in cardiovascular regulation (Barnes & Roberts, 1991; Polidori *et al*., 1991; Scheucher *et al*., 1991), mediated by central cholinergic pathways, and that the central nucleus of the amygdala is an important site for this integration.

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