Research Report

Cardiovascular effects of acetylcholine microinjection into the ventrolateral and dorsal periaqueductal gray of rats

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ABSTRACT

In the present study, we describe the cardiovascular effects of local acetylcholine (Ach) microinjection into both the ventrolateral (vlPAG) and dorsal (dPAG) periaqueductal gray areas of anesthetized rats and the possible local receptors involved with these responses. Microinjection of Ach (9, 27, 45 or 81 nmol/50 nL) into the vlPAG caused dose-related depressor responses. These hypotensive responses were blocked by local pretreatment with increasing doses of the nonselective muscarinic antagonist atropine (1, 3 or 9 nmol/50 nL). The microinjection of Ach into the dPAG caused no significant cardiovascular responses in anesthetized rats. In conclusion, the present findings suggest that a cholinergic system present in the vlPAG, but not in the dPAG, is involved with cardiovascular system control. Moreover, these cardiovascular responses evoked by Ach are mediated by muscarinic receptors.

1. Introduction

The periaqueductal gray area (PAG) is a mesencephalic region that integrates behavioral and cardiovascular responses in rodents (Huang et al., 2000; Jenck et al., 1989; Nashold et al., 1969). The PAG is functionally subdivided into four longitudinal columns along its rostrocaudal axis: the dorsomedial, dorsolateral, lateral and ventrolateral columns (Bandler et al., 1991).

PAG neurons present projections to numerous structures involved in cardiovascular regulation, among them the rostral ventrolateral medulla (RVLM) (Chen and Aston-Jones, 1995; Farkas et al., 1998; Van Bockstaele et al., 1989). Particulary, electrical stimulation of the dorsal PAG (dPAG) increases arterial pressure through a sympathoexcitatory action (Kubo et al., 1999). However, electrical stimulation of the dorsal,

dorsolateral or lateral PAG evokes hypertension, tachycardia and hindlimb vasodilatation in anesthetized rats (Hamalainen and Lovick, 1997; Lovick, 1992a). Additionally, microinjection of DL-homocysteic acid into the dPAG of anesthetized rats has been reported to cause hypertension and tachycardia, whereas depressor and bradycardiac responses have been observed after its injection into the ventrolateral PAG of anesthetized rats (vlPAG) (Bandler et al., 1991; Huang et al., 2000; Lovick, 1985, 1992a; Rossi et al., 1994). Therefore, pressor responses are evoked by dorsal and lateral columns in PAG stimulation, whereas depressor and bradycardiac responses are evoked after stimulation of the ventrolateral PAG (Carrive and Bandler, 1991; Carrive et al., 1989; Lovick, 1992a; Rossi et al., 1994).

Interestingly, Pelosi and Correa (2005) reported that the microinjection of noradrenaline (NA) into either the vlPAG or

the dPAG evoked hypertensive and bradycardiac responses in unanesthetized rats (Pelosi et al., 2008).

It has been described that cholinergic systems of several brain regions are involved in cardiovascular modulation, among them those in the lateral septal area (Scheucher et al., 1987), the posterior hypothalamus (Brezenoff and Xiao, 1989), the nucleus of the solitary tract (Sundaram et al., 1989) and the medial prefrontal cortex (Crippa et al., 1999).

There are results showing the presence of both cholinergic synapses and muscarinic receptors in the PAG (Wamsley et al., 1981). Because a cholinergic system is present in the PAG, and this brain area is involved in central cardiovascular modulation, it is possible to suggest that such PAG cholinergic neurotransmission could also modulate the cardiovascular system. However, there are no reports on the cardiovascular effects of local injection of Ach into the PAG, and particularly into the dPAG or the vlPAG at different rostrocaudal coordinates, in the rat brain.

Therefore, the present work examined the cardiovascular effects of local Ach microinjection into the vlPAG and dPAG columns of anesthetized rats and the subtype of cholinergic receptors that mediate these responses.

2. Results

2.1. Cardiovascular response to the microinjection of different doses of Ach into the different rostrocaudal coordinates of the vlPAG of anesthetized rats

The basal levels of both MAP and HR of the rats used to generate the dose–response curves were, respectively, 91 ± 3 mmHg and 390 ± 8 bpm ($n=20$).

Microinjections of Ach (9, 27, 45 and 81 nmol/50 nL) into the rostral, medial and caudal portions of the vlPAG of anesthetized rats caused dose-related MAP decreases ($r^2=0.92$, *$P<0.05$) (Fig. 1). However, no significant HR changes were observed even at the highest dose of Ach (81 nmol/50 nL) (before: HR $= 390 \pm 12$ bpm and after: HR $= 392 \pm 11$ bpm; $n=5$, $t=0.58$, $P>0.05$). The estimated ED_{50} was approximately 45 nmol/50 nL for the depressor response.

The depressor response (ΔMAP $= -17 \pm 2.5$ mmHg, $n=6$) evoked by microinjection of Ach (45 nmol/50 nL) into the vlPAG was significantly different from those effects observed by injection of 50 nL of ACSF ($n=5$; $t=9$, $P<0.05$). In addition, the microinjection of Ach (45 nmol/50 nL) into the dorsal raphe nucleus and laterodorsal tegmental nucleus (outside the vlPAG) did not cause significant changes in either MAP (before: 93 ± 2.7 mmHg and after: 92.5 ± 3.mmHg; $n=5$, $P>0.05$, $t=0.7$) or HR (before: 391 ± 5.4 bpm and after: 394 ± 5 bpm; $n=5$, $t=0.9$, $P>0.05$).

2.2. Cardiovascular response to the microinjection of different doses of Ach into the different rostrocaudal coordinates of the dPAG of anesthetized rats

The basal levels of both MAP and HR of the rats used to generate the dose–response curves were respectively 93 ± 3 mmHg and 394 ± 7 bpm ($n=12$).
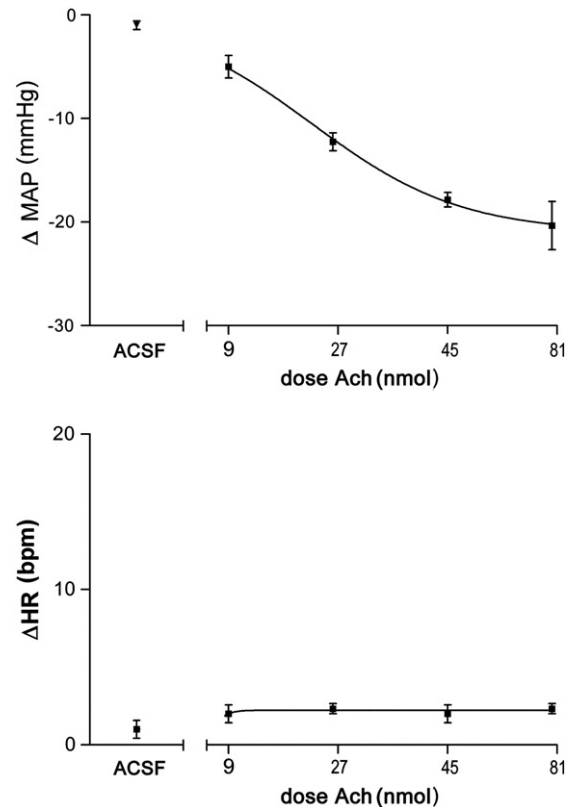

Fig. 1 – Changes in mean arterial pressure (ΔMAP) and heart rate (ΔHR) in response to the microinjection of Ach (9, 27, 45 and 81 nmol/50 nL $n=20$) in the different rostrocaudal coordinates of the vlPAG of anesthetized rats. Columns represent the means and bars the SEM. Dose–effect curves were generated by nonlinear regression analysis and were statistically significant. (ΔMAP, $r^2=0.92$; *$P>0.05$).

Microinjection of Ach (9, 27, 45 or 81 nmol/50 nL) into the rostral, medial or caudal portions of the dPAG did not affect either MAP ($r^2=0.3$, $P>0.05$) or HR ($r^2=0.4$, $P>0.05$).

2.3. Effect of local pretreatment with ACSF or different doses of atropine on blood pressure response to Ach microinjection into the vlPAG of anesthetized rats

2.3.1. Vehicle
Pretreatment of the vlPAG with 50 nL of ACSF ($n=5$) did not affect basal levels of either MAP (before: 92 ± 4.4 mmHg and after: 94 ± 1.2 mmHg, $t=0.64$, $P>0.05$) or HR (before: 405 ± 9.2 bpm and after: 403 ± 6.8 bpm, $t=0.45$, $P>0.05$). Moreover, the pretreatment with ACSF did not affect the hypotensive response evoked by Ach (45 nmol/50 nL) into the vlPAG (ΔMAP before ACSF $= -17.3 \pm 2$ mmHg and ΔMAP after ACSF $= -16.9 \pm 2.3$ mmHg; $n=5$, $t=0.8$, $P>0.05$).

2.3.2. Atropine
Inhibition of MAP responses by the microinjection of Ach (45 nmol/50 nL) into the vlPAG after local pretreatment with different doses of the nonselective muscarinic receptor antagonist atropine (1, 3 and 9 nmol, $n=4$ for each dose).
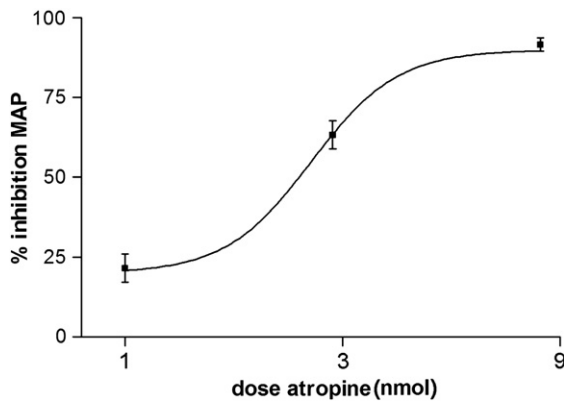
Fig. 2 – Inhibition of MAP responses (expressed as %) by the microinjection of Ach (45 nmol/50 nL) into the vlPAG after local pretreatment with different doses of atropine antagonist (1, 3 and 9 nmol, $n=4$ for each dose). Curves were generated by nonlinear regression analysis. Symbols represent means and vertical lines indicate the S.E. mean.

Pretreatment of the vlPAG with 1 nmol/50 nL did not affect basal levels of either MAP (MAP before atropine: 90 ± 1.7 mmHg and after: 91 ± 2.1 mmHg; $n=12$, $t=1.1$, $P>0.05$) or HR (before atropine: 398 ± 9 bpm and after: 399 ± 6 bpm; $n=12$, $t=0.9$, $P>0.05$).

Pretreatment of the vlPAG with 3 nmol/50 nL did not affect basal levels of either MAP (MAP before atropine: 90 ± 2.5 mmHg and after: 93 ± 3 mmHg; $n=12$, $t=1.1$, $P>0.05$) or HR (before atropine: 402 ± 7.2 bpm and after: 399 ± 6.5 bpm; $n=12$, $t=0.9$, $P>0.05$).

Pretreatment of the vlPAG with 9 nmol/50 nL did not affect basal levels of either MAP (MAP before atropine: 92 ± 2.3 mmHg and after: 93 ± 2 mmHg; $n=12$, $t=1.1$, $P>0.05$) or HR (before atropine: 391 ± 5.7 bpm and after: 387 ± 6.8 bpm; $n=12$, $t=0.9$, $P>0.05$).

Local pretreatment with atropine (1, 3 and 9 nmol/50 nL) caused a dose-related inhibition ($r^2=0.9$) of depressor responses to Ach microinjection into the vlPAG (Fig. 2). The higher dose of atropine inhibited $92\pm3\%$ of the Ach depressor response.

In addition, pretreatment with 9 nmol/50 nL injected into the dorsal raphe nucleus and laterodorsal tegmental nucleus (outside the vlPAG) did not cause inhibition of depressor responses to Ach in the vlPAG (ΔMAP before: -18 ± 2.7 mmHg and after: -16.5 ± 3 mmHg; $n=4$, $P>0.05$, $t=0.7$).

Recordings from a representative anesthetized rat showing the effects of injection of Ach (45 nmol/50 nL) into the vlPAG on both the mean or pulsatile arterial pressure as well as the heart rate, before and 10 min after local pretreatment of the vlPAG with 1 nmol/50 nL of atropine (A), 3 nmol/50 nL (B) and 9 nmol/50 nL (C) are presented in Fig. 3.

2.4. Effect of systemic (i.v.) pretreatment with atropine on the blood pressure response to Ach microinjection into the vlPAG of anesthetized rats

The systemic i.v. administration of the same dose of atropine (9 nmol) microinjected into the vlPAG did not affect basal levels of either MAP (before atropine: 90 ± 2.4 mmHg and after: 92.3 ± 2.3 mmHg; $n=6$ $t=1$, $P>0.05$) or HR (before atropine: 394 ± 9 bpm and after: 397 ± 7 bpm; $n=6$, $t=0.84$, $P>0.05$). Systemic pretreatment with atropine did not affect the hypotensive response evoked by microinjection of 45 nmol of Ach into the vlPAG (ΔMAP before atropine$=-18\pm5$ mmHg and ΔMAP after atropine$=-19\pm4$ mmHg; $t=0.5$, $P>0.05$, $n=6$).

2.5. The distribution of injection sites

The distribution of injection sites in the dPAG, vlPAG and outside the vlPAG of all animals used are presented in Fig. 4 A and B, respectively. Photomicrographs illustrating sites of injection in the dPAG and vlPAG are presented in Fig. 5A and B, respectively.

3. Discussion

In the present study, we report that microinjection of Ach into the rostral, medial and caudal portions of the vlPAG of anesthetized rats evoked dose-dependent hypotensive responses. However, no significant cardiovascular changes were observed after its injection into the rostral, medial or caudal portions of the dPAG.

Mapping of PAG areas in which chemical stimulation evoked cardiovascular responses was performed in both cats and rats and indicated that the PAG is organized as rostro-caudal columns (Carrive and Bandler, 1991; Lovick, 1985, 1992a). Such organization may explain why different cardio-vascular responses were observed when Ach was microinjected into different portions of the PAG. The depressor responses observed when Ach was microinjected into the vlPAG were similar to those reported after the injection of DL-homocysteic acid into the same area (Bandler et al., 1991; Huang et al., 2000; Lovick, 1985, 1992a; Rossi et al., 1994).

The fact that no significant HR changes were observed after its microinjection into the vlPAG could be a consequence of an impaired baroreflex response. Baroreflex activity has been reported to be blunted under anesthesia (Crippa et al., 2000; Fluckiger et al., 1985; Shimokawa et al., 1998), thus reducing the range of ΔHR changes and resulting in smaller reflex responses.

Studies using tracing techniques have indicated that several brain regions, including the PAG, provide afferent inputs to the RVLM (Van Bockstaele et al., 1991). The PAG is thought to be involved in cardiovascular control, perhaps via a relay in the RVLM (Carrive et al., 1989; Keay et al., 2000; Lovick, 1992b; Verberne and Struyker Boudier, 1991). Neurons in the vlPAG have been retrogradely labeled when a tracer was injected into the RVLM and hypotensive responses observed after its stimulation seem to be mediated by an inhibition of those pressor neurons (Carrive and Bandler, 1991). In addition, the vlPAG is also connected with depressor regions in the caudal midline medulla and caudal ventrolateral medulla, which may in part contribute to the vlPAG-mediated hypo-tension (Henderson et al., 1998).

According to Bandler and Shipley (1994), the vlPAG participates in the organization of more passive responses that tend to reduce the physiological and emotional impact of
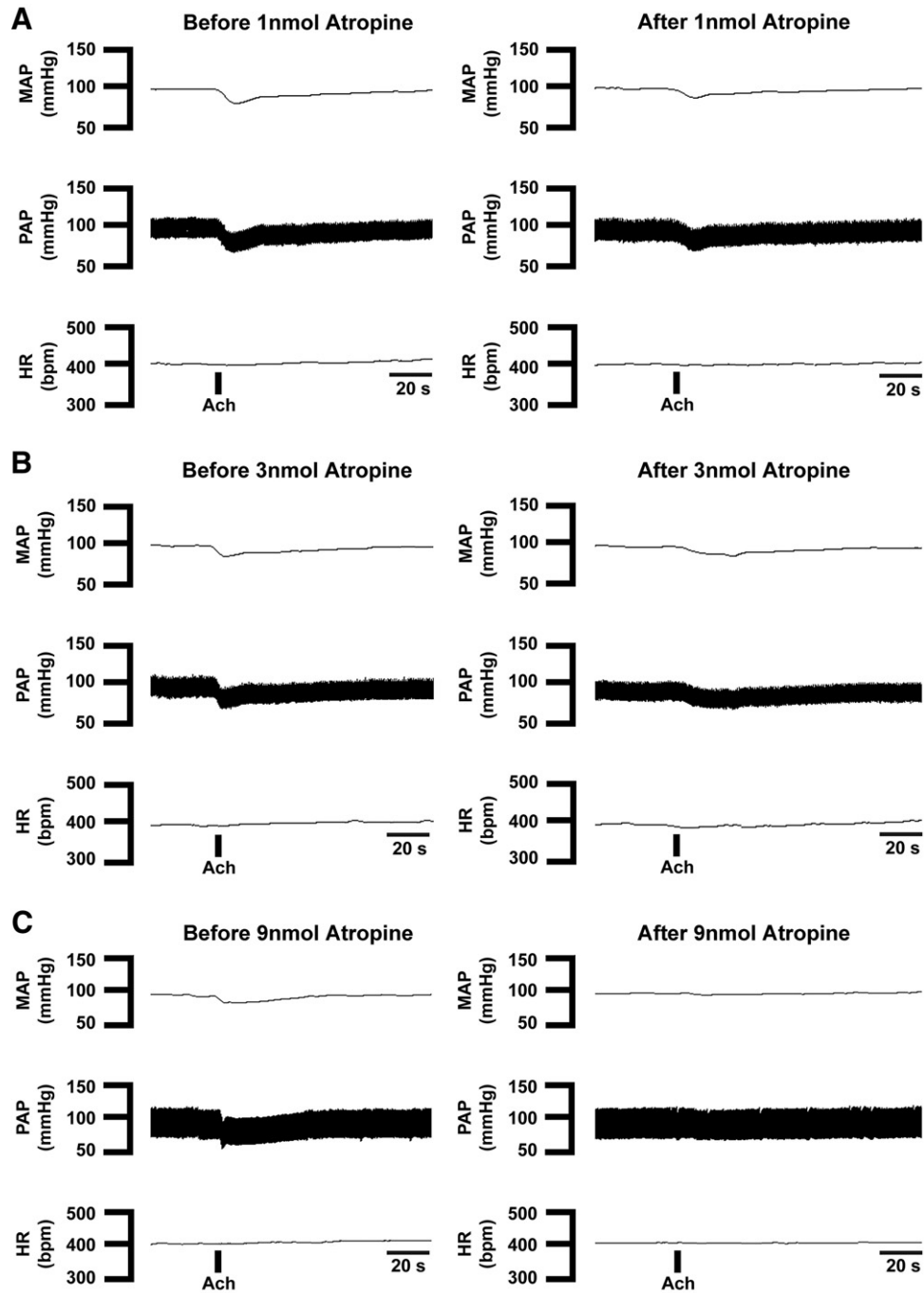
Fig. 3 – Recordings of a representative anesthetized rat showing the effects on mean arterial pressure (MAP), pulsatile arterial pressure (PAP), and heart rate (HR) of the injection of 45 nmol/50 nL Ach into the vlPAG, before and 10 min after local pretreatment of the vlPAG with 1 nmol/50 nL of atropine (A), 3 nmol/50 nL (B) and 9 nmol/50 nL (C).

an inescapable stimulus. This region of the PAG also coordinates autonomic correlates of defense reactions (Depaulis et al., 1994; Zhang et al., 1990). The activation of neurons in the vlPAG evokes a decrease in the arterial pressure as well as changes in the vasoconstrictor sympathetic tonus (Carrive and Bandler, 1991). Monassi and colleagues (1997) reported that the microinjection of a cholinergic agonist into the vlPAG increased the duration of tonic immobility episodes. The cholinergic system in the PAG has

also been reported to be activated in subordinate rats during encounters that result in social defeat (Kroes et al., 2007). Conversely, stimulation of the vlPAG evokes submissive behavior. In most cases these behavioral changes are accompanied by a decrease in the arterial pressure (Carli, 1974).

Although there is no clear evidence of the mechanism involved in the mediation of the hypotensive response to the injection of Ach into the vlPAG, this mechanism may involve an inhibition of the sympathetic nervous system. Neurons in the
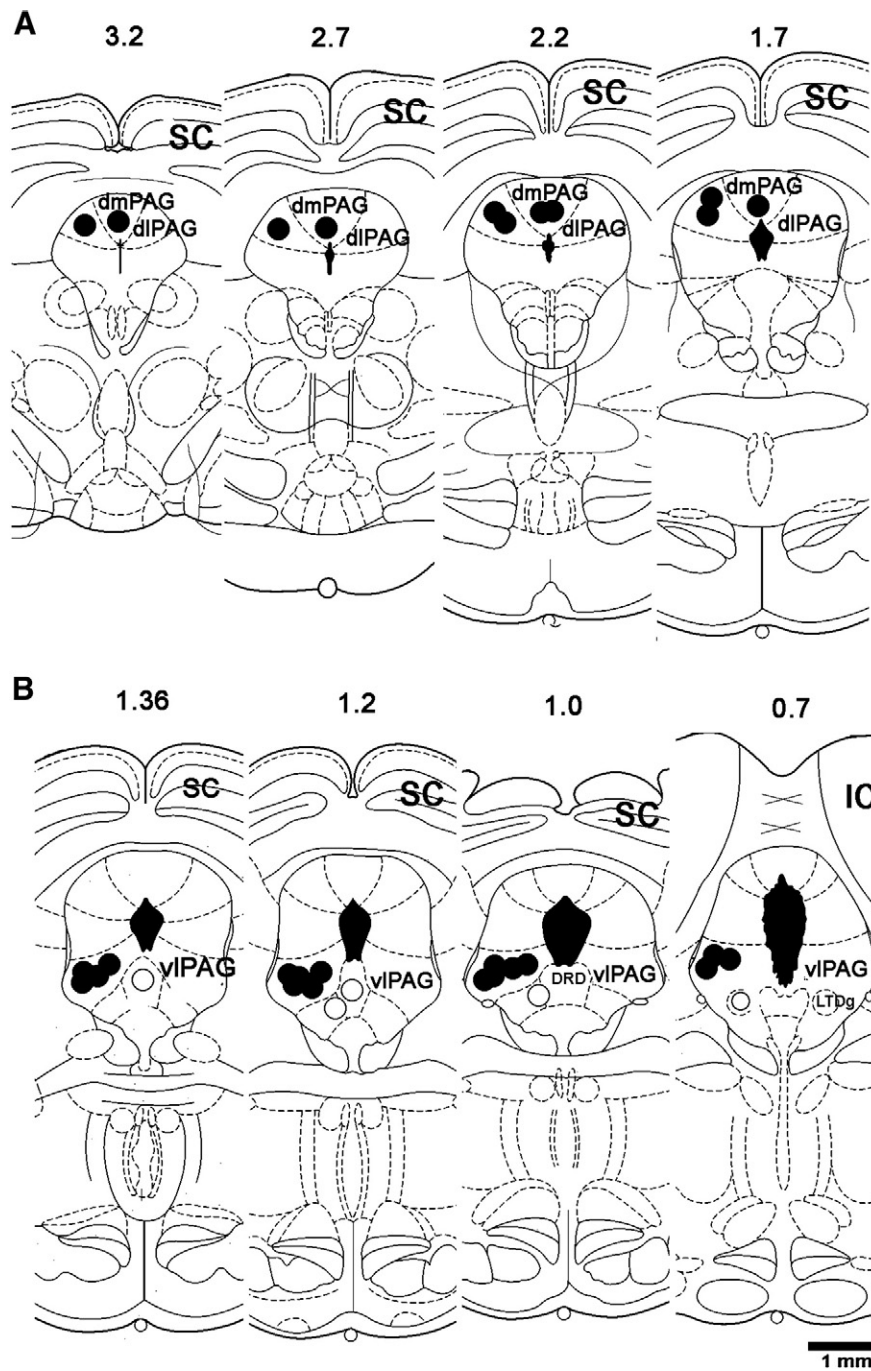
Fig. 4 – Diagrammatic representation of rat brain frontal sections modified from the atlas of Paxinos and Watson (1997), indicating the sites of microinjection: A) exclusively into the dPAG (closed circles), B) vlPAG of anesthetized rats (closed circles) and outside of vlPAG (open circles). SC, superior colliculus; IC, inferior colliculus; DRD, dorsal raphe nucleus; LDTg, laterodorsal tegmental nucleus; IA, distance from the interaural line.

dPAG also have been shown to project to the sympathoexcitatory region of the RVLM (Carrive et al., 1988). Experimental evidence indicates that the microinjection of Ach into the dPAG causes a pressor response in anesthetized rats (Pizzirusso et al., 1998) However, in the present report no significant cardiovascular changes were observed after the microinjection of Ach into this area. One possible explanation is that in Pizzirusso's study, Ach could be reaching areas outside the dPAG as a result of the higher volume used (100 nL). Additionally, earlier studies

have shown that cardiovascular responses can be evoked after chemical stimulation of the superior colliculus, a site very close to the dPAG (Keay et al., 1988; Pelosi et al., 2007).

There is a dense plexus of cholinergic nerve terminals in the PAG (Woolf et al., 1990). Acetylcholine's physiological effects result from the activation of either ligand-gated nicotinic cholinergic receptors (nAchRs) or G protein-coupled receptors (mAchRs). There are five subtypes of mAchRs: the M_2 and M_4 subtypes that are coupled via Gi/o proteins and the M_1, M_3, and
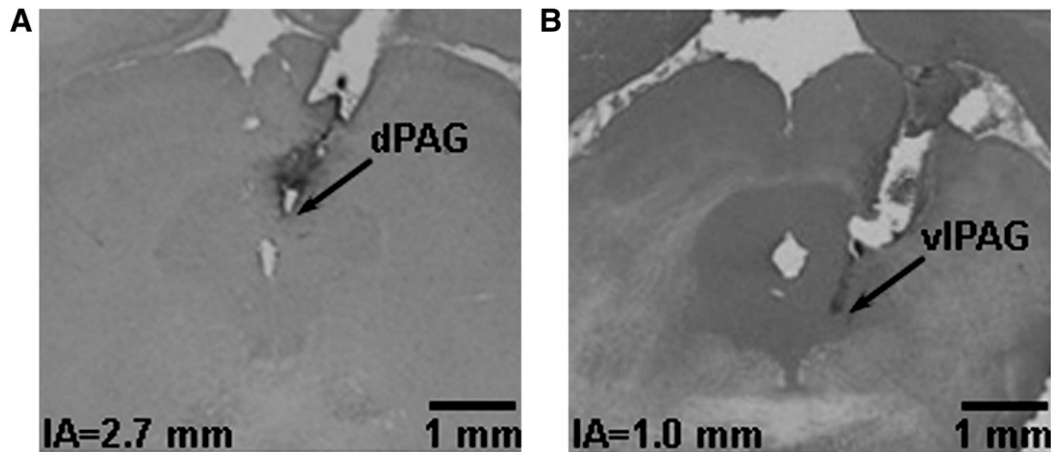
Fig. 5 – Photomicrograph of coronal rat brain sections showing examples of injection sites in the dPAG (A) and vlPAG (B): dPAG, dorsal periaqueductal gray; vlPAG, ventrolateral periaqueductal gray; IA, distance from interaural line.

M_5 subtypes that are coupled via Gq proteins (Caulfield, 1993). The midbrain PAG contains a range of mAchR subtypes (Aubert et al., 1996; Yasuda et al., 1993), and so it becomes relevant to assess the involvement of these receptors in the modulation of the cardiovascular response to the microinjection of Ach into the vlPAG. First, we pretreated the vlPAG with the nonselective muscarinic receptor antagonist atropine, which blocked the hypotensive effect of Ach, thus suggesting that muscarinic receptors within the vlPAG mediate the response. The injection of atropine into the vlPAG caused no effect on baseline blood pressure, which may indicate that vlPAG cholinergic mechanisms do not exert a tonic influence on cardiovascular control in anesthetized rats. More specific antagonists such as 4-DAMP and pirenzepine should be used in future studies to identify the subtype of muscarinic receptor that mediates the hypotensive response to the injection of Ach into the vlPAG.

Because Ach is a potent vasodilator, there is a possibility that the hypotensive effect observed after its microinjection into the vlPAG could be due to drug spreading from its injection site to the systemic circulation. However, the idea that Ach is indeed activating receptors in the vlPAG is favored by the observation that an i.v. injection of 9 nmol atropine, which blocked the effect of Ach when applied to the vlPAG, did not affect the response to the injection into the vlPAG. In addition, the microinjection of Ach into the dPAG did not evoke significant cardiovascular changes, thus suggesting that the effect observed after its microinjection into the vlPAG is not consequent to a spreading into the systemic circulation.

In conclusion, our results indicate that a cholinergic system within the vlPAG is involved in the control of cardiovascular responses, acting through the activation of local muscarinic receptors. The results also suggest that the dPAG's cholinergic mechanism is not involved in the cardiovascular control.

4. Experimental procedures

4.1. Animals

Experimental procedures were carried out following protocols approved by the ethical review committee of the School of Medicine of Ribeirão Preto, University of São Paulo. Male Wistar rats weighing 220–260 g ($n=38$) were used in the present experiment. Animals were housed in plastic cages in a temperature-controlled room (25 °C), under a 12:12 h light–dark cycle. Animals had free access to water and standard laboratory chow, except during the experimental period. The Institution's animal ethics committee approved housing conditions and experimental protocols (protocol 168/2007).

4.2. Surgical preparation

For implantation of stainless steel guide cannulas in the vlPAG or the dPAG, animals were anesthetized with tribromoethanol (250 mg/kg i.p., Aldrich Chemical Co. Inc., USA). After local anesthesia with 2% xylocaine, the skull was surgically exposed and stainless steel guide cannulas (24 G) were implanted 1 mm above the injection sites using a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Stereotaxic coordinates for cannula implantation in the vlPAG or the dPAG were selected from the brain atlas of Paxinos and Watson (1997). The following coordinates were used: vlPAG: AP=+1.2 mm from the interaural line, L=+2.5 mm from the medial suture and V=−5.1 mm deep from the skull with a lateral inclination of 18°; dPAG: AP=+2.7 mm from the interaural line, L=+1.5 mm from the medial suture and V=−4.8 mm deep from the skull with a lateral inclination of 26°. Cannulas were fixed to the skull with dental cement and one metal screw. A tight-fitting mandrel was kept inside the guide cannula to avoid its occlusion. After surgery, animals were treated with a polyantibiotic preparation of streptomycins and penicillins i.m. (Pentabiotico®, Fort Dodge, Brazil) to prevent infection and with the nonsteroidal anti-inflammatory flunixine meglumine (2.5 mg kg^{-1} s.c.; banamine®, Schering Plough, Brazil) for postoperative analgesia. The cannula was chronically implanted to be used for microinjections in anesthetized rats. This approach was taken to allow potential integration with studies conducted in unanesthetized rats standardized in our laboratory. Animals were allowed to recover for 48 h.

After the animals were anesthetized with urethane, a catheter (a 4 cm segment of PE-10 heat-bound to a 13 cm segment of PE-50, Clay Adams, Parsippany, NJ, USA) was inserted into the abdominal aorta through the femoral artery

for the acute recording of blood pressure and heart rate values. The absence of somatic motor reflexes in response to tail pinching or blinking after a low-pressure corneal stimulation was assumed as indicative of deep anesthesia and analgesia. Experiments were initiated 1 h after the onset of anesthesia.

4.3. Measurement of cardiovascular response

Arterial pressure (MAP) and heart rate (HR) signals were recorded using an amplifier (model 7754A, Hewlett Packard, USA) coupled to a computerized acquisition system (MP100, Biopac, USA).

4.4. Drug injection

A volume of 50 nL was injected using a 1 µl syringe (KH7001; Hamilton, USA) connected to an injection needle (33-gauge) by a piece of PE-10 tubing. The microinjection needle was 1 mm longer than the guide cannula. The volume was controlled by checking the movement of an air bubble inside the PE-10 tubing.

4.5. Drugs

Acetylcholine (SIGMA) and atropine (SIGMA) were dissolved in sterile artificial cerebrospinal fluid (ACSF; composition: NaCl 100 mM; Na_3PO_4 2 mM; KCl 2.5 mM; $MgCl_2$ 1 mM; $NaHCO_3$ 27 mM; $CaCl_2$ 2.5 mM; pH = 7.4).

4.6. Experimental design

The first group of animals received injections of increasing doses of Ach (9, 27, 45 or 81 nmol/50 nL) into the rostral, medial and caudal portions of the vlPAG to generate a dose–response curve. Each rat received up to two microinjections with a 10 min interval between them. Resulting data points were fitted to a dose–response curve. The dose of 45 nmol/50 nL was used in the following protocols and 50 nL of ACSF was microinjected as vehicle control. Numbers of rats used, n = 20.

The second group of animals received injections of increasing doses of Ach (9, 27, 45 or 81 nmol/50 nL) into the rostral, medial or caudal portions of the dPAG to generate a dose–response curve. Each rat received up to two microinjections with a 10 min interval between them. Resulting data points were fitted to a dose–response curve. The dose of 45 nmol/50 nL was used in the following protocols and 50 nL of ACSF was microinjected as vehicle control. Number of rats used, n = 12. These doses were established from data in the literature (Pizzirusso et al., 1998).

The third group of animals was used to evaluate the involvement of muscarinic receptors in the cardiovascular response to the injection of Ach into the vlPAG. Different doses of the nonselective muscarinic receptor antagonist atropine (1, 3 or 9 nmol/50 nL) were microinjected into the vlPAG 10 min before microinjection of Ach. Each animal received only one dose of atropine. Number of rats used, n = 16. These doses were established from data in the literature (Crippa et al., 1999).

In the last part of the study, we determined whether the cardiovascular response was due to a central effect of Ach. Animals received intravenously the same dose of atropine as injected into the vlPAG (9 nmol) 10 min prior to the injection of 45 nmol of Ach into that area. Number of rats used, n = 6.

4.7. Histological procedure

At the end of the experiments, 50 nL of 1% Evan's blue dye was injected into the vlPAG or the dPAG as a marker of the injection site. Animals were submitted to intracardiac perfusion with 0.9% NaCl followed by 10% formalin. Brains were removed and post-fixed for 48 h at 4 °C and serial 40 µm-thick sections were cut using a cryostat (CM1900, Leica, Germany). Brain sections were stained with 1% neutral red for light microscopy analysis. The actual location of microinjection sites in the area was determined after the analysis of serial sections and represented according to the rat brain atlas of Paxinos and Watson (1997).

4.8. Statistical analysis

Nonlinear regression analysis was used to compare MAP and HR results from different Ach doses microinjected into the vlPAG or the dPAG. Baseline MAP and HR values were compared using the paired Student's t test (before treatment vs. after treatment). Percentages of response inhibition by vlPAG pretreatment with muscarinic antagonists were analysed utilizing nonlinear regression analysis. We used Prism software (GraphPad, USA) to perform statistical analysis. *P < 0.05 was assumed to be statistically significant.

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