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Role of brainstem adenosine A_1 receptors in the cardiovascular response to hypothalamic defence area stimulation in the anaesthetized rat

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1 The role of centrally located adenosine A_1 receptors in the cardiovascular changes associated with the hypothalamic defence response has been investigated by *in vitro* autoradiography and the intraventricular application of an A_1 receptor antagonist.

2 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), a highly selective adenosine A_1 antagonist and its vehicle, ethanol, were administered directly into the posterior portion of the fourth ventricle of α-chloralose anaesthetized, paralysed and artificially ventilated rats.

3 DPCPX (0.01 to 0.3 mg kg^{-1}) caused a dose-dependent decrease in the magnitude of the evoked pressor response (from −13 to −23 mmHg) elicited on hypothalamic defence area stimulation at a dose 10 fold lower than that required to produce an equivalent effect following systemic administration whilst ethanol, the vehicle, had no effect.

4 *In vitro* autoradiography revealed a heterogeneous distribution of adenosine A_1 binding sites in the lower brainstem of rats. Image analysis showed the ventrolateral medulla to have the highest density of A_1 receptors. Intermediate levels of binding were seen in caudal regions of the nucleus tractus solitarii and the hypoglossal nucleus.

5 These data imply that a proportion of the cardiovascular response to hypothalamic defence area stimulation are produced by the activation of adenosine A_1 receptors localized close to the surface of, or adjacent to, the fourth ventricle in the immediate vicinity of the injection site.

Keywords: Adenosine A_1 receptors; *in vitro* autoradiography; cardiovascular response; hypothalamic defence area

Introduction

During the past decade adenosine has become widely accepted as having a neuromodulatory role in both the peripheral and central nervous system (CNS) primarily by actions resulting in reduction of neuronal excitability (Hedner *et al.*, 1982; Wessberg *et al.*, 1984). Adenosine is present in the CNS at pharmacologically active concentrations (Barraco *et al.*, 1987) which are markedly elevated during periods of hypoxia (Winn *et al.*, 1981) and increased neuronal excitation (Lloyd *et al.*, 1993). Evidence from a variety of sources has indicated that administration of adenosine and its analogues into brain regions concerned with cardiovascular control elicits circulatory effects. Such findings are suggestive of a role of adenosine in cardiovascular control, perhaps by its ability to modify the chemoreceptor and baroreceptor reflex pathways (Dawid-Milner *et al.*, 1994).

Stimulation of the hypothalamic defence area (HDA) produces an increase in blood pressure, heart rate and respiratory minute volume (Hilton & Redfern, 1986). These changes are mediated partially by the potentiation of the chemoreceptor reflex (Silva-Carvalho *et al.*, 1993) and the attenuation of the baroreceptor reflex, involving a GABAergic mechanism in the nucleus tractus solitarii (Jordan *et al.*, 1988). The effects of adenosine on the baroreceptor and the chemoreceptor reflexes resemble the changes observed in the performance of these reflexes as a consequence of HDA stimulation (see Silva-Carvalho *et al.*, 1993). Recent work from our laboratory has indicated an involvement of adenosine A_1 receptors, located in the CNS, in the cardiovascular changes evoked during HDA stimulation in both the rat (St Lambert *et al.*, 1994a,b) and cat (Silva-Carvalho *et al.*, 1993). However, the exact sites of action within the CNS of adenosine with regard to these evoked effects remain unknown.

Radiolabelled adenosine and xanthine derivatives have been used to visualize and quantify adenosine A_1 receptors in rodent, cat and dog brain. The present study was undertaken to first localize adenosine A_1 receptors, with *in vitro* autoradiography, in the lower brainstem of the rat by use of a highly selective adenosine A_1 ligand, [^{3}H]-DPCPX. Secondly the involvement of adenosine A_1 receptors in the defence response was investigated further by means of injections of DPCPX into the posterior portion of the fourth ventricle of rats.

Methods

Experiments were performed on 12 male Sprague Dawley rats (300-350 g). Anaesthesia was induced with sodium pentobarbitone (40 mg kg^{-1}, i.p.) and supplemented as necessary with α-chloralose (5 mg kg^{-1}, i.v.).

Surgical procedures

The femoral artery and vein were cannulated for the measurement of arterial blood pressure and the administration of drugs respectively. The trachea was cannulated below the larynx and the animals breathed spontaneously until positioned in a stereotaxic frame, after which they were paralysed with gallamine triethiodide (4 mg kg^{-1}, i.v., supplemented with 3 mg kg^{-1} h^{-1}) and ventilated artificially with O_2-enriched air. End tidal CO_2, recorded with a fast response CO_2 analyzer, and was maintained in the range of 4-5 vol% by adjusting respiratory frequency. Blood flow to hindlimb skeletal muscle was measured with an electromagnetic flow probe (Carolina

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Medical Electronics) placed around the femoral artery after ligation of the paw. Rectal temperature was maintained at 37–38°C by a servo-controlled heating pad. The hypothalamus was stimulated electrically (1 ms pulses, 50–200 μA at 100 Hz for 5 s) with a concentric bipolar electrode (SNE 100, Rhodes Medical Electrodes). The hypothalamic defence area was located on the basis of the characteristic cardio-respiratory response observed in the rat evoked on stimulation as described in our previous studies (St Lambert *et al.*, 1994b). The rat skull was adjusted to a 45° angle from the horizontal plane and the atlanto occipital membrane exposed and pierced with a Hamilton syringe for the administration of drugs into the posterior portion of the fourth ventricle. The drug was administered at a rate of 4 μl per 10 s.

Experimental protocol

In the first group of rats, ($n=6$), the effect of cumulative doses of DPCPX (0.01 to 0.3 mg kg^{-1}) and its vehicle ethanol (8, 16, 12 and 16 μl) were examined in the HDA-induced cardiovascular responses. A control response to HDA stimulation was characterized prior to drug administration. DPCPX was administered cumulatively at 0.01, 0.02, 0.07 and 0.2 mg kg^{-1} with HDA stimulated after each successive dose. A period of 5 min was allowed before repeat HDA stimulation. Since DPCPX was dissolved in absolute ethanol cumulative vehicle dose-response curves to ethanol (8, 16, 12, 16 μl) were also constructed in the same animal. The volume of absolute ethanol injected was the same as the volumes required to dissolve the various doses of DPCPX.

In another group of animals ($n=6$) the effect of a bolus dose of DPCPX (0.3 mg kg^{-1}) or its vehicle ethanol (25 μl) on the cardiovascular changes observed during the HDA response was examined. The drugs were administered directly into the posterior portion of the fourth ventricle 5 min before the stimulation of the defence area.

Histology

At the end of the experiment, stimulation sites were marked by passing 500 μA d.c. current through the electrode for 10–20 s, and an equivalent volume of dye injected into the posterior portion of the fourth ventricle. Frozen sections were cut at the level of the hypothalamus and lower brainstem, counterstained with neutral red and examined under a light microscope for localization of stimulation sites.

In vitro *autoradiography*

Adult male Sprague Dawley rats (300–350 g) were anaesthetized with pentobarbitone sodium and perfused with 0.1% formalin in phosphate sucrose buffer. Brainstems were removed rapidly, frozen on dry ice and stored at −70°C until required. Serial coronal sections 20 μm thick were cut with a microtome cryostat and thaw mounted onto gelatin coated slides.

The slide mounted brainstem sections were pre-incubated in 50 ml of 170 mM Tris-HCl, pH 7.4 at 25°C for 15 min. The slides were transferred into a fresh incubation medium containing 50 ml of 170 mM Tris-HCl, 0.8 nM [^{3}H]-DPCPX and 0.2 u ml^{-1} adenosine deaminase for 90 min. The slides were washed twice for 2 min in ice-cold buffer, dipped in ice-cold water and dried in cold air. The sections were exposed to hyperfilm ^{3}H, with appropriate standards, for 10–14 days at 4°C after which the film was developed and binding sites identified. Non-specific binding was assessed by incubating in the presence of 30 μM **R**-N^6 phenylisopropyladenosine (**R**-PIA). The images on the ^{3}H-sensitive film were analyzed with a Vidas imaging analysis system (Kontron, Watford). Specific brainstem areas were measured and binding was estimated (d.p.m. × 10^3 per mm^2) from curves that were generated using ^{3}H microscales (Amersham) co-exposed with tissue sections.

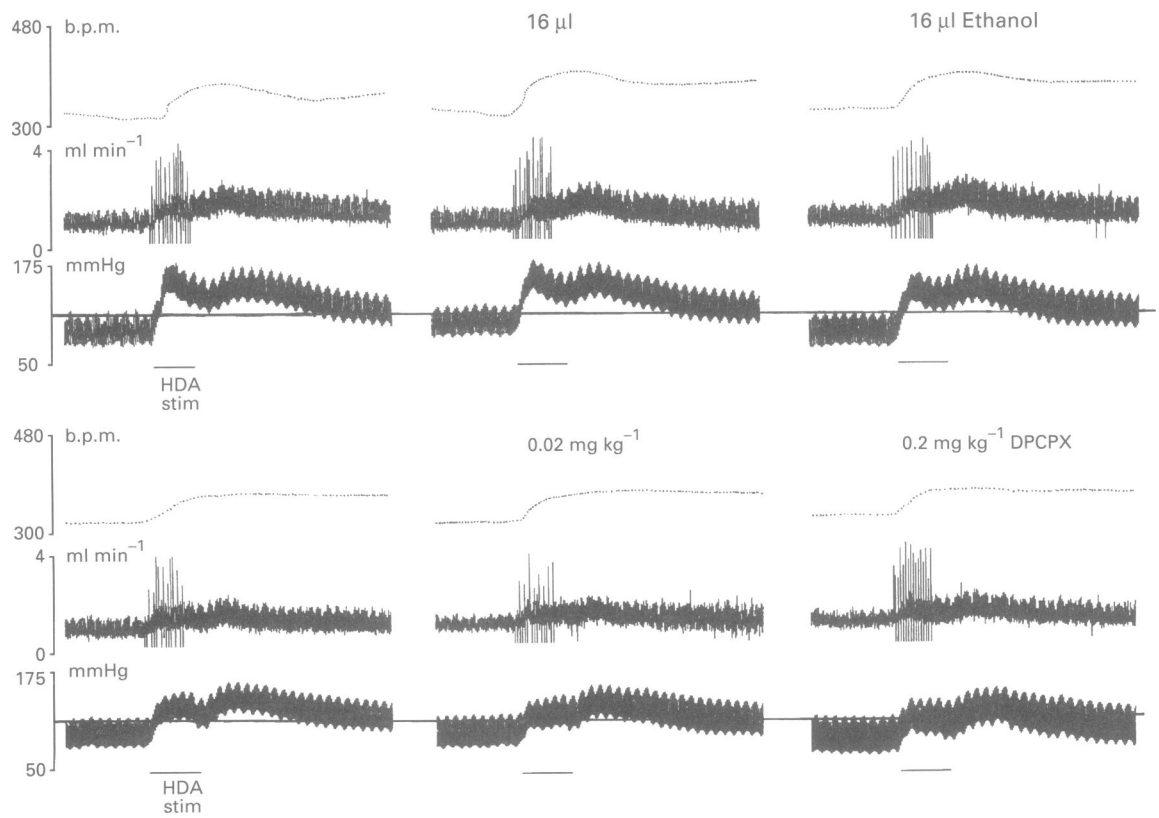

Figure 1 The effect of 2 cumulative doses of DPCPX (0.02 and 0.2 mg kg^{-1}) and its vehicle, ethanol (16 μl) on the hypothalamic defence response following administration into the posterior portion of the fourth ventricle. The lines drawn across the blood pressure traces indicate 100 mmHg.

Specific binding was established by subtracting non-specific from specific binding. Underlying tissue was stained with neutral red for histological analysis.

Analysis of data

All data are expressed as the means ± s.e.mean. Baseline values for all variables were taken immediately before a stimulus. Stimulation of the HDA resulted in a biphasic pressor response, and the changes in mean arterial pressure (MAP) and heart rate (HR) were therefore divided into primary and secondary phases (see St Lambert *et al.*, 1994b). The primary response was taken as the peak change observed at the end of the 5 s period of stimulation, and the secondary response 5–10 s later. Stimulus evoked changes in heart rate were measured as the peak response observed during the 5 s stimulation of the defence area.

The effects of cumulative doses of DPCPX (0.01, 0.02, 0.07 and 0.2 mg kg^{-1}) and absolute ethanol (8, 16, 12 and 16 μl) on resting MAP and HR were compared using repeated measures ANOVA, with *post-hoc* Dunnett Multiple Comparisons Test. However stimulus evoked responses in MAP and HR were compared with the response to the appropriate vehicle control (absolute ethanol) at matched time intervals by using Students' paired *t* test. In addition, the effect of a bolus dose of DPCPX (0.3 mg kg^{-1}) and ethanol (25 μl) on resting levels and stimulus evoked changes in MAP and HR were analyzed statistically by Student's paired *t* test. Differences were considered significant when $P < 0.05$.

Drugs and solution

The following compounds were used: gallamine triethiodide (Flaxedil) and sodium pentobarbitone (Sagatal) (May and Baker Ltd Dagenham); 8-cyclopentyl-1,3,-dipropylxanthine (DPCPX) dissolved in 100% ethanol and **R**-N^6-phenylisopropyladenosine (Research Biochemicals Incorporated Natick); α-chloralose, di-sodium tetraborate 10-hydrate and Tris- (hydroxymethyl) methylamine (BDH Ltd. Poole); [^{3}H]-DPCPX (specific activity 109 Ci $mmol^{-1}$) from New England Nuclear and adenosine deaminase (Sigma Chemicals).

Results

The administration of cumulative doses of DPCPX (0.01, 0.02, 0.07 and 0.2 mg kg^{-1}), dissolved in absolute ethanol, into the posterior portion of the fourth ventricle caused insignificant changes in resting arterial MAP or HR with the exception of 0.2 mg kg^{-1} DPCPX which caused a significant reduction in MAP (92.3 ± 6.6 mmHg vs 77.9 ± 3.9 mmHg, $P < 0.05$). Cumulative doses of ethanol (8, 16, 12, 16 μl) had no effect on resting MAP or HR. The i.v. bolus administration of DPCPX (0.3 mg kg^{-1}) and ethanol (25 μl) had no effect on resting mean arterial blood pressure or HR.

Electrical stimulation of the HDA evoked an initial increase in MAP (1°) which was often followed by a second increase in pressure (2°) which was maintained for at least 20 s after the cessation of the stimulation (see St Lambert *et al.*, 1994b). On stimulation of the HDA a marked increase in femoral hindlimb

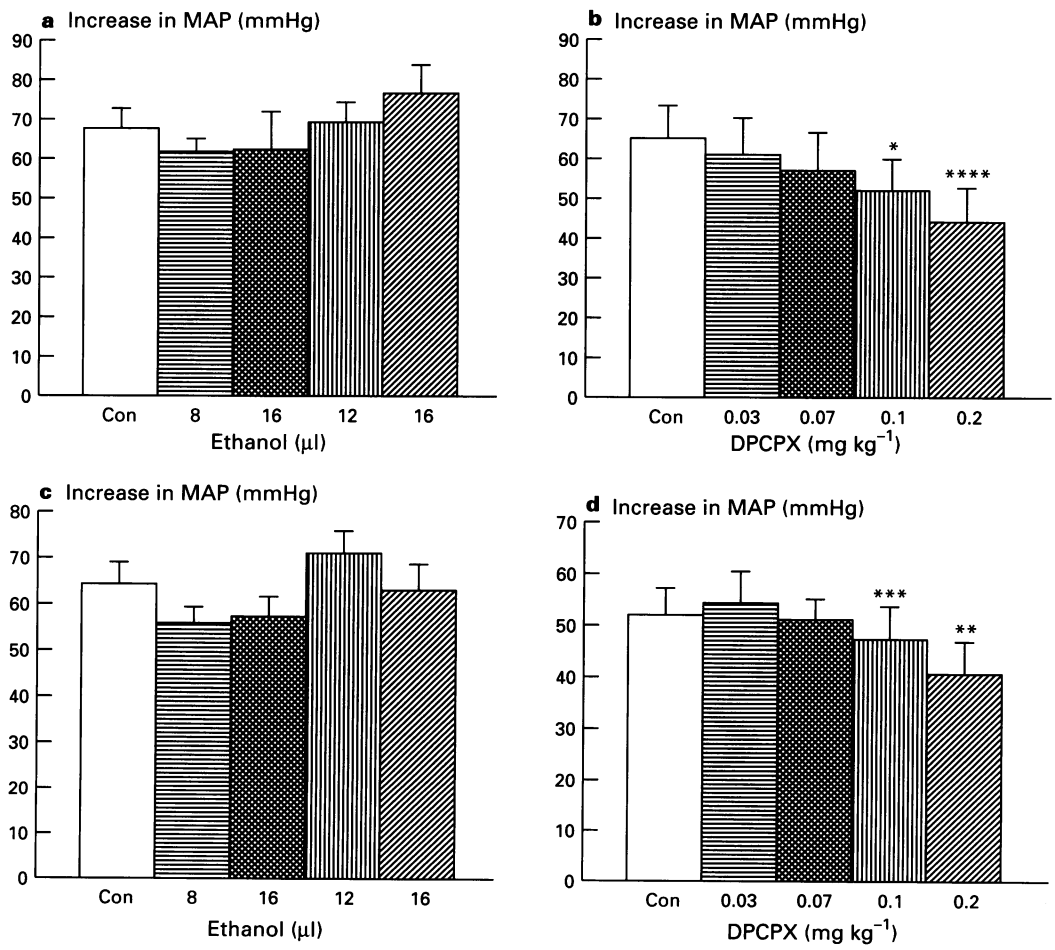

Figure 2 The effect of cumulative doses of ethanol and DPCPX on increase in mean arterial pressure observed during the primary (a and b) and secondary (c and d) component of the pressor response evoked by hypothalamic defence area stimulation. Each column represents the mean ($n = 6$) ± s.e.mean.

blood flow was observed (1.32 ± 0.04 ml min^{-1} to 1.72 ± 0.06 ml min^{-1} prior to the administration of ethanol, $P < 0.001$ and 1.37 ± 0.04 ml min^{-1} to 1.63 ± 0.07 ml min^{-1} prior to the administration of DPCPX, $P < 0.001$). Cumulative doses of DPCPX delivered into the fourth ventricle caused a dose-dependent decrease in the magnitude of both the primary and secondary component of the pressor response to stimulation of the defence area (Figures 1 and 2). A level of significance was reached at 0.07 and 0.2 mg kg^{-1} for both the primary (69.2 ± 4.8 mmHg vs 51.6 ± 8.0 mmHg, $P < 0.02$ and 76.4 ± 6.8 mmHg vs 44.3 ± 8.6 mmHg; $P < 0.0001$, respectively) and secondary increase in blood pressure (70.5 ± 4.9 mmHg vs 46.8 ± 6.3 mmHg and 63.0 ± 5.6 mmHg vs 40.2 ± 6.2 mmHg; $P < 0.005$, respectively). DPCPX also caused a significant decrease in the magnitude of the evoked tachycardia at 0.01 and 0.07 mg kg^{-1} (56.8 ± 6.9 b.p.m. vs 44.7 ± 7.1 b.p.m. and 60.8 ± 5.9 b.p.m. vs 41.5 ± 6.3 b.p.m., $P < 0.05$ respectively). In contrast ethanol, the vehicle, had no effect on the magnitude of either component of the pressor response or the evoked tachycardia (Figures 1 and 2).

The administration of a bolus dose of DPCPX (0.3 mg kg^{-1}) into the posterior portion of the fourth ventricle in a second group of animals ($n = 6$) decreased significantly both components of the evoked pressor response (primary phase, 57.8 ± 7.1 mmHg vs 33.5 ± 3.6 mmHg, $P < 0.05$; secondary phase 71.67 ± 2.39 mmHg vs 55.8 ± 3.19 mmHg, $P < 0.02$) whilst leaving the heart rate unaffected (Figure 3). Ethanol, the vehicle, had no effect on the cardiovascular changes observed during the defence response (Figure 3).

The location of stimulation sites from where responses were reduced significantly by DPCPX administered into the fourth ventricle were examined. Histological analysis showed them to fall within regions of the hypothalamus known to evoke the defence response (Yardley & Hilton, 1986).

In vitro *autoradiography*

Image analysis revealed a heterogeneous distribution of adenosine A_1 binding sites in the brainstem sections following an incubation with 0.8 nM [^{3}H]-DPCPX (Table 1). The highest density of A_1 binding sites was found in the ventrolateral medulla (RVLM), throughout its rostral-caudal extent, followed by the hypoglossal nucleus (see Table 1). Intermediate levels of binding were seen in the region of the caudal nucleus tractus solitarii (NTS) (-5.7 mm to -4.7 mm from interaural line, Paxinos & Watson, 1986). The density of adenosine A_1 binding sites was low in the more rostral region of the NTS and the trigeminal tract. Non-specific binding was low since brainstem slices incubated with [^{3}H]-DPCPX and 30 μM **R**-PIA showed autoradiographic intensities which could not be distinguished from film background.

Discussion

The results of the present study confirm previous observations that adenosine A_1 receptors located in the central nervous system are activated during the defence reaction that is evoked by electrical stimulation at restricted sites within the hypothalamus (St Lambert *et al.*, 1994b; Thomas & Spyer 1994). Indeed, the threshold dose of DPCPX required to produce a significant attenuation of the cardiovascular component of the

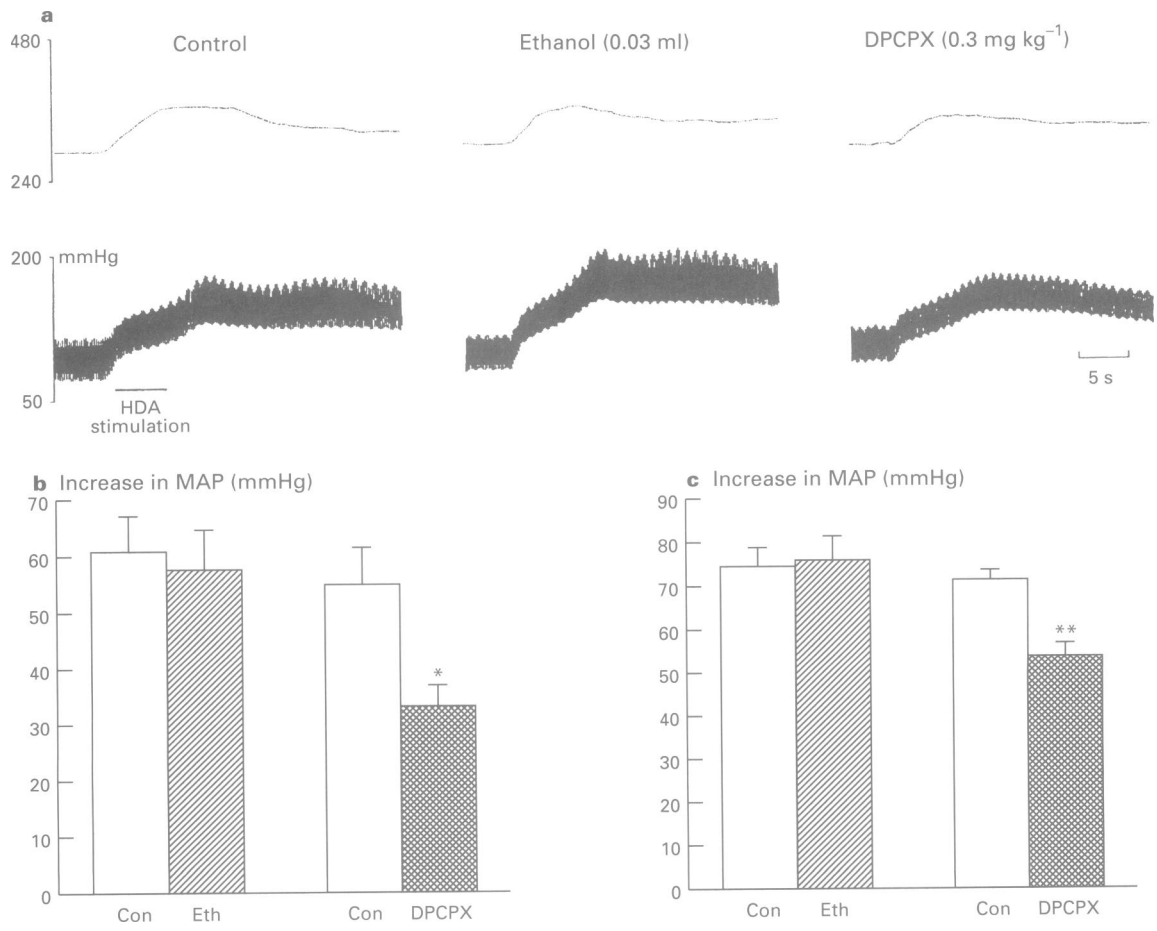

Figure 3 Original traces showing the effect of a bolus dose of ethanol and DPCPX on the blood pressure and heart rate component of the defence response (a). Histograms illustrating the effects of DPCPX and ethanol on the mean primary (b) and secondary (c) increases in MAP following HDA stimulation. Columns represent mean ($n = 6$) $\pm$ s.e.mean.

Table 1 [^{3}H]-DPCPX binding determined by densitometric analysis: values expressed in terms of d.p.m. $\times 10^3$ per mm^2 derived from co-expressed ^{3}H microscales

	Total binding	*Non-specific binding*	*Total minus non-specific*	*No. of counts*
Cerebellum	32.92±4.71	5.33±1.17	27.59±4.15	$n=74$
Caudal NTS	17.26±4.24	6.82±1.08	13.01±3.89	$n=45$
Rostral NTS	12.91±3.10	6.38±6.60	8.23±2.85	$n=36$
Hypoglossal	18.46±4.19	4.60±1.08	13.82±3.75	$n=79$
VLM	20.14±4.01	5.05±2.06	15.09±3.72	$n=72$
Trigeminal tract	9.35±3.54	4.40±1.55	4.94±2.61	$n=78$

defence response following injection into the fourth ventricle was 10 fold lower than that to produce an equivalent effect following intravenous administration (St Lambert *et al.*, 1994b). This observation coupled with the fact that the onset of action was quite rapid suggests a restricted site of action close to the point of administration. It is likely that DPCPX acted at loci on the surface of or adjacent to the fourth ventricle in the immediate vicinity of the injection site. Indeed, close to the point of administration are regions of the brainstem such as the subpostremal NTS which lack the blood brain barrier. In these regions drug penetration is likely to be high and hence such areas represent potential sites of action. However, due to the highly lipophilic nature of DPCPX we cannot rule out a site of action at other brainstem sites. Possible sites are discussed below. It is unlikely however, that DPCPX affected spinal neurones involved in sympathetic control since the diffusion of dye, administered into the fourth ventricle at the end of each experiment, was localized primarily on the lateral walls and floor of the caudal portion of the fourth ventricle.

Stimulation of the HDA produces a pattern of responses which includes an increase in blood pressure and heart rate (St Lambert *et al.*, 1994a,b). DPCPX, a highly potent and selective A_1 antagonist (Lohse *et al.*, 1987), when administered into the posterior portion of the fourth ventricle close to the area postrema caused a dose-dependent decrease in the magnitude of the evoked pressor response. DPCPX is a highly selective adenosine A_1 antagonist with a 700 fold greater selectivity for the A_1 receptor than the A_2 receptor (Lohse *et al.*, 1987). However, at doses such as 0.3 mg kg^{-1}, the maximum dose used in this study, it is likely to block the A_2 adenosine receptors in addition to the A_1 receptors. In spite of this, we are confident that the neuromodulatory effect of DPCPX observed during the defence response is predominantly mediated by an action at the A_1 receptor. Support for this comes from the fact that the ratio of A_1 to A_2 receptors in the brainstem is 7:1 (Barraco *et al.*, 1991a,b). Moreover, recent observations from our laboratory have demonstrated that microinjections of a selective adenosine A_1 antagonist into the caudal NTS attenuates significantly the pressor response evoked upon HDA stimulation (St Lambert & Spyer, unpublished observations).

The experiments required careful control since DPCPX was dissolved in absolute ethanol, which in itself could cause changes in cardiovascular variables (Sun & Reis, 1992). However, in this study ethanol injected into the fourth ventricle had no effect on resting levels of either arterial blood pressure or heart rate or on the cardiovascular changes associated with the defence response. In addition, DPCPX had no effect on baseline levels of arterial blood pressure or heart rate indicating that adenosine is unlikely to have any tonic effect on cardiovascular activity through actions on areas sensitive to its application by an intracerebroventricular route. One possible site of action of DPCPX is the NTS since this is relatively close (<300 μm) to the fourth ventricle and contains a substantial amount of adenosine A_1 receptors (St Lambert *et al.*, 1994a; Table 1). It has long been recognised that the NTS is a major relay nucleus for baroreceptor and chemoreceptor afferent inputs (Spyer, 1994). Preliminary studies in the cat have shown that both the baroreceptor and chemoreceptor reflex may be modulated by the central action of DPCPX (Silva-Carvalho *et al.*, 1993) in that this A_1 antagonist appears to cause the facilitation of the baroreceptor reflex and the attenuation of the chemoreceptor-evoked pressor response (Silva-Carvalho *et al.*, 1993). It has also been shown that extracellular levels of adenosine are markedly elevated during brief expose to hypoxia (Winn *et al.*, 1981), and there is mounting evidence that adenosine levels are elevated in the NTS itself (Barraco *et al.*, 1991a,b). Moreover, microinjection of adenosine into the caudal NTS of rats elicits a dose-related decrease in blood pressure (Barraco *et al.*, 1988). Recent studies from our laboratory have shown that microinjections of the adenosine A_1 antagonist, 8-cyclopentyl-1,3-dimethylxanthine into the caudal NTS can attenuate significantly the biphasic pressor response evoked on HDA stimulation (unpublished observations). Taken together these data clearly implicate a neuromodulatory role for adenosine in cardiorespiratory control exerted within the NTS.

Another possible site of action is the RVLM since this region has the highest concentration of adenosine A_1 binding sites in the brainstem (St Lambert *et al.*, 1994a, Table 1) and its sympatho-excitatory neurones are excited by stimulation within the hypothalamic defence area (McAllen 1986a,b). Further support for the involvement of the RVLM comes from the fact that DPCPX had a more profound effect on the pressor response observed during the defence response than on the accompanying tachycardia. Recent work from our laboratory has illustrated that microinjections of adenosine into the RVLM can alter cardiovascular activity, and moreover augment the magnitude of the evoked pressor response observed on hypothalamic defence area stimulation (Thomas & Spyer, 1994). Furthermore, microinjection of the adenosine antagonist, 8-SPT, reduces both pressor and tachycardia responses to HDA stimulation (Thomas & Spyer, unpublished observations).

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