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Short Communication

Adenosine A1 receptors mediate inhibition of cAMP formation in vitro in the pontine, REM sleep induction zone

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Abstract

Microinjection of adenosine A1 receptor agonist or an inhibitor of adenylyl cyclase into the caudal, oral pontine reticular formation (PnOc) of the rat induces a long-lasting increase in REM sleep. Here, we report significant inhibition of forskolin-stimulated cAMP in dissected pontine tissue slices containing the PnOc incubated with the A1 receptor agonist, cyclohexaladenosine (10^{-8} M). These data are consistent with adenosine A1 receptor agonist actions on REM sleep mediated through inhibition of cAMP.

Theme: Neural basis of behavior
Topic: Biological rhythms and sleep

Keywords: Pontine reticular formation; Pontine slice; Adenylyl cylase; Cyclohexaladenosine; Forskolin

The caudal aspects of the oral pontine reticular formation (PnOc) of the rat comprise a REM sleep induction zone in which the microinjection of a variety of agents including adenosine agonists produce a long-lasting increase in REM sleep [1,3,4,11,16]. Increased levels of adenosine are associated with increases in neural activity [9], and adenosine levels in the PnOc may be a mechanism regulating expression of REM sleep following different experiences of wakefulness. Some of these experiences may include increased wake time, stress, and learning.

Agonist-ligands acting at both adenosine A1 and A2a receptor subtypes can mediate the REM sleep response through independent mechanisms. The A2a receptor agonist, CGS21,680, increases acetylcholine release when dialyzed into the PnOc of the mouse [6], and microinjection in rat increases REM sleep that is blocked by the pre-application of the muscarinic antagonist atropine [13]. These findings indicate that the A2a receptor mechanism to increase REM sleep requires the participation of the cholinergic system. Elevations in REM sleep produced by the A1 receptor agonist, cyclohexaladenosine (CHA), are insensitive to atropine, suggesting an alternative mechanism of action [13]. Microinjection in the PnOc of the adenylyl cyclase inhibitor SQ22,536 also results in long-lasting increases in REM sleep [12]. In that A1 adenosine receptors, and not A2a, are coupled to G proteins producing inhibition of adenylyl cyclase, the synthetic enzyme for cAMP [8,14], we hypothesize that A1 receptors mediate adenosine effects in the brainstem by reducing the production of cAMP. Here, we report on the dose–response relationship of an adenosine agonist selective for A1 receptors to inhibit forskolin increases in cAMP in tissue slices containing the pontine, REM sleep induction zone.

The animal protocol was conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Long–Evans hooded rats ($N = 6$) (Harlan) between 100 and 200 g were decapitated under ketamine anesthesia (150 mg/kg), and the brains were

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quickly removed and chilled in a gassed (95% O_2, 5% CO_2) dissection medium. The dissection medium consisted of a 1 mM solution of kynurenate and artificial cerebrospinal fluid (ACSF, in mM, 126 NaCl; 25 $NaHCO_3$; 1.2 NaH_2PO_4; 2.5 KCl; 2.5 $CaCl_2$; 1.2 $MgCl_2$; and 11 glucose, pH 7.3) Three consecutive 300-μm coronal sections were cut through the pons using a Vibratome (Ted Pella) from approximately 8.0 to 8.9 mm posterior to the Bregma suture [15]. These sections were then further dissected into right and left rectangular pieces ($\sim 2 \times 3$ mm) each occupied principally by cells of the PnOc, see Fig. 1. Rostral sections contained part of the ventral tegmental nucleus of Gudden and the reticulotegmental pontine nucleus. All sections contained part of either the raphe medianus or pontis at the margin of the medial cut.

Dissected tissue samples were transferred to a multiwell plate and individually incubated in ACSF (2 ml) under constant gassing at 37°C for at least 1 h (1–1.5 h). This was followed by adding solutions of drug (all from RBI) to the incubation medium of the tissue samples. The experimental conditions were (in final concentrations in ACSF) ACSF alone for an additional 25 min; ACSF for 10 min followed by adding the water soluble derivative of the adenylyl cyclase activator, forskolin (10^{-5} M) for 15 min; the adenosine A1 receptor agonist, CHA (10^{-9}, 10^{-8}, or 10^{-7} M) for 10 min, followed by forskolin (10^{-5} M) for 15 min; and finally, the adenosine A1 receptor antagonist, cyclopentaltheophyline (CPT) (10^{-7} M) for 10 min, followed by CHA (10^{-8} M) for 10 min followed by forskolin (10^{-5} M) for 15 min. Each experimental condition was independently repeated on at least four tissue samples.

Following drug incubations, tissue slices were quickly blotted on buffer-moistened filter paper to remove excess media, placed in Eppendorf-type tubes, capped, frozen on powdered dry ice, and maintained in a −70 °C deep freezer until assayed for cAMP. At the time of assay, tissue samples were thawed individually and sonicated briefly in acid alcohol (ethanol: 1NHCl, 99:1), then centrifuged (Beckman Microfuge) for 5 min. The supernatants were transferred to clean tubes, dried completely, and tissue pellets were analyzed for protein (see below). The residues were dissolved in the manufacturer supplied assay buffer (Cayman), the same as for diluting cAMP standards. Sample and standard aliquots were acetylated individually by successive addition of a triethylamine/acetic anhydride solution. Samples and standards were aliquoted into the 96-well microplate in triplicate along with non-specific and maximum-binding wells. Cyclic AMP acetylcholinesterase tracer was added to all wells, except total and blank wells. Cyclic AMP antiserum was added to all wells, except the total, non-specific and blank wells and the plate incubated for 18 h at room temperature. Plate wells were washed 5 times with wash buffer. Aliquots of Ellman's reagent were added to each well and tracer to total activity wells. Color development was carried out for 1 h, and the plate was read in a microplate reader (Bio-Rad 3550) at 450 nm.

The tissue pellets were dissolved in 0.5 N NaOH, aliquots were assayed for protein using a modified Bradford

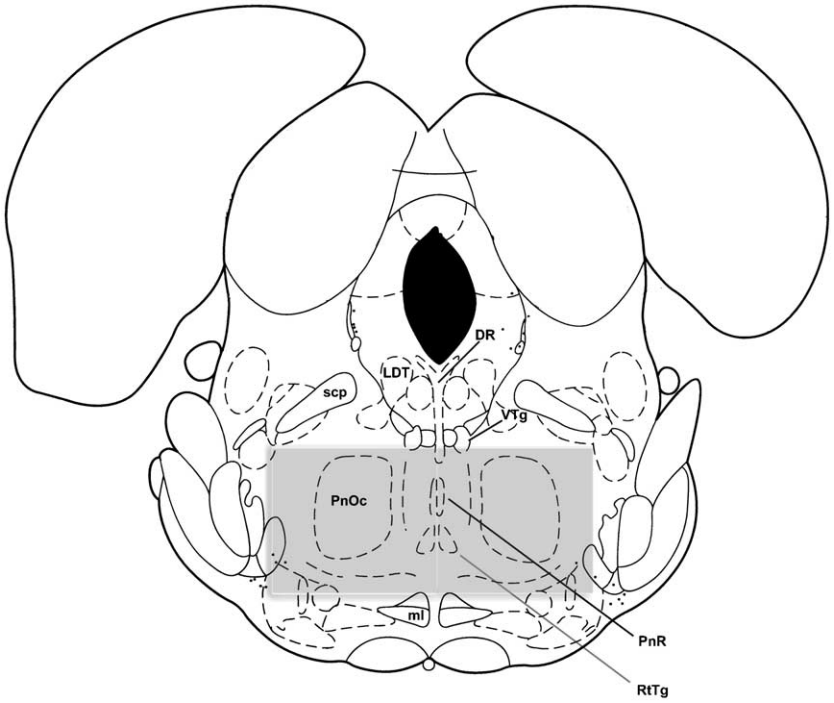

Fig. 1. Schematic diagram illustrating the dissection of tissue samples containing the REM sleep induction zone from coronal pontine slices. Grey areas indicate right and left rectangular pieces ($\sim 2 \times 3$ mm) each occupied principally by cells of the PnOc. Abbreviations: LDT—laterodorsal tegmental nucleus; DR—dorsal raphe nucleus; scp—superior cerebellar peduncle; RtTg—reticulotegmental nucleus of the pons; PnOc—oral pontine reticular nucleus (caudal part); PnR—pontine raphe nucleus; Vtg—ventral tegmental nucleus.