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Binding of an adenosine A_1 receptor agonist and adenosine A_1 receptor antagonist to sheep pineal membranes

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Abstract

The pineal organ of vertebrates produces melatonin and adenosine. In lower vertebrates, adenosine modulates melatonin production. We report herein that 2-chloro-cyclopentyl-[^{3}H]-adenosine ([^{3}H]CCPA: adenosine A_1 receptor agonist) and [^{3}H]-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX: adenosine A_1 receptor antagonist), bind specifically to sheep pineal membranes. Binding of [^{3}H]CCPA reached equilibrium at 90 min and dissociation revealed the presence of two components. Saturation analysis suggested the presence of a single population of binding sites ($K_d = 1.67 \pm 0.06$ nM, $B_{max} = 2386$ fmol/mg protein). Binding was sensitive to GTP and GTPγS. Binding of [^{3}H]DPCPX reached equilibrium at 60 min and dissociation was monophasic. Saturation analysis revealed a single population of binding sites ($K_d = 5.8 \pm 1.12$ nM, $B_{max} = 1116$ fmol/mg protein). The specificity of the [^{3}H]-analogues used and the rank order potency of the competitors tested in the competition experiments suggested the presence of A_1 receptors. Future investigations are necessary to elucidate the significance of the differences observed between the binding properties of the adenosine A_1 receptor agonist and adenosine A_1 receptor antagonist. © 1997 Elsevier Science B.V.

Keywords: (Sheep); Pineal; Adenosine; Adenosine receptor; Melatonin

1. Introduction

The pineal hormone melatonin plays a central role in controlling seasonal reproduction in mammals (Bittman et al., 1983; Reiter, 1991). Our first knowledge of the mechanisms involved in the regulation of melatonin synthesis derived principally from studies on the rat pineal gland. In this species, norepinephrine, released at night from sympathetic nerve endings, acts on β_1- and α_1-adrenergic receptors to stimulate the activity of the arylalkylamine *N*-acetyltransferase (NAT). This enzyme, which converts serotonin to *N*-acetylserotonin, is rate limiting in the melatonin metabolic pathway (Klein, 1985; Sugden, 1989).

Although norepinephrine is widely considered the major signal in terms of regulation of pineal activity, growing evidence strengthens the hypothesis that the regulation of pineal melatonin synthesis depends also on a variety of other first messengers (Nordio et al., 1989, 1992). Among these, the nucleoside adenosine deserves special attention. The role of adenosine as an autocrine/paracrine mediator of cell to cell communication is now well established in a number of systems (Phillis and Wu, 1981; Williams, 1987; Stone, 1991). Based on the development of selective ligands and the isolation of multiple cDNA clones four cell surface adenosine receptors have been identified (Palmer and Stiles, 1995). The A_1 and A_3 receptor subtypes are negatively linked to the adenylyl cyclase and modulation of the phospholipase C activity has also been reported in some cases; the A_{2A} and A_{2B} receptor subtypes are positively linked to the adenylyl cyclase and the A_{2B} receptors may also mediate activation of calcium channels via phospholipase C (Cooper and Londos, 1988; Stone, 1991; Palmer and Stiles, 1995). Intracellularly, the nucleoside is at a crossroads of several metabolic pathways (Fredholm and Sollevi, 1986). In the pineal, there is evidence suggesting that adenosine is synthesized in noticeable amounts, at least as a degradation product of phosphorylated nucleosides (including cAMP) and of *S*-adenosylmethionine as a cofactor of the hydroxyindole-O-methyltransferase (Falcón

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et al., 1988a,b, 1991, 1992; Nikodijevic and Klein, 1989). This enzyme catalyses the conversion of *N*-acetylserotonin to melatonin (Klein, 1985; Sugden, 1989). In addition, in pike, trout and chicken pineal, adenosine modulates melatonin secretion in a biphasic fashion, possibly through A_1 and A_2 receptors (Falcón et al., 1988a, 1991, 1992, 1995). The final effect of the nucleoside might depend both on its local concentration and on the relative expression of its receptors throughout a light/dark cycle (Falcón et al., 1988a, 1991, 1992, 1995). In the rat, some studies reported the involvement of A_{2B} receptors in the control of melatonin production, whereas others concluded in the absence of effect (Gharib et al., 1989; Sarda et al., 1989; Nordio et al., 1989, 1992). The cloning of the A_3 adenosine receptor allowed to identify expression of this receptor subtype in the sheep pineal, using reverse transcriptase-polymerase chain reaction (Linden, 1994). On the basis of these considerations and of preliminary results, suggesting a biphasic modulation of melatonin production by cultured ovine pineal cells, we investigated whether adenosine binding sites were present in ovine pineal organs. Binding assays were run using membrane preparations from sheep pineals together with the selective adenosine A_1 receptor agonist ([^{3}H]-2-chloro-cyclopentyladenosine: [^{3}H]CCPA) or adenosine A_1 receptor antagonist ([^{3}H]-cyclopentyl-1,3-dipropylxanthine: [^{3}H]DPCPX) as radioligands.

2. Materials and methods

2.1. Chemicals

Radiolabeled 2-chloro-N^6-cyclopentyl[2,3,4,5-^{3}H]-adenosine ([^{3}H]CCPA; 42.8 Ci/mmol) and cyclopentyl-1,3-dipropylxanthine,8-[*dipropyl*-2,3-^{3}H(N)] ([^{3}H]DPCPX; 120 Ci/mmol) were purchased from Dupont NEN (Les Ulis, France). Adenosine deaminase type VI (ADA), bovine serum albumin (BSA), 2-chloroadenosine (CLAD), cyclohexyladenosine (CHA), cyclopentyladenosine (CPA), 5′-(*N*-cyclopentyl)-carboxamidoadenosine (CPCA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), guanosine triphosphate (GTP), guanosine-5′-O-(3-thiotriphosphate) (GTPγS), methylcarboxamidoadenosine (MECA), *N*-ethylcarboxamido-adenosine (NECA), phenylisopropyladenosine (PIA), phenyl-methylsulfonylfluoride (PMSF) and sucrose were purchased from Sigma (L'Isle d'Abeau, France). N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine (DPMA) was purchased from RBI (Bioblock, France).

2.2. Preparation of sheep pineal membranes

Sheep of both sexes, originating from the region Poitou Charentes, were killed early in the morning (between 06.00 and 09.00) in a slaughter house. Pineal organs were removed within 10 min and immediately frozen in liquid nitrogen. They were stored at −80°C until preparation of the membranes. All steps of the preparation were run at +4°C. Frozen pineals (40 to 50) were thawed and homogenized using an ultraturax tissumixer, in 50 ml of Tris–HCl buffer (pH 7.6). The buffer contained $MgCl_2$ (5 mM), PMSF (1 mM) and sucrose (0.32 M). The resulting homogenate was centrifuged for 10 min at $800 \times g$. The pellet was discarded and the supernatant was recentrifuged for 20 min at $80\,000 \times g$. The supernatant was eliminated and the pellet was resuspended in Tris buffer devoid of sucrose and of PMSF. After another centrifugation as above, the resulting pellet was resuspended in buffer so that the protein concentration was between 200 and 600 μg/ml. Protein concentration was measured using the method of Bradford (1976) with BSA as a standard. The final membrane preparation was stored at −80°C until binding assay was run.

2.3. Binding assays

Aliquots (800 μl) of membrane suspensions were incubated with 100 μl of either [^{3}H]CCPA or [^{3}H]DPCPX and 100 μl of Tris buffer containing 20 IU/ml of ADA and, where appropriate, competing compounds at the desired concentration. Samples were routinely incubated for 90 min (excepting in the time-course experiments, see below) at room temperature (20°C), under vigorous shaking. Selection of the incubation time was dictated by the time-course association of [^{3}H]CCPA and of [^{3}H]DPCPX. Reaction was terminated by rapid filtration under vacuum (using a Millipore harvester) of the assay tube content, over Whatman GF/B glass fibre filters. Filters were rinsed with four times 4 ml of ice-cold buffer. Filter disks were then placed into vials with 8 ml of ECOSCINT A (Fressinet) scintillation cocktail. After overnight shaking, radioactivity was estimated using a Beckman rack β scintillation counter, with a 55% efficiency.

The amount of radioligand bound was 10% or less of added ligand. Specific binding was defined as total binding minus binding occurring in the presence of 50 μM of CPA (for [^{3}H]CCPA binding) or of 50 μM of DPCPX (for [^{3}H]DPCPX binding). Specific binding represented 90 to 95% of total binding at nanomolar concentrations of [^{3}H]ligand.

Each curve corresponds to data obtained from 40 to 50 pooled pineal organs and each plot corresponds to triplicate determinations (both for the total and non-specific binding); all experiments were run in duplicate or in triplicate.

2.4. Analysis of binding data

Equilibrium saturation analysis: Data were fitted to the equation of a rectangular hyperbola, according to the one- or the two-sites models (Limbird, 1986).

Time-course association: Data were fitted to a rectangu-

lar hyperbola equation and analysed as a pseudo-first order reaction described by the equation $\ln([B_e]/[B_e]-[B]) = K_{obs}(t)$, where B_e is the binding at equilibrium, B the binding at time t and K_{obs} is the observed association rate constant (Limbird, 1986).

Time-course dissociation: Data were fitted to a single, or to a double, exponential decay. Data were also analysed as a first order reaction described by the equation $\ln([B]/[B_e]) = -K_{-1}(t)$, where B_e and B are as above indicated and K_{-1} is the dissociation rate constant (Limbird, 1986).

Competitive displacement by unlabeled analogs: Data were analysed according to the four-parameter logistic equation, from which the Hill coefficient and the IC_{50} were determined (Limbird, 1986).

Statistics: Data are described as a better fit by one model of ligand binding than another when a partial F-test comparing the two models indicated significant improvement in residual sum of squares as described previously (Wells, 1992).

3. Results

3.1. Time-course of association and dissociation binding

[^{3}H]CCPA bound specifically to sheep pineal membranes. At 20°C, the specific binding of 2.1 nM of [^{3}H]CCPA reached equilibrium within 90 min, and remained constant for the following 90 min (Fig. 1). The kinetics of the association appeared monophasic, with a

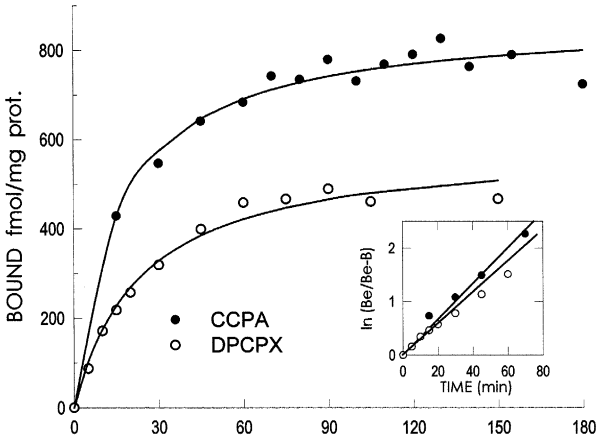

Fig. 1. Time course of association of [^{3}H]CCPA and [^{3}H]DPCPX binding to sheep pineal membranes. Membranes were incubated in the presence of [^{3}H]CCPA (2.1 nM) or of [^{3}H]DPCPX (2.5 nM) as described in Section 2. Non-specific binding was measured in the presence of 50 μM of CPA and DPCPX, respectively, and was constant throughout the association reaction. Inset shows the pseudo-first order replot of the association data, as explained in the text. Each curve corresponds to data obtained from 40 to 50 pooled pineal organs; each plot corresponds to triplicate determinations. Lines through data points were computer generated. CPA = N^6-cyclopentyl-adenosine; CCPA = 2-chloro-N^6-cyclopentyl-adenosine; DPCPX: cyclopentyl-1,3-dipropylxanthine.

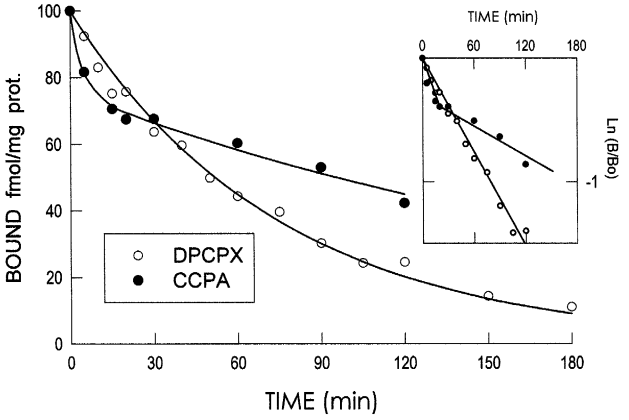

Fig. 2. Time-course of CPA-induced [^{3}H]CCPA dissociation and DPCPX induced [^{3}H]DPCPX dissociation. Membranes were first incubated for 90 min at 20°C in the presence of 2.1 nM of [^{3}H]CCPA or 2.5 nM of [^{3}H]DPCPX to allow binding to reach equilibrium. Non-specific binding was determined in the presence of 50 μM of CPA or DPCPX, respectively. After 90 min, 50 μM of the corresponding unlabelled adenosine analogue were added in all test tubes, in a negligible volume (1% of total incubation volume). Non-specific binding was constant throughout the duration of the dissociation reaction. Inset depicts the first order replot of dissociation data, as explained in the text. Each curve corresponds to data obtained from 40 to 50 pooled pineal organs; each plot corresponds to triplicate determinations. Lines through data points were computer generated. CPA = N^6-cyclopentyl-adenosine; CCPA = 2-chloro-N^6-cyclopentyl-adenosine; DPCPX: cyclopentyl-1,3-dipropylxanthine.

$t_{1/2}$ value of 15.42 ± 2.37 min. At equilibrium, the non-specific binding did not exceed 6% of the total [^{3}H]CCPA bound. When the data were analysed as a pseudo-first order reaction (see Section 2) a straight line was obtained (inset in Fig. 1) with a slope ($= K_{obs}$) of 0.03 ± 0.003 min^{-1}.

The time-course of the CPA induced dissociation of specific [^{3}H]CCPA binding was studied, after equilibrium was reached (90 min), by adding 50 μM of CPA to the incubation media. As shown in Fig. 2, the [^{3}H]CCPA binding was displaceable. The data fitted better a double ($r = 0.98$) than a single ($r = 0.55$) exponential decay ($F = 19.88$, $p < 0.005$). The low affinity sites (26.38 ± 6.57% of the total specific binding sites) had a dissociation rate constant $k_{-1L} = 0.13 \pm 0.06$ min^{-1} ($t_{1/2} = 5.28$ min). The high affinity sites (72.9 ± 5.74% of the total specific binding sites) had a dissociation rate constant $k_{-1H} = 0.0041 \pm 0.001$ min^{-1} ($t_{1/2} = 168.4$ min). The presence of two components also appeared when $\ln([B]/[B_e])$ (inset in Fig. 2), or $\log([B]/[B_e])$ (not shown), were plotted as a function of time. The dissociation rate constants, calculated from the slopes of the lines obtained in Fig. 2 were in reasonable agreement with those found above: $k_{-1L} = 0.02 \pm 0.003$ min^{-1} and $k_{-1H} = 0.0044 \pm 0.001$ min^{-1}.

Non-specific binding was not significantly modified during the association or during the dissociation of [^{3}H]CCPA binding.

Time-course association of [^{3}H]DPCPX (2.5 nM) was similar to that described above for [^{3}H]CCPA. Specific

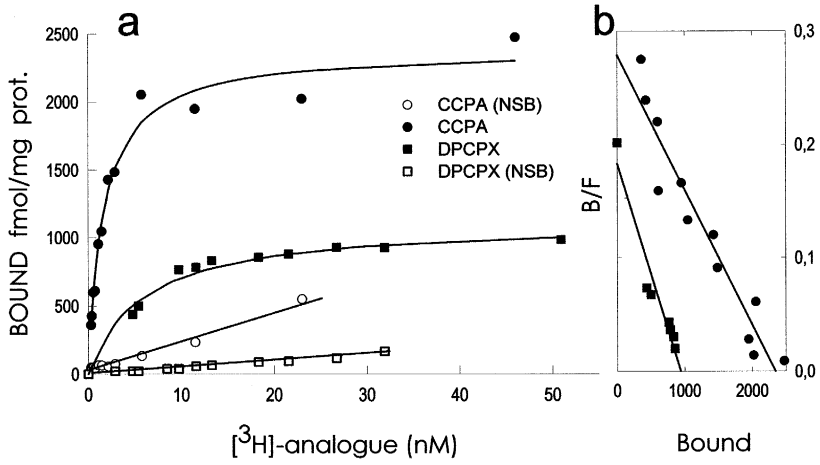

Fig. 3. Equilibrium saturation binding of [^{3}H]CCPA and [^{3}H]DPCPX binding to sheep pineal membranes. (a) Membranes were incubated in the presence of increasing concentrations of the labelled compounds, as indicated in Section 2. The specific binding (filled symbols) is defined as total minus non-specific binding (open symbols). Non-specific binding was determined in the presence of 50 μM of CPA or DPCPX, respectively. (b) Scatchard replot of the experimental data, and the best least-squares regression lines. Each curve corresponds to data obtained from 40 to 50 pooled pineal organs; each plot corresponds to triplicate determinations. Lines through data points were computer generated. CPA = N^6-cyclopentyl-adenosine; CCPA = 2-chloro-N^6-cyclopentyl-adenosine; DPCPX: cyclopentyl-1,3-dipropylxanthine; F = free radioligand concentration in the assay.

binding reached equilibrium within 60 min, with a $t_{1/2}$ value of 23.8 ± 3 min and a K_{obs} of 0.025 ± 0.001 min^{-1} (Fig. 1). In contrast, the time-course of the DPCPX induced dissociation of [^{3}H]DPCPX was resolved by a single exponential decay, with a $t_{1/2}$ of 52 ± 1.4 min and a dissociation rate constant of 0.013 ± 0.000 min^{-1} (Fig. 2).

3.2. Equilibrium saturation analysis

The specific binding of [^{3}H]CCPA and of [^{3}H]DPCPX (Fig. 3) were saturable, while non-specific binding increased linearly with increasing ligand concentrations. Both, the equation based on the mass action principle and the Scatchard replotting of the data revealed one class of binding sites. The maximal number of binding sites and dissociation constants were 2386 ± 86 fmol/mg protein and 1.67 ± 0.2 nM for [^{3}H]CCPA, and 1116 ± 63 fmol/mg protein and 5.8 ± 1.12 nM for [^{3}H]DPCPX (mean ± S.E., n = 3).

At the concentration of 1.9 nM, the specific [^{3}H]CCPA binding increased linearly with increasing protein concentrations (90, 180 and 360 μg of proteins gave, respectively, 38 ± 0.1, 82 ± 3 and 163 ± 2 fmol/assay; mean ± S.E., n = 3).

Table 1
Relative potencies of adenosine analogs as inhibitors of [^{3}H]CCPA and [^{3}H]DPCPX binding

	[^{3}H]CCPA				[^{3}H]DPCPX			
	IC_{50} (nM)	Hill slope	F	$P <$	IC_{50} (nM)	Hill slope	F	$P <$
Agonist								
CPA	0.27 ± 0.03	0.52 ± 0.07	44.2	0.001	4.47 ± 0.15	0.36 ± 0.06	25	0.005
PIA	1.36 ± 0.01	0.57 ± 0.07	28.7	0.001	0.12 ± 0.008	0.2 ± 0.04	47.6	0.001
CCPA	—	—	—	—	15.01 ± 0.25	0.44 ± 0.04	37.7	0.001
CHA	1.68 ± 0.02	0.49 ± 0.07	32.6	0.001	—	—	—	—
CLAD	15.44 ± 0.23	0.64 ± 0.07	11.1	0.01	—	—	—	—
DPMA	—	—	—	—	67.59 ± 0.14	0.78 ± 0.2	1.13	NS
NECA	18.48 ± 0.17	0.79 ± 0.07	5.21	0.05	112 ± 0.3	0.62 ± 0.12	5.3	0.05
MECA	> 2000	1	—	NS	—	—	—	—
Antagonist								
DPCPX	—	—	—	—	2.41 ± 0.03	0.54 ± 0.07	15.14	0.005
XAC	0.27 ± 0.03	0.52 ± 0.07	44.2	0.001	0.44 ± 0.008	0.53 ± 0.09	11.7	0.005

Binding was measured in the presence of 1.9 ± 0.1 nM [^{3}H]CCPA, or 2.5 ± 0.1 nM [^{3}H]DPCPX. The experimental data (each obtained from 40–50 pooled pineal organs) were fitted to the four parameter logistic equation by means of a computer-assisted analysis. F values and levels of significance (P) were obtained after comparison of the cooperative model (Hill slope < 1) with the one-site model (Hill slope = 1) as indicated in Section 2. Abbreviations: CLAD, 2-chloroadenosine; CHA, cyclohexyladenosine; CPA, cyclopentyladenosine; CPCA, 5′-(*N*-cyclopentyl)-carboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DPMA, N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine; MECA, methylcarboxamidoadenosine; NECA, *N*-ethylcarboxamido-adenosine; PIA, phenylisopropyladenosine; XAC, xanthine amine congener.

3.3. Effects of GTP and GTPγS on [^{3}H]CCPA binding

The specific binding of [^{3}H]CCPA was susceptible to inhibition by GTP and by GTPγS. GTP was active only at the concentration of 10^{-3} M, which induced a 50% inhibition (data not shown). The GTPγS-induced reduction in [^{3}H]CCPA binding was apparent over a wider range of concentrations (from 10^{-6} to 10^{-3} M) reaching also 50% inhibition at the concentration of 10^{-3} M (data not shown).

3.4. Equilibrium competition profiles of [^{3}H]CCPA and [^{3}H]DPCPX binding

The binding of [^{3}H]CCPA was displaceable in the presence of adenosine analogues. After fitting the data to the four-parameter logistic equation (Table 1), comparison of the IC_{50} gave the following order of potency: XAC ≫ CPA > PIA = CHA ≫ CLAD > NECA ≫ MECA. When compared to the one-site model, the cooperative model significantly improved the fitting for all the compounds tested, except for MECA (Table 1).

Also the binding of [^{3}H]DPCPX was displaced by the adenosine agonists and antagonists. When the data were fitted to the four parameter logistic equation (Table 1), comparison of the IC_{50} gave the following order of potency: PIA > XAC > DPCPX > CPA > CPCA ≫ DPMA ≫ NECA. When compared to the one-site model, the cooperative model significantly improved the fitting for all the compounds tested, except for DPMA (Table 1).

4. Discussion

The present results provide the first evidence that A1 selective radioligands, namely [^{3}H]CCPA and [^{3}H]DPCPX, bind to mammalian (sheep) pineal membrane preparations. Binding was saturable, reversible and of high affinity. Computer-assisted analysis of saturation data revealed apparently one category of binding sites for, both, [^{3}H]CCPA ($K_d = 1.67 \pm 0.2$ nM) and [^{3}H]DPCPX ($K_d = 5.8 \pm 1.12$ nM). The K_d values found in this study compare well with those measured for the binding of [^{3}H]CPA, [^{3}H]CCPA and [^{3}H]DPCPX to membrane preparations from mammalian brains, including sheep (Cooper and Londos, 1988; Klotz et al., 1991). In the sheep pineal, the maximal number of binding sites found with [^{3}H]DPCPX was also comparable to the number of binding sites in rat brain membranes (Cooper and Londos, 1988; Klotz et al., 1991). This contrasted with the twice higher values obtained after [^{3}H]CCPA binding to sheep pineal membrane preparations. These differences between the adenosine receptor agonist and antagonist might be related to a more complex binding of [^{3}H]CCPA than [^{3}H]DPCPX. It has previously been shown that the binding of [^{3}H]DPCPX increases in the presence of GTP (Parkinson and Fredholm, 1992). Future experiments are necessary to determine whether this also holds true in the sheep pineal. Alternatively, it might be that the agonist binds to more than one category of sites, whereas the antagonist binds preferentially to one category of sites.

The kinetic studies also revealed some differences between the binding of [^{3}H]CCPA and of [^{3}H]DPCPX. The time-course associations of the agonist and antagonist were resolved by a simple bimolecular reaction with a $t_{1/2}$ of 15.42 and 23 min, respectively. Under our experimental conditions, maximal binding was achieved within 60 to 90 min. The time-course dissociation of [^{3}H]DPCPX was also monophasic with a $t_{1/2}$ of 52 min. From the dissociation and association rate constants a calculated K_d of 2.5 nM was obtained, in good agreement with the value measured from the saturation studies. In contrast, the time-course dissociation of the labeled agonist was very slow and resolved by a double exponential decay, revealing a small (25%) proportion of low-affinity sites ($t_{1/2}$ of 5.28 min) and a high proportion of high-affinity sites ($t_{1/2}$ of 168.4 min). The biphasic pattern of [^{3}H]CCPA dissociation might reflect the presence of two different types of binding sites or one class of binding sites exhibiting different affinity states. Biphasic patterns of dissociation have also been described after binding of adenosine analogs to rat brain membranes (Bruns et al., 1986; Yeung et al., 1987). According to some authors, this might be due to the fact that the association rate constant changes little in response to conformation changes of the receptor; conversely, a high affinity binding to the adenosine receptors is stabilized by adenosine agonists and by Mg^{2+} ions in the incubation medium (Linden, 1991; Yeung et al., 1987). Stability may also be increased by endogenous adenosine, located in cryptic binding sites, which cannot be removed by a short treatment with ADA (Linden, 1991; Prater et al., 1992). According to various authors, the effects of Mg^{2+} ions to enhance high affinity agonist binding may be the result of activating Mg^{2+}-sensitive GTPase activity, GTP increasing the dissociation rate of prebound agonists (Cooper, 1988; Linden, 1991; Parkinson and Fredholm, 1992). Also in CHO (Chinese hamster ovary) cells (Cohen et al., 1996), (i) the dissociation of [^{3}H]DPCPX was monophasic, (ii) the dissociation of [^{3}H]CHA and of [^{3}H]PIA was biphasic consisting of a slow component ($K_{-1} = 0.03$ min^{-1}, very similar to that found in the sheep (0.02 min^{-1})) and a very slow, pseudoirreversible component. The slow and very slow components where converted into a fast component upon addition of GTP (Cohen et al., 1996).

In the G protein coupled receptors, the binding of an agonist is decreased in the presence of GTP (and related compounds) (Yeung et al., 1985; Cooper, 1988). In sheep pineal membranes, GTP or GTPγS induced a dose-dependent decrease of [^{3}H]CCPA binding. GTPγS was more potent than GTP, most probably because the former is less

susceptible to hydrolysis than the later. However, none of these compounds could induce a complete suppression of the binding, including at the highest concentration used. Such a phenomenon has already been observed in studies with rat and bovine brain membranes (Nanoff et al., 1995) and is referred to as 'the tight coupling mode'. According to Nanoff et al. (1995) it is attribuable to a membrane protein which stabilizes the agonist/receptor/G-protein complex. In addition, it has also been shown that the GTP or Gpp(NH)p effect of prompting low affinity states was partly or completely overcome when Mg^{2+} was present in the media (Cooper, 1988; Woods and Blazynski, 1991). In membrane preparations from CHO cells, the presence of saponin decreased the binding of [^{3}H]CHA and increased the CHA-stimulated binding of [^{35}S]-GTPγS to G proteins (Cohen et al., 1996). The authors suggested that there was a limited access of GTP or analogs to G proteins, which was overcome by treatment with saponin. Altogether, a number of reasons might explain that in the present study GTP and GTPγS never induced a complete reversal of the [^{3}H]CCPA binding to sheep pineal membranes.

The binding of [^{3}H]CCPA and [^{3}H]DPCPX to sheep pineal membranes was displaced in the presence of adenosine analogs. The order of potency of the different compounds was similar to that typically described for the A_1 receptor. In addition, DPCPX is one of the antagonists displaying the higher specificity for this receptor subtype (Linden, 1994). The displacement curve exhibited low Hill coefficients suggesting, here again, complex interactions which need further experimentation to be explained (see above). The particularly high potency of R-PIA to displace [^{3}H]DPCPX binding should not look odd. This compound was also very potent in displacing [^{3}H]DPCPX binding to bovine brain membranes (Gonzalez-Calero et al., 1992), but much less potent to displace it in the rat brain (Bruns et al., 1987). According to Linden (1994) there are marked species dependent differences in the affinities of xanthines for A_1 receptors among mammals.

The present study provides strong support to the idea that A_1 receptors are present in preparations from sheep pineal membranes. Future investigations using a molecular biology approach together with more radioligand studies should help to support this point. The presence of A_1 receptors in the sheep pineal should not be surprising. Indeed, in the homologous pineal organ of fish and birds, A_1 agonists inhibit cAMP production and/or melatonin biosynthesis (Falcón et al., 1988a,b, 1992). The question remains: (i) which pineal cell type(s) express(es) these receptors in the sheep (blood vessel cells, sympathetic endings which innervate the pineal and/or pinealocytes stricto sensu are potential candidates) and (ii) what role does adenosine play in the pineal gland? Preliminary investigations, using dissociated sheep pinealocytes in culture, indicated that adenosine analogs inhibited melatonin production at picomolar concentrations, and stimulated this production at the nanomolar concentrations.

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References

Bittman, E.L., Dempsey, R.T., Karsch, F.J., 1983. Pineal melatonin secretion drives the reproductive response to daylength in the ewe. Endocrinology 113, 2276–2283.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bruns, R.F., Lu, G.H., Pugsley, T.A., 1986. Characterization of the A_2 adenosine receptor labeled by [^{3}H]-NECA in rat striatal membranes. Mol. Pharmacol. 29, 331–346.

Bruns, R.F., Fergus, J.H., Badges, E.W., Bristol, J.A., Santay, L.A., Hartman, J.D., Hays, S.J., Huang, C.C., 1987. Binding of A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. Naunyn Schmiedebergs Arch. Pharmacol. 335, 59–63.

Cohen, F.R., Lazareno, S., Birdsall, N.J.M., 1996. The effects of saponin on the binding and functional properties of human adenosine A_1 receptor. Br. J. Pharmacol. 117, 1521–1529.

Cooper, D.M.F., 1988. Structural studies of A_1 adenosine receptors. In: Cooper, D.M.F., Londos, C. (Eds.), Adenosine Receptors. Alan R. Liss, New York, NY, pp. 63–74.

Cooper, D.M.F., Londos, C., 1988. Adenosine Receptors. Alan R. Liss, New York, NY.

Falcón, J., Brun, J., Claustrat, B., Collin, J.P., 1988a. Melatonin production in organ cultured chicken pineal: Modulation by adenosine and its analogs. Pfluegers Arch. 413, 93–95.

Falcón, J., Besse, C., Guerlotté, J., Collin, J.P., 1988b. 5′-nucleotidase activity in the pineal organ of the pike. An electron microscopic study. Cell Tissue Res. 251, 495–502.

Falcón, J., Thibault, C., Martin, C., Brun, J., Claustrat, B., Collin, J.P., 1991. Regulation of melatonin production by catecholamines and adenosine in a photoreceptive pineal organ. An in vitro study in the pike and the trout. J. Pineal Res. 11, 123–134.

Falcón, J., Thibault, C., Bégay, V., Zachmann, A., Collin, J.P., 1992. Regulation of the rhythmic melatonin secretion by fish pineal photoreceptor cells. In: Ali, M.A. (Ed.), NATO Advance Study on Rhythms in Fish. Plenum Press, New York, NY, pp. 167–198.

Falcón, J., Van Camp, G., Collin, J.P., 1995. Adenosine A_2 receptor-mediated stimulation of cyclic AMP in cultured chicken pineal cells. J. Pineal Res. 19, 72–78.

Fredholm, B.B., Sollevi, A., 1986. Cardiovascular effects of adenosine. Clin. Physiol. 6, 1–21.

Gharib, A., Reynaud, D., Sarda, N., Vivien-Roels, B., Pevét, P., Pacheco, H., 1989. Adenosine analogs elevate *N*-acetylserotonin and melatonin in rat pineal gland. Neurosci. Lett. 106, 345–349.

Gonzalez-Calero, G., Cubero, A., Klotz, K.-N., 1992. G-protein-coupled A_1 adenosine receptors in coated vesicles of mammalian brain: Characterization by radioligand binding and photoaffinity labelling. Cell. Signal. 4, 737–745.

Klein, D.C., 1985. Photoneural regulation of the mammalian pineal gland. In: Evered, D., Clark, S. (Eds.), Photoperiodism, Melatonin and the Pineal. A CIBA Foundation Symposium, vol. 119. Pitman, London, pp. 38–56.

Klotz, K.N., Vogt, H., Tawfik-Schlieper, H., 1991. Comparison of A1 adenosine receptors in brain from different species by radioligand binding and photoaffinity labelling. Naunyn Schmiedebergs Arch. Pharmacol. 343, 196–201.

Limbird, L.E., 1986. Cell Surface Receptors: A Short Course on Theory and Methods. Martinus Nijhoff, Boston, MA.

Linden, J., 1991. The adenosine receptor molecule. In: Stone, T. (Ed.), Adenosine in the Nervous System. Academic press, San Diego, CA, pp. 103–118.

Linden, J., 1994. Cloned adenosine A_3 receptors: Pharmacological properties, species differences and receptor function. Trends Pharmacol. Sci. 15, 298–306.

Nanoff, C., Mitterauer, T., Roka, F., Hohenegger, M., Freissmuth, M., 1995. Species differences in A_1 adenosine receptor/G protein coupling: Identification of a membrane protein that stabilizes the association of the receptor/G protein complex. Mol. Pharmacol. 48, 806–817.

Nikodijevic, O., Klein, D.C., 1989. Adenosine stimulates adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate accumulation in rat pinealocytes: Evidence for a role of adenosine in pineal neurotransmission. Endocrinology 125, 2150–2157.

Nordio, M., Santana, C., Guerrero, J.M., Reiter, R.J., 1989. Effects of either phosphatidylserine or an adenosine analog on the in vitro and in vivo melatonin synthetic activity of the rat pineal gland. Adv. Pineal Res. 3, 93–97.

Nordio, M., Conte, D., Nonaka, K.O., Reiter, R.J., Isidori, A., 1992. Failure to stimulate melatonin synthesis in the rat pineal gland with an adenosine analog. Psychoneuroendocrinology 17, 249–254.

Palmer, T.M., Stiles, G.L., 1995. Adenosine receptors. Neuropharmacology 34, 683–694.

Parkinson, F.E., Fredholm, B.B., 1992. Magnesium-dependent enhancement of endogenous agonist binding to A1 adenosine receptors: A complicating factor in quantitative autoradiography. J. Neurochem. 58, 941–950.

Phillis, J.W., Wu, P.H., 1981. The role of adenosine and its nucleotides in central synaptic transmission. Prog. Neurobiol. 16, 187–239.

Prater, M.R., Taylor, H., Munshi, R., Linden, J., 1992. Indirect effect of guanine nucleotides on antagonist binding to A_1 adenosine receptors: Occupation of cryptic binding sites by endogenous vesicular adenosine. Mol. Pharmacol. 42, 765–772.

Reiter, R.J., 1991. Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. Endocr. Rev. 12, 151–180.

Sarda, N., Gharib, A., Reynaud, D., Ou, L., Pacheco, H., 1989. Identification of adenosine receptor in rat pineal gland: Evidence for A_2 selectivity. J. Neurochem. 53, 733–737.

Stone, T., 1991. Adenosine in the Nervous System. Academic Press, London.

Sugden, D., 1989. Melatonin biosynthesis in the mammalian pineal gland. Experientia 45, 922–932.

Wells, J.W., 1992. Analysis and interpretation of binding at equilibrium. In: Hulme, E.C. (Ed.), Receptor–Ligand Interactions, a Practical Approach. IRL Press, Oxford, pp. 289–395.

Williams, M., 1987. Purine receptors in mammalian tissues: Pharmacology and functional significance. Annu. Rev. Pharmacol. Toxicol. 27, 315–345.

Woods, C.L., Blazynski, C., 1991. Characterization of adenosine A1-receptors binding sites in bovine retinal membranes. Exp. Eye Res. 53, 325–331.

Yeung, S.-M.H., Fossom, L.H., Gill, D.L., Cooper, D.M.F., 1985. Magnesium ion exerts a central role in the regulation of inhibitory adenosine receptors. Biochem. J. 229, 91–100.

Yeung, S.-M.H., Perez-Reyes, E., Cooper, D.M.F., 1987. Hydrodynamic properties of adenosine Ri receptors solubilized from rat cerebral cortical membranes. Biochem. J. 248, 635–642.