Comparison of A_1 adenosine receptors in brain from different species by radioligand binding and photoaffinity labelling

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Summary. Radioligand binding to A_1 adenosine receptors at brain membranes from seven species was investigated. The antagonist 8-cyclopentyl − 1,3-[^{3}H]dipropylxanthine ([^{3}H]DPCPX) bound with affinities between 0.17 nM in sheep brain and 2.1 nM in guinea pig brain. Competition of several antagonists for [^{3}H]DPCPX binding showed that the most potent compounds were DPCPX with K_i values of 0.05 nM in bovine brain and 1.1 nM in guinea pig brain and xanthine amine congener (XAC) with K_i values of 0.03 nM in bovine brain and 5.5 nM in guinea pig brain. The differences in affinity of the agonist radioligand 2-chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) were less pronounced, ranging from a K_D value of 0.12 nM (hamster brain) to 0.42 nM (guinea pig brain). Agonist competition for [^{3}H]DPCPX binding of photoaffinity labelling, however, exhibited marked species differences. N-Ethylcarboxamidoadenosine (NECA) and S-N^6-phenylisopropyladenosine (S-PIA) showed 20 to 25-fold different K_D values in different species. NECA had a particularly high affinity in guinea pig brain and was only two-fold less potent than R-PIA. Thus, the difference from the "classical" A_1 receptor profile (R-PIA > -NECA > S-PIA) is not sufficient to speculate that A_1 receptor subtypes may exist that are coupled to different effector systems. Our data show that these difference can easily be explained by species differences.

Key words: A_1 adenosine receptors − Species differences − Radioligand binding − Photoaffinity labelling

Introduction

Adenosine receptors have been characterized in different tissues by both functional and binding studies (for review

see Lohse et al. (1988a). On the basis of their coupling to adenylate cyclase, two subtypes have been distinguished (van Calker et al. 1978). The A_1 subtype inhibits adenylate cyclase via the inhibitory guanine nucleotide-binding protein G_i, while the A_2 receptor stimulates cyclase via G_s. Several reports have recently shown that adenosine receptors are also coupled to other effector systems (Belardinelli and Isenberg 1983; Kurachi et al. 1986; Kurtz 1987; Arend et al. 1988) and, therefore, it is also reasonable to distinguish receptor subtypes on the basis of the rank order of potency of agonists and antagonists (Londos et al. 1980). The "classical" agonist rank order of potency for an A_1 receptor is R-PIA->NECA > S-PIA, with pronounced stereoselectivity for the PIA (N^6-phenylisopropyladenosine) diastereomers, while the A_2 receptor is defined by NECA > R-PIA > S-PIA and a low stereoselectivity for the PIA diastereomers.

With these criteria, adenosine receptors of some tissues − e.g. cardiac receptors − are not easily referred to the A_1 or A_2 subtype. In several functional studies with guinea pig hearts NECA has been shown to be as potent as R-PIA (Brückner et al. 1985; Leung et al. 1986; von der Leyen et al. 1989). Similar results have also been obtained in binding experiments in guinea pig atria (Tawfik-Schlieper et al. 1989). The different pharmacological profile compared with the rat brain receptor and the different effector coupling led to the speculation that the cardiac adenosine receptor may represent an A_1 receptor subtype.

It has been shown in rat heart, on the other hand, that R-PIA is about seven times more potent than NECA in the depression of heart rate (Oei et al. 1988). Binding studies with rat ventricular myocytes (Martens et al. 1988) and membranes from rat ventricular myocytes (Martens et al. 1987) showed a pharmacological profile which closely resembled the classical agonist profile. The notion, however, that cardiac A_1 receptors may represent a subclass is mainly based on the data from guinea pig hearts. It is important, therefore, to exclude the idea that the observed differences are only species differences. Binding

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Table 1. Binding parameters of brain membranes from different species for [^{3}H]DPCPX binding

Species	K_D (nM)	B_{max} (fmol/mg)
Sheep	0.17 (0.11 − 0.27)	2150 ± 165
Bovine	0.22 (0.15 − 0.31)	1350 ± 130
Hamster	0.50 (0.39 − 0.63)	4090 ± 720
Rat	0.51 (0.35 − 0.77)	1090 ± 140
Rabbit	0.72 (0.35 − 1.45)	1970 ± 240
Pig	0.76 (0.40 − 1.47)	590 ± 80
Guinea pig	2.08 (1.08 − 4.00)	1600 ± 300

Data shown are means from 3 to 4 separate saturation experiments in each case. K_D values are shown with 95% confidence limits in brackets and B_{max} values are given ± SEM

Table 2. Competition of antagonists for [^{3}H]DPCPX binding (data from representative experiments)

Species	K_i (nM)			
	DPCPX	XAC	CPT	Theophylline
Sheep	0.10	0.09	2.9	9050
Bovine	0.05	0.03	1.4	6330
Hamster	0.14	0.31	4.3	4550
Rat	0.18	0.49	6.3	5600
Rabbit	0.21	0.45	6.4	4710
Pig	0.23	1.48	4.5	5980
Guinea pig	1.06	5.49	26.1	7060

CPT, cyclopentyl-theophylline; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, xanthine amino congener

Table 3. Binding parameters of brain membranes from different species for [^{3}H]CCPA binding

Species	K_D (nM)	B_{max} (fmol/mg)
Sheep	0.15 (0.12 − 0.18)	1920 ± 250
Bovine	0.22 (0.16 − 0.29)	1100 ± 20
Hamster	0.12 (0.11 − 0.13)	1630 ± 60
Rat	0.20 (0.17 − 0.23)	880 ± 30
Rabbit	0.24 (0.22 − 0.26)	1440 ± 30
Pig	0.31 (0.24 − 0.42)	500 ± 80
Guinea pig	0.42 (0.31 − 0.58)	1190 ± 110

Data shown are means from 3 to 4 separate saturation experiments. K_D-values are shown with 95% confidence limits in brackets and B_{max} values are shown ± SEM

studies at A_1 receptors in brain membranes showed that species differences exist (Bruns et al. 1980; Murphy and Snyder 1982; Ferkany et al. 1986), in particular for 8-substituted xanthine derivatives (Hamilton et al. 1985; Schwabe et al. 1985; Jacobson et al. 1986). We now investigate whether different agonist profiles at A_1 receptors in several species may account for the different functional data reported by several groups compared with the classical A_1 profile. We compared adenosine receptors from brain membranes as the typical source for A_1 receptors from seven species by means of radioligand binding. A_1 receptors were defined by labelling with the highly A_1 selective ligands [^{3}H]DPCPX (antagonist) and [^{3}H]CCPA (agonist) and the consistently observed high stereoselectivity for the PIA diastereomers. In addition, the A_1 receptors from different sources were compared by photoaffinity labelling with R-2-azido-N^6-^{125}I-p-hydroxyphenylisopropyladenosine (^{125}I-AHPIA, Klotz et al. 1985). Comparison of the pharmacological agonist profiles from brain membranes with binding and functional data from other tissues in the respective species should facilitate in distinguishing new receptor subtypes and species differences.

Materials and methods

Materials. [^{3}H]CCPA was purchased from Du Pont − New England Nuclear (Dreieich, FRG) and [^{3}H]DPCPX from Amersham Buchler (Braunschweig, FRG). AHPIA was iodinated to give ^{125}I-AHPIA with a specific radioactivity of about 74 TBq/mmol (Klotz et al. 1985). DPCPX (8-cyclopentyl-1,3-dipropylxanthine) and CCPA were synthesized as previously described (Lohse et al. (1987, 1988 b, respectively). 8-Cyclopentyl-theophylline (CPT) was from RBI (Natick, Mass, USA). Xanthine amino congener (XAC) was a kind gift of Dr. Entzeroth (Thomae, Biberach, FRG). All other chemicals were of the highest purity available.

Preparation of membranes. Brain membranes from all species were prepared as described earlier (Lohse et al. 1984) from whole brain, with the exception of bovine brain from which only the cortex was used (no difference has been observed for the K_D values for the agonist [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA) in different calf-brain areas; Murphy and Snyder 1982).

Radioligand binding. Binding of [^{3}H]DPCPX was performed according to Lohse et al. (1987) and binding of [^{3}H]CCPA was performed as described recently (Klotz et al. 1989). In competition experiments 0.2 − 0.5 nM [^{3}H]DPCPX with 30 − 50 μg protein in a total volume of 250 μl was used. Nonspecific binding of [^{3}H]DPCPX and [^{3}H]CCPA was determined in the presence of 10 μM R-PIA and 1 mM theophylline, respectively. Adenosine deaminase was present in all binding assays at a concentrations of 0.2 U/ml. Each value was determined in duplicate. Data were analysed by nonlinear curve-fitting with the program SCTFIT as described by Lohse et al. (1987). Two affinity states were assumed in competition experiments when curve fitting according to a two-site model significantly improved the fit ($p < 0.001$).

Photoaffinity labelling. Photoaffinity labelling of A_1 receptors in brain membranes was performed with ^{125}I-AHPIA at a concentration of about 200 pM as described elsewhere (Klotz et al. 1985; Klotz and Lohse 1986). In brief, membranes (approximately 250 μg) were incubated for 2 − 3 h with ^{125}I-AHPIA (500000 cpm) in a total volume of 750 μl in the dark in the presence and absence of 1 mM theophylline to define non-specific labelling. Then membranes were UV-irradiated and prepared for sodiumdodecyl sulfate − polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. The gels were stained with Coomassie Blue and then dried for autoradiography (Klotz et al. 1985).

Results

Saturation experiments with brain membranes from different species showed that marked differences in affinity
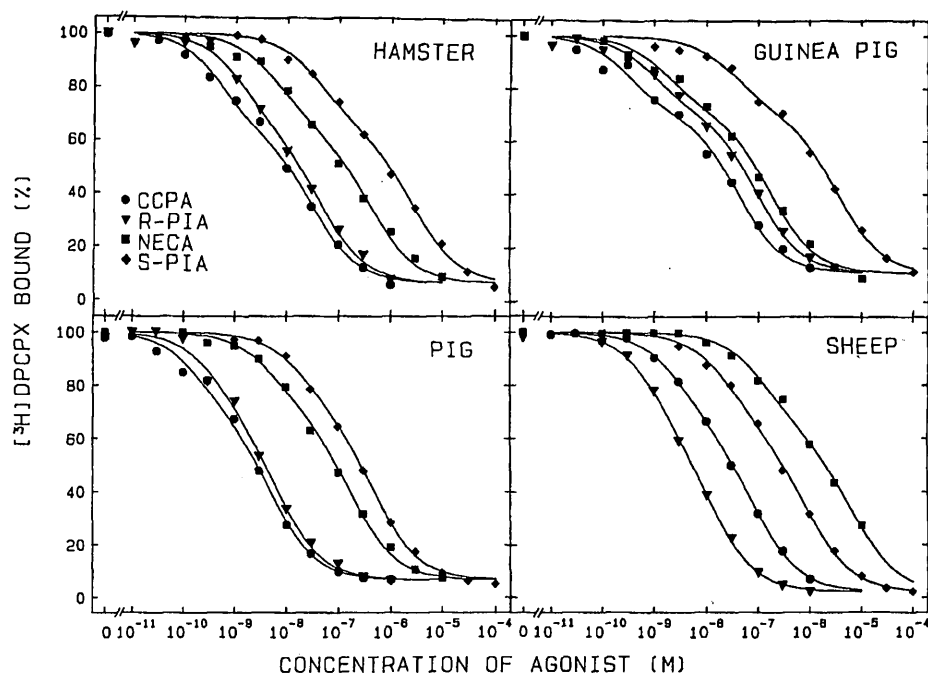
Fig. 1. Competition of agonists for [³H]DPCPX binding. Membranes from different brains were incubated with 0.2–0.5 nM [³H]DPCPX and increasing concentrations of agonists at 25°C. Data are shown from representative experiments. K_i values for agonists from multiple experiments are summarized in Table 4 for all species. $CCPA$, 2-chloro-N^6-[³H]cyclopentyladenosine; $NECA$, N-ethylcarboxamidoadenosine; R-PIA, R-N^6-phenylisopropyladenosine; S-PIA, S-N^6-phenylisopropyladenosine

Table 4. Competition of agonists for [³H]DPCPX binding. K_i values (nM) are given for high and low affinity states (K_H and K_L, respectively) for each agonist. B_{max} values are sums of high and low affinity states (fmol/mg protein) and % R_H shows the percentage of receptors in the high affinity state

Species	CCPA		R-PIA		S-PIA		NECA		B_{max}	% R_H
	K_H	K_L	K_H	K_L	K_H	K_L	K_H	K_L		
Sheep	0.98	39.3	0.36	6.9	8.3	366	32.9	2333	2030	51
Bovine	0.15	7.7	0.03	1.6	1.3	69	13.4	466	1700	52
Hamster	0.39	23.2	0.94	35.9	30.5	1763	4.2	277	2220	57
Rat	0.23	16.1	0.51	20.8	29.1	1490	3.7	170	960	56
Rabbit	0.20	8.5	0.31	13.5	10.3	534	5.3	85	1680	46
Pig	0.15	4.0	0.27	4.5	16.8	386	5.3	161	630	40
Guinea pig	0.59	39.7	0.89	66.6	33.1	2985	1.8	129	1480	42

Data are mean values from 3 to 6 experiments

and receptor density exist for the A_1 receptor-selective antagonist [³H]DPCPX (Table 1). The affinity was the highest at sheep brain receptors with a K_D value of 0.17 nM and the lowest with 2.1 nM at the guinea pig receptor. The B_{max} values ranged from about 600 fmol/ mg in pig brain membranes up to 4000 fmol/mg protein in hamster brain membranes (Table 1). The marked species differences for 8-substituted xanthine derivatives was also documented by antagonist competition for [³H]DPCPX binding (Table 2). Theophylline had similar affinities in all species under investigation, while DPCPX, XAC and CPT exhibited 20- to 100-fold higher K_D values in guinea pig brain compared to bovine brain.

The species difference was less pronounced for the affinity of the A_1 receptor-selective agonist [³H]CCPA. A_1 receptors from hamster brain had a K_D value of 0.12 nM, and at guinea pig brain receptors a K_D value of 0.42 nM was measured (Table 3). The receptor densities determined with [³H]CCPA were lower than those values

obtained with [³H]DPCPX, because only high affinity binding was tested (Table 3).

The pharmacological profile of agonists for the receptors from different species was determined in competition experiments. Competition for binding of the antagonist [³H]DPCPX resulted in biphasic curves, which are characteristic for the presence of receptors in high- and low-affinity states for agonists. Figure 1 gives an example for the profiles of four different species. Strikingly, the differences between the three agonists CCPA, R-PIA and NECA are only very small at A_1 receptors from guinea pig brain membranes compared to membranes from other species. The difference between R-PIA and NECA is less than two-fold in guinea pig brain, compared to 20-fold in pig brain, for example. In sheep and bovine brain a different rank order of potency of agonists was found. In these species, R-PIA is more potent than CCPA, and S-PIA is more potent than NECA. The sum of high and low affinity B_{max} values calculated from the competition
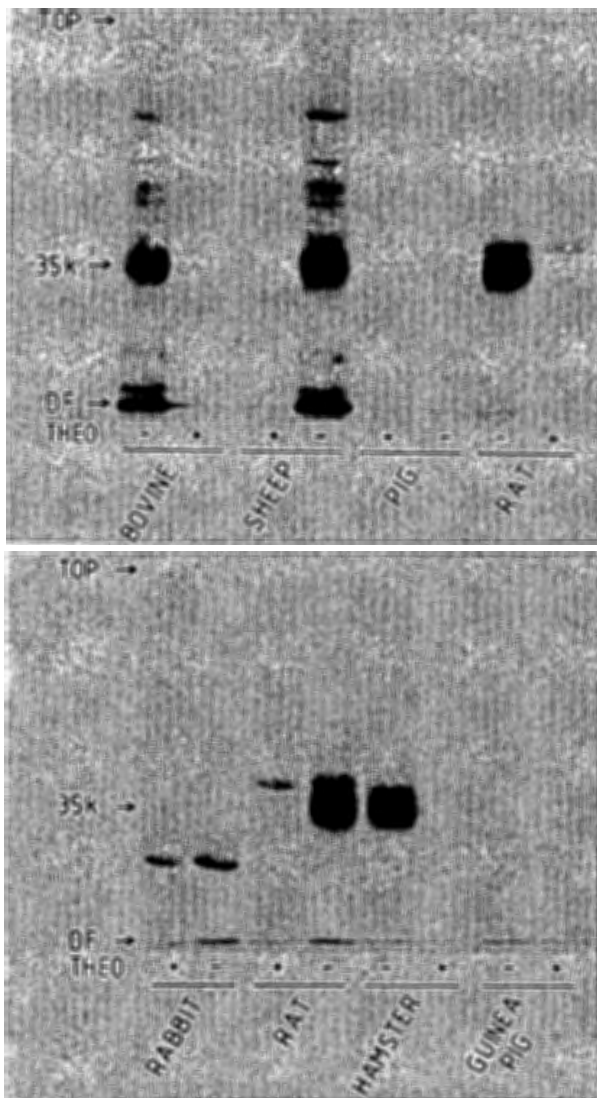
(Table 4) and from guinea pig atria (Tawfik-Schlieper et al. 1989) suggest that the same adenosine receptors exist in both nervous and cardiac tissue.

A recent report by Leid et al. (1989) also showed an atypical agonist profile of the solubilized porcine atrial A_1 receptor. It is conspicuous that NECA and S-PIA are almost equipotent at this receptor. The same characteristic profile was detected for the A_1 receptor in pig brain membranes (Table 4).

In sheep and bovine brain not only were different potencies for particular agonists found, but even a different rank order of agonist potency was observed. Strikingly, R-PIA was found to be more potent than CCPA, and S-PIA was more potent than NECA. These results show that fundamental differences may exist for agonist profiles at A_1 receptors from different species.

Species variations can be visualized by photoaffinity labelling with ^{125}I-AHPIA. The dramatic differences in the photoincorporation yield, which are not related to receptor binding of the photoaffinity ligand (Table 5), confirm the existence of species-dependent variations in the agonist-binding domain of A_1-receptors.

In the past, evidence accumulated that A_1 receptors may be coupled not only with adenylate cyclase but also with other effector systems (Belardinelli and Isenberg 1983; Kurachi et al. 1986; Kurtz 1987; Arend et al. 1988), probably via different guanine nucleotide binding proteins. The copurification of A_1 receptors with different G protein α subunits from bovine brain (Munshi and Linden 1989) supports the idea that A_1 receptors couple to different G proteins. This may possibly allow different potencies for one agonist in mediating different intracellular signals via a single receptor subtype. Therefore, the existence of different receptor subtypes involved in mediating different signals, should also be evidenced with radioligand binding studies. In contrast to functional agonist potencies, which are in addition dependent on parameters like "spare receptors" (Lohse et al. 1986), binding paramenters describe only the receptor recognition site. Receptor classification on the basis of the signal transduction process has also been regarded as unsafe for α-adrenergic receptors (McGrath and Wilson 1988). The "variable receptor affinity hypothesis" provides another explanation for selective tissue responses, which may be dependent on the microenvironment of the receptors in different membranes (Bevan et al. 1989). All these factors may have contributed to the variations of the pharmacological profile for A_1 type receptors, which led to the proposal of an A_3 receptor (Ribeiro and Sebastiao 1986). The classification as an A_3 receptor was based on functional data and mainly on different positions in the rank order of potency of NECA and 2-chloroadenosine, compared with the classical A_1 profile.

Another problem for classification based on functional studies emerges when ligands like NECA and 2-chloroadenosine are used. These compounds have considerable potencies at A_2 receptors, which might influence the determination of the A_1 potency in different tissues to various degrees depending on the densities of A_1 and A_2 receptors. This problem can be overcome in binding experiments with the highly A_1 selective radioligands [^3H]DPCPX and [^3H]CCPA, which do not label A_2 receptors.

Our data clearly show that comparing agonist profiles from different tissues with the classical profile from rat brain is not sufficient to propose new A_1 receptor subtypes. The discrimination of receptor substypes requires comparison of binding and functional data within the same species. Functional data, however, may not match the binding profiles, because they are the matter of modulation via a variety of different mechanisms, e.g. spare receptors or interaction of ligands with A_2 receptors. We propose using the brain A_1 receptor as the reference receptor and, therefore, provide binding data for brain membranes from a series of species. The highly A_1 selective CCPA, the PIA enantiomers and NECA seem to be reasonable means of defining agonist profiles in binding studies.

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