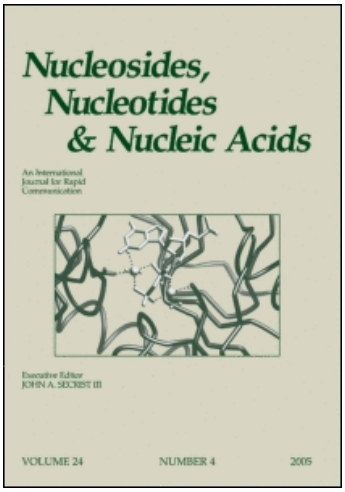

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A Functional Screening of Adenosine Analogues at the Adenosine A_{2B} Receptor: A Search for Potent Agonists

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A FUNCTIONAL SCREENING OF ADENOSINE ANALOGUES AT THE ADENOSINE A_{2B} RECEPTOR: A SEARCH FOR POTENT AGONISTS

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Abstract. Various adenosine analogues were tested at the adenosine A_{2B} receptor. Agonist potencies were determined by measuring the cyclic AMP production in Chinese Hamster Ovary cells expressing human A_{2B} receptors. 5'-*N*-Substituted carboxamidoadenosines were most potent. 5'-*N*-Ethylcarboxamidoadenosine (NECA) was most active with an EC_{50} value of 3.1 μM. Other ribose modified derivatives displayed low to negligible activity. Potency was reduced by substitution on the exocyclic amino function (N^6) of the purine ring system. The most active N^6-substituted derivative N^6-methyl-NECA was 5 fold less potent than NECA. C8- and most C2-substituted analogues were virtually inactive. 1-Deaza-analogues had a reduced potency, 3- and 7-deazaanalogues were not active.

Introduction

The adenosine A_{2B} receptor is one of the four known subtypes of adenosine receptors, the others being named A_1, A_{2A} and A_3. The A_1 and A_3 receptor subtypes inhibit the enzyme adenylate cyclase, diminishing the production of the second messenger cyclic AMP. On the contrary, both A_2 subtypes stimulate adenylate cyclase to produce cyclic AMP. A subdivision of A_2 receptors was first proposed by Daly *et al.* based upon

observations of lower EC_{50} values on cyclic AMP accumulation in rat striatum than in rat brain slices[1]. Bruns *et al.* designated the high affinity striatal A_2 receptor as the A_{2A} subtype, whereas they referred to the low affinity subtype, existing throughout the brain, as to A_{2B}[2]. Molecular cloning and expression have been reported of all four subtypes of adenosine receptors of several species, including the A_{2B} receptor from both rat and human brain[3,4]. By the cloning techniques it had also become possible to determine tissue distributions of the receptors. In the human body, low expression of the A_{2B} receptor was found in kidney, moderate levels were observed in heart, brain and lungs, and high densities were measured in the colon[5].

Through A_{2B} receptors, adenosine inhibits the Tumour Necrosis Factor-α (TNF-α) production by monocytes[6,7]. TNF-α is a pro-inflammatory cytokine that triggers the production cascade of other cytokines and acute-phase proteins[8]. When produced in large quantities—*e.g.*, when the immune system is overactivated due to a challenge by pathogenic bacteria—TNF-α can lead to septic shock and eventually to death[9,10]. A future therapeutic purpose for adenosine A_{2B} receptor agonists could thus be the treatment of septic shock.

However, no A_{2B} selective compounds have been reported yet. One of the most potent agonists on this subtype is 5'-*N*-ethylcarboxamidoadenosine (NECA). EC_{50} values on the production of cyclic AMP are in the low micromolar range, considerably higher than on the other subtypes[11-14].

As a start of our search to find agonists with increased potency and selectivity, we screened adenosine analogues with a great variety of substituents and modifications on their ability to generate cyclic AMP in a CHO cell line stably transfected with human adenosine A_{2B} receptor cDNA[4].

Results

Cyclic AMP production was measured at compound concentrations of 100 μM. Cyclic AMP production is shown as percentage of the production by 100 μM NECA.

A number of compounds were taken further into investigation by determining their EC_{50} values.

NECA (**1**) and 5'-*N*-cyclopropylcarboxamidoadenosine (NCPCA; **2**) were found to have EC_{50} values on cyclic AMP production of 3.1 and 5.3 μM, respectively (TABLE 1).

More 5'-carboxamidoadenosines are listed in TABLE 2. Compounds **3-8** are N^6-(3-pentyl)-substituted. In this series, the 5'-*N*-benzyl-substituted analogue (**8**) did not show activity. Cyclic AMP production decreased according to the following substituent order: cyclopropyl > isopropyl > methyl > allyl > 3-pentyl. NECA lost its activity when the ethyl substituent was modified into a 2-aminoethyl group (compound **9**).

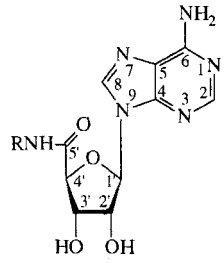
Adenosines substituted at the C2-position of the purine are shown in TABLE 3. Several 2-thio-, 2-amino- and 2-halo-analogues were evaluated. C2-substution of adenosine diminished potency. In the 2-halo series, 2-chloroadenosine (**18**) was the most potent compound with an EC_{50} value of 24 μM; potency decreased when an iodo or a fluoro substituent was present instead of a chloro substituent. 2-Thio- and 2-amino-substituted adenosines, including the A_{2A} agonist CV 1808, displayed low potency.

In the C8-amino-substituted series (TABLE 4), only the 8-amino-analogue itself showed some activity, albeit marginal (9%).

N^6-substituted analogues were categorized into four groups as shown in TABLES 5-8. In TABLE 5, NECA analogues are listed; analogues of 5'-*N*-methylcarboxamidoadenosine (MECA) are shown in TABLE 6; adenosine derivatives without modifications in the ribose are displayed in TABLE 7; some N^6-functionalized congeners are listed in TABLE 8.

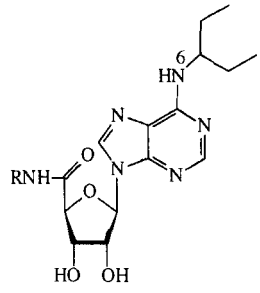
In the series of the NECA analogues in TABLE 5, the N^6-methyl-substituted member (**26**) was the most active one with an EC_{50} value of 19 μM. The other analogues, most of them bearing N^6-(substituted)-benzyl groups, had activities varying from 14-95%. The MECA analogues in TABLE 6 generally displayed a considerably lower activity than the NECA analogues (0-23%). In the adenosine series (TABLE 7), EC_{50} values of compounds **55-60** were determined. The A_1 selective agonist N^6-cyclopentyladenosine (CPA, **55**) had an EC_{50} value of 203 μM. The N^6-phenyl-(**56**), N^6-benzyl-(**57**), N^6-phenylethyl-(**58**) and N^6-(*R*)-phenylisopropyl-substituted (R-PIA, **59**) adenosines were more active, with EC_{50} values in the range of 53-96 μM. The activity of N^6-(4-sulfophenyl)adenosine (SPA, **60**) was in this same range (52 μM). Adenosine derivatives with other N^6-substituents were also tested. N^6-furyl adenosine (**61**) gave a 3% response.

TABLE 1. Activities of NECA and NCPCA on cAMP production in CHO cells expressing human A_{2B} receptors



		R	EC_{50} (μM)
1	NECA	ethyl	3.1±2.4
2	NCPCA	cyclopropyl	5.3±1.6

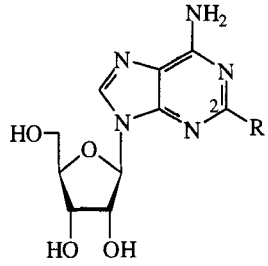
TABLE 2. Activities of N^6-(3-pentyl), 5'-*N*-disubstituted carboxamidoadenosines on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



	R	% stimulation
3	methyl	63%
4	allyl	41%
5	isopropyl	69%
6	cyclopropyl	78%
7	3-pentyl	6%
8	benzyl	0%
9	2-NH_2-ethyl*	1%

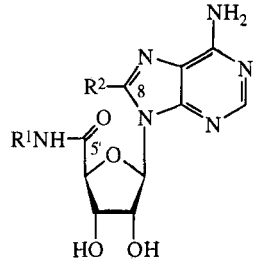
*N^6-unsubstituted analogue

TABLE 3. Activities of C2-substituted adenosines on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



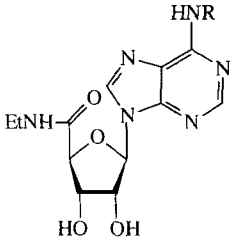
	R	% stimulation or EC_{50} (μM)
10	SH	1%
11	SCH_2CH_2CN	0%
12	S-cyclohexyl	3%
13	S-hexadecyl	5%
14	SBn	3%
15	NHPh (CV1808)	10%
16	NHPhEt	3%
17	F	23%
18	Cl	24±11μM
19	I	2%

TABLE 4. Activities of C8-substituted NECA and MECA analogues on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



	R^1	R^2	% stimulation
20	Et	Br	0%
21	Et	NH_2	9%
22	Et	EtNH	0%
23	Et	Me_2N	0%
24	Me	MeNH	0%
25	Me	Me_2N	0%

TABLE 5. Activities of N^6-substituted NECA analogues on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



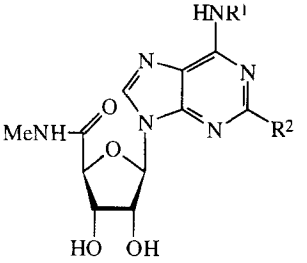
	R	EC_{50} (μM) or % stimulation
26	methyl	19 ± 6μM
27	3-CH_3O-phenyl	63%
28	3- CH_3-benzyl	16%
29	4-CH_3O-benzyl	14%
30	3- CH_3O-benzyl	91%
31	3-F-benzyl	80%
32	3-Cl-benzyl	84%
33	3-Br-benzyl	95%
34	3-I-benzyl	71%
35	2-NO_2-benzyl	87%
36	3-NO_2-benzyl	57%
37	4-NO_2-benzyl	41%
38	benzyl*	24%

*5'-*N*-**cyclopropyl** analogue

Analogues with substituted benzyl groups had activities of 12-35% (**62-65**). N^6-alkyladenosines (**71-74**) were less potent than the N^6-phenyl(alkyl)adenosines mentioned before. N^6,N^6-dialkyladenosines (**75,76**) were inactive. The N^6-(4-sulfo-phenyl)alkyladenosines **68-70** appeared to be far less active than SPA (**60**). N^6-amino adenosine (6-hydrazino purine riboside, **77**) was not active.

The N^6,C2-disubstituted compounds **45-47** and **66,67** were found to have reduced activities in comparison with the C2-unsubstituted analogues **44** and **72**. Analogues with large substituents, such as the (adenosin-N^6-yl)alkyl-substituted compounds **78** and **79** in

TABLE 6. Activities of N^6-substituted MECA analogues on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



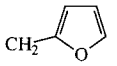
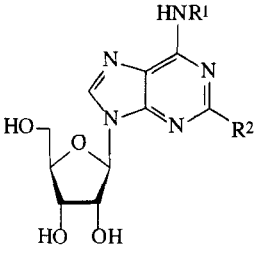
	R^1	R^2	% stimulation
39	3-CH_3-benzyl	H	13%
40	3-CF_3-benzyl	H	0%
41	4-Cl-benzyl	H	1%
42	3-Br-benzyl	H	16%
43	4-Br-benzyl	H	3%
44	3-I-benzyl (IB-MECA)	H	10%
45	3-I-benzyl (Cl-IB-MECA)	Cl	3%
46	3-I-benzyl	SMe	0%
47	3-I-benzyl	NHMe	0%
48	4-SO_3H-benzyl	H	0%
49	3-I-4-NH_2-benzyl (I-AB-MECA)	H	18%
50	3-NO_2-benzyl	H	4%
51	(*R*)-1-phenyl-ethyl	H	23%
52	(*S*)-1-phenyl-ethyl	H	15%
53		H	0%
54	benzyl	H	15%

TABLE 7 and the functionalized congeners **80-82** in TABLE 8, had low activities (10-20%).

Deaza derivatives (TABLE 9) showed negligible potencies, except the 1-deaza analogues.

7-Xanthinyl adenosines (FIG. 1) lacked potency, as well as the ribose modified adenosines in FIG. 2. Compounds that were not categorized, most of them having more than one modification, are depicted in FIG. 3. These all had low activities.

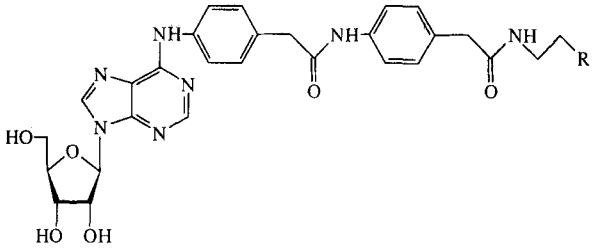
TABLE 7. Activities of N^6-substituted adenosines on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



	R^1	R^2	EC_{50} (μM) or % stimulation
55	cyclopentyl (CPA)	H	203±97 μM
56	phenyl	H	63±19 μM
57	benzyl	H	96±32 μM
58	phenylethyl	H	67±4 μM
59	(*R*)-phenylisopropyl (R-PIA)	H	53 ±8μM
60	4-SO_3H-phenyl (SPA)	H	52 ±17μM
61		H	3%
62	4-NH_2-benzyl	H	12%
63	2-CH_3-benzyl (metrifudil)	H	21%
64	2-Cl-benzyl	H	35%
65	3-I-benzyl	H	18%
66	3-I-benzyl	Cl	6%
67	3-I-benzyl	NH_2	6%
68	4-SO_3H-phenylpropyl	H	1%
69	4-SO_3H-phenylbutyl	H	2%
70	4-SO_3H-phenyldecyl	H	4%
71	n-butyl	H	23%
72	n-decyl	H	3%
73	4-NH_2-butyl	H	10%
74	10-NH_2-decyl	H	0%
75	*N,N*-dimethyl*	H	0%
76	*N,N*-dipropyl*	H	0%
77	NH_2	H	0%
78	(adenosin-N^6-yl)butyl	H	15%
79	(adenosin-N^6-yl)decyl	H	13%

* N^6,N^6-disubstituted analogue

TABLE 8. Activities of N^6 functionalized congeners on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



	R	% stimulation
80	NHAc	13%
81	$N(CH_3)_3$	17%
82	OH	19%

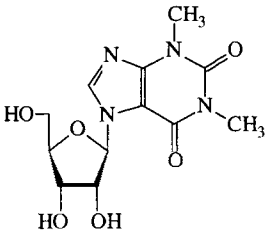
TABLE 9. Activities of deazaadenosine analogues on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.

	analogue	% stimulation or EC_{50} (μM)
83	1-deazaadenosine	10%
84	2-chloro-1-deazaadenosine	20%
85	3-deazaadenosine	1%
86	1,3-dideazaadenosine	1%
87	7-deazaadenosine	0%
88	1,7-dideazaadenosine	0%
89	1-deaza-NECA	16 ± 5 μM

Discussion

Our aim was to analyze compounds for A_{2B} agonism. Therefore we tested modified adenosine analogues. In this approach our focus was on stimulation of the receptor. We did not do experiments to determine antagonistic activities.

Until today the reference ligand for the adenosine A_{2B} receptor has been the nonselective adenosine agonist NECA. Pierce *et al.* and Alexander *et al.* reported NECA



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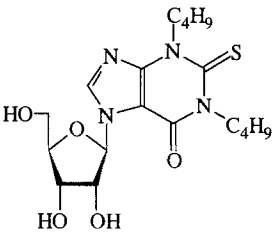

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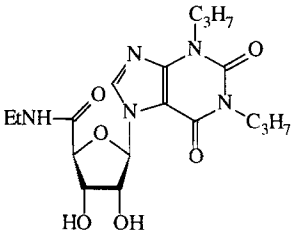

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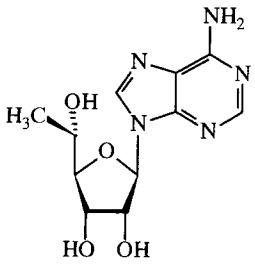
FIG. 1. 7-Xanthinyl analogues (no activities on cAMP production in CHO cells expressing human A_{2B} receptors). Compounds were tested at 100 μM.



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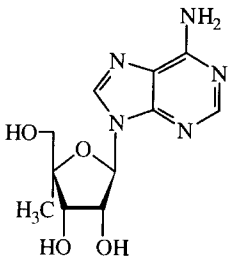

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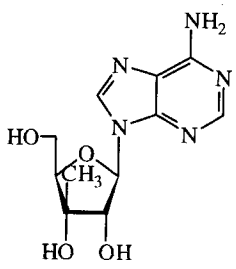

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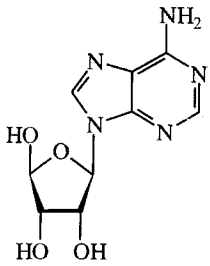

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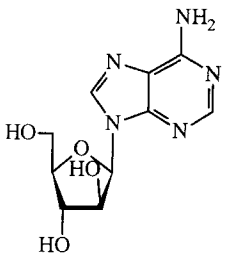

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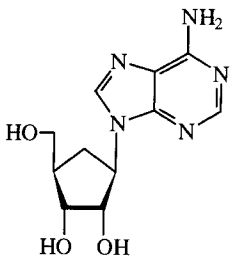

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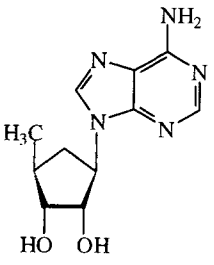

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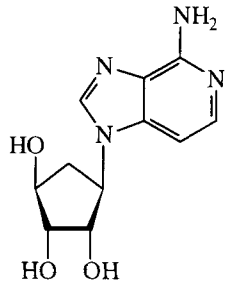
FIG. 2. Activities of ribose modified adenosines on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.



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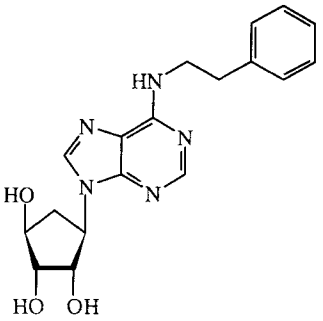

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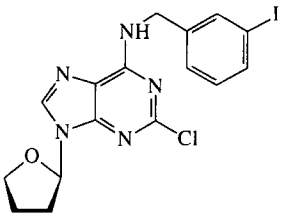

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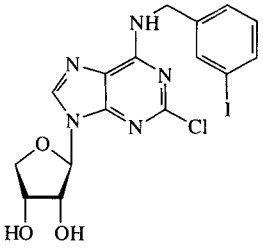

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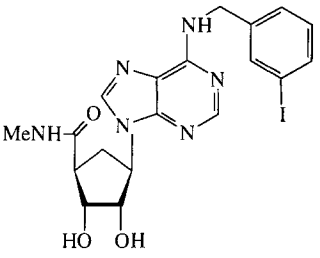

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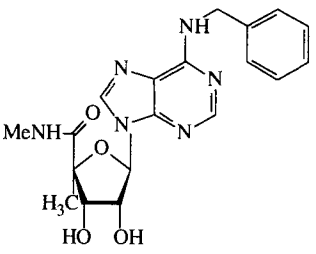

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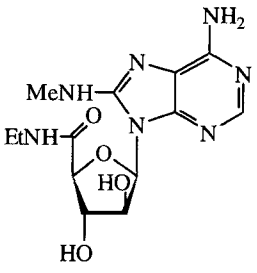

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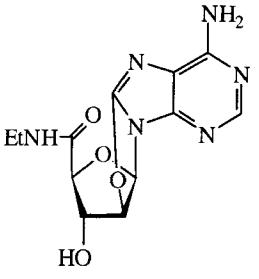

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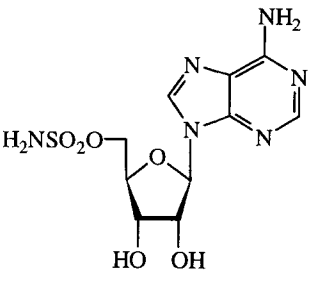

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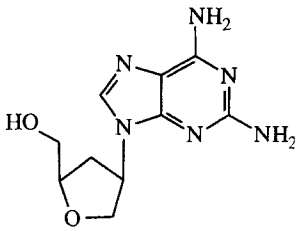

109 (0%)

FIG. 3. Activities of variously modified analogues on cAMP production in CHO cells expressing human A_{2B} receptors. Compounds were tested at 100 μM. Activities are given as percentages of stimulation with 100 μM NECA.

to have an EC_{50} value of approximately 1 μM on the production of cyclic AMP in CHO-K1 cells[4,14]. These cells were also used here as the test system. EC_{50} values of 3.1 and 5.3 μM were determined for NECA and its 5'-*N*-cyclopropyl analogue NCPCA, respectively (TABLE 1).

Regarding the relatively high potency of the carboxamido analogues NECA and NCPCA, the compounds in TABLE 2 are interesting. Although the N^6-(3-pentyl) group reduced activity, still a trend can be observed. The 5'-*N*-cyclopropyl-substituted analogue was the most potent ligand in this series, followed by the isopropyl-, the methyl-, the allyl-, the (3-pentyl)- and the benzyl-substituted compounds. A (3-pentyl) and a benzyl group in this position are largely unfavourable, probably because of steric or electronic interactions.

Substitution at the C2-position (TABLE 3) with appropriate groups is known to enhance A_{2A} selectivity. The relatively A_{2A} selective agonist CV 1808 (**15**) showed only low activity in the A_{2B} assay. This was also observed with other 2-amino-substituted adenosines and with 2-thio-analogues. Of the halogen atoms in the 2-position, chloro (in compound **18**) appeared to be a relatively good substituent. The activity (24 μM), however, did not equal that of NECA. Recently Alexander *et al.* reported 2-chloroadenosine to have an EC_{50} value of 5.3 μM on the same cell line we used here[14]; the A_{2A} reference compound 2-[*p*-(2-carboxyethyl)-phenylethylamino]-NECA (CGS 21680, not tested in our study) showed a 40% response at a concentration of 100 μM and it was inactive at concentrations of 10 μM. Results from both this and our study suggest that C2 is not a suitable site of modification in order to gain A_{2B} potency. Probably there is little space in the receptor for a substituent in this position.

Substitution at the C8-position (TABLE 4) led to virtually inactive compounds. Bruns reported responses for 8-methylamino- and 8-dimethylaminoadenosine of 33% and 5% respectively[11]. These responses are higher than the activities we measured with the MECA and NECA analogues. The 5'-*N*-methylcarboxamido moiety in 8-methylamino-MECA (**24**) probably causes the lower activity in comparison with Bruns' adenosine analogue. More recently it has been shown that C8-substitution yields partial agonists for the A_1 receptor[15].

N^6-Substitution with appropriate groups has led to A_1 and A_3 selective agonists[16,17]. In the N^6-substituent series of MECA (TABLE 6), compounds have reduced activities

compared to their NECA analogues (TABLE 5). It is concluded that an ethyl substituent on the 5'-carboxamido group is favoured over a methyl substituent. The selective A_3 agonist 2-chloro-N^6-(3-iodobenzyl)-MECA (Cl-IB-MECA, **45**)[18] was included in the MECA series. This compound and the other 2-substituted derivatives **46**, **47** (TABLE 6), **66** and **67** (TABLE 7) displayed lower activities than their C2-unsubstituted analogues **44** and **72**. These results also indicate that C2-substitution diminishes A_{2B} potency.

N^6 should be monosubstituted, because the disubstituted analogues **75** and **76** were inactive. The potency order for the N^6-benzyl-substituted derivatives (TABLES 5, 6 and 7) was: $2 \geq 3 > 4$ substitution. However, no trends in the type of benzyl substituents could be discovered. In the adenosine series (TABLE 7) optima in potency were found for N^6-phenyl (**56**) and N^6-(*R*)-phenylisopropyl (**59**) substitution (50-60 μM). The N^6-benzyl substituted analogue (**57**) showed the lowest activity (96 μM). Metrifudil (**63**, N^6-[(2-methylphenyl)methyl]adenosine) has been reported to be a moderately A_{2B} selective agonist, albeit five times less potent than NECA on guinea pig aorta contraction[19]. Here we report a 21% response for metrifudil. These results suggest that substituted N^6-phenyl and N^6-phenylisopropyl groups might lead to more active agonists than substituted benzyl groups.

The NH_2 group in the adenosine molecule is even allowed to bear rather large substituents (TABLES 7 and 8), not causing a total loss of activity (diadenosines **78** and **79**, functionalized congeners **80-82**). There must be some space in the receptor in this position. A part of the N^6-substituent might occupy a region outside the receptor, as has been hypothesized for the adenosine A_1 receptor[20].

From the results of the deazaadenosines in TABLE 9 it is obvious that the purine nitrogen atoms are essential for potency of adenosine A_{2B} receptor agonists, especially N3 and N7. When N1 was substituted for CH, a response was left, such as in 1-deazaadenosine (**90**), 2-chloro-1-deazaadenosine (**91**) and 1-deaza-NECA (**96**). 1-Deaza-NECA[21] was moderately potent with an EC_{50} value of 16 μM. When the other nitrogen atoms were substituted, the response was negligible. In Bruns' study 7-deazaadenosine was found to be inactive, whereas 1- and 3-deazaadenosine were not evaluated. 2-Azaadenosine on the contrary (not tested in our assay) was slightly less active than NECA[11]. All results together suggest that the purine skeleton should not be altered.

Similar observations have been done for analogues in TABLE 9 in binding experiments on the rat brain A_1 receptor and on the platelet A_{2A} receptor[22].

Derivatives in which the purine ring system was modified into a (substituted) xanthinyl group are depicted in FIG. 1. All three hybrides gave very low responses. This accounts again for the importance of an intact purine ring system for A_{2B} agonists.

Low responses were also found for adenosines with modified ribose parts (FIG. 2) and with various modifications (FIG. 3). It should be mentioned that adenosine deaminase was added to the assay to avoid false hits caused by endogenous adenosine. The use of adenosine deaminase, however, may have inactivated some of the derivatives in FIG. 2. Especially suspect are **94**, **95**, **97** and **98**, all having (besides a free 6-amino function) an unsubstituted hydroxymethylene group which has been proven necessary for adenosine deaminase substrates to be hydrolysed[23]. 5'-Deoxyasteromycin (**99**), not a substrate for adenosine deaminase, did not show activity, whereas Bruns found a fairly good agonistic activity for asteromycin **98**[11]. Apparently, removal of the 5'-hydroxyl group is detrimental for A_{2B} receptor activity. This is also the case when the hydroxymethylene group is removed (**103**, compare with **66**), or when a methyl group is added on C-5' (**93**). Adding a methyl group on C-4' also lowers activity (**94** and **105**, compare with **54**). Substituting the ribose ring oxygen for a methylene group does not cause an increase of activity (**98** and **104**). Summarizing, for enhanced potency it is not allowed to add methyl groups in various positions in the ribose part, neither to remove the 5'-hydroxyl group or the 5'-methylene group, nor substituting the ring oxygen for a methylene group.

Conclusions and evaluation

By the study presented here, we gained more insight in the pharmacologic profile of the human adenosine A_{2B} receptor. Of all compounds, NECA was most potent with an EC_{50} value of 3.1 μM, and still the most active agonist reported yet. Concerning the medicinal chemistry of the human A_{2B} receptor we conclude that 1) 5'-*N*-carboxamidoadenosines show highest potencies; NECA analogues are most potent in this series, MECA derivatives are less active; the rest of the ribose moiety should not be changed; 2) C2- and C8-substitution lower agonist activity; 3) N^6-substitution reduces potency, but less severely than C2 and C8 substitution; 4) the purine ring system should not be modified into deazapurines or xanthines.

Two sites of substitution might be promising in order to increase agonist potency. The first site is the 5'-carboxamido moiety. Analogues with novel substituents on the carboxamido group could be synthesized and tested. Secondly, there appears to be space in the N^6-region of the receptor. Appropriate substituents in this part of the molecule, possibly combined with a 5'-carboxamido function, might yield more potent and selective agonists.

Experimental section

Materials

Test system

A Chinese Hamster Ovary (CHO.K1) cell line stably transfected with human adenosine A_{2B} receptor cDNA[4,14] was used to test the adenosine analogues.

Compounds

[^{3}H]-cyclic AMP was obtained from NEN (Du Pont de Nemours, 's-Hertogenbosch, the Netherlands).

Test compounds came from the following persons or companies: NECA (**1**) and 2-chloroadenosine (**18**) were purchased from Sigma; NCPCA (**2**) and compounds **3-8** and **27** were a gift of Dr. R.A. Olsson; compounds **15** and **16** were a gift of Takeda Chem. Ind. Ltd., Japan; compounds **19**, **56** and **58** were synthesized according to literature procedures, with minor modifications[24-26]; CPA (**55**) and R-PIA (**59**) were purchased from Boehringer Mannheim, Mannheim, Germany; compound **57** was a gift from Dr. A. van Aerschot, Leuven, Belgium; SPA (**60**) was a gift from Research Biochemicals Inc., Massachusetts, USA; compound **62** was a gift from Dr. J. Linden; compound **64** was a gift from Dr. O. Saiko and Prof. H.P. Wolf, Merck, Darmstadt, Germany; compounds **71-76, 78** and **79** have been synthesized and published before by Van Galen *et al.*[27]; one of the authors, G.C., provided the deaza compounds **83-89**; one of the authors, K. A. J., provided compounds **9-14, 17, 20-54, 61, 63-70, 77, 80-82, 90-109**.

Cell culture

CHO-K1 cells were grown under 5% CO_2/95% O_2 humidified atmosphere at a temperature of 37°C in DMEM supplemented with Hams F12 nutrient mixture (1/1), 10% newborn calf serum, 2mM glutamine and containing 100 IU penicillin and streptomycin. Cells were cultured in 10 cm ∅ round plates and subcultured when grown confluent (approximately after 72 hours). PBS/EDTA containing 0.25% trypsine was used for detaching the cells from the plates. Experimental cultures were grown overnight as a monolayer in 24 wells tissue culture plates (400 µL/well; 0.8×10^6 cells/well).

Cyclic AMP generation

Cyclic AMP generation was performed in DMEM/HEPES buffer (DMEM containing 50mM HEPES, pH 7.4, 37°C). To each well, washed twice with DMEM/HEPES buffer, 125 µL adenosine deaminase (final concentration 10 U/mL) and

125 µL rolipram (final concentration 100 µM) were added. After incubation for 40 minutes at 37°C, 100 µL of the ligand solution (final concentration 100 µM; maximal DMSO concentration 3%) was added. After 15 minutes, incubation at 37°C was terminated by removing the medium and adding 200 µL of 0.1 M HCl. Wells were stored at -20°C until assay.

Cyclic AMP determination

The amounts of cyclic AMP were determined after a protocol with cAMP binding protein[28] with the following minor modifications. As a buffer was used 150 mM K_2HPO_4/ 10mM EDTA/ 0.2% BSA FV at pH 7.5. Samples (20 µL) were incubated for 90 minutes at 0°C. Incubates were filtered over GF/C glass microfibre filters in a Brandel M-24 Cell Harvester. The filters were additionally rinsed with 4 times 2 ml 150 mM K_2HPO_4/ 10mM EDTA (pH 7.5, 4°C). Punched filters were counted in Packard Emulsifier Safe scintillation fluid after 2 hours of extraction.

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