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Adenosine derivatives with N^6-alkyl, -alkylamine or -alkyladenosine substituents as probes for the A_1-receptor

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Three series of N^6-substituted adenosine derivatives were synthesized, having in common an unbranched alkyl chain with lengths varying from 2 to 12 methylene units, but differing in their ω-alkyl substituents: N^6-*n*-alkyladenosines (I), N^6-ω-amino-alkyladenosines (II) and α ω,di-(adenosin-N^6-yl)alkanes (III). The compounds of the latter series combine two functional groups in one molecule. A_1-receptor affinity of these compounds was measured as inhibition of [^{3}H]PIA binding to calf brain membranes. With relatively short chain lengths, compounds in series I are the most potent. In this series, optimum acitivity is reached with N^6-*n*-pentyladenosine (K_i=0.50 nM). With short chain lengths, compounds in series II and III are 6–20-fold less potent than their congeners in series I. The potency order however is reversed with higher chain lengths. While affinity in series II and III increases strongly, reaching an optimum with the nonyl derivatives, affinity in series I decreases sharply with alkyl chains larger than 8 methylene units. Highest affinity is found with 9-amino-nonyladenosine (K_i=0.32 nM). In general, the ω-aminoalkyl derivatives are somewhat more potent than the corresponding di-adenosinyl derivatives. Implications for the possible topography of the N^6 region of the A_1-receptor and the area further removed from N^6 are dicussed.

Adenosine A_1-receptor; N^6 region

1. INTRODUCTION

Adenosine has numerous physiological actions, including vasodilation and hypotension, muscle relaxant and central depressant effects and inhibition of platelet aggregation [1]. These actions are thought to be mediated via membrane bound receptors. Presently, the existence of two adenosine receptor subtypes, A_1 and A_2, is generally accepted. At the A_1-receptor, N^6-*R*-1-phenyl-2-propyladenosine (*R*-PIA) is more potent than 5′-*N*-ethylcarboxamideadenosine (NECA). There is a marked stereoselectivity for the *R*- and *S*-stereoisomers of PIA: at the A_1-receptor, *R*-PIA is at least 10-fold more potent than *S*-PIA. At the A_2-receptor, NECA is more potent than *R*-PIA and stereoselectivity for the isomers of PIA is less than 10-fold [2].

Substitutions at or modifications of the adenine ring or the ribose moiety in adenosine usually lead to compounds with less activity, or no activity at all. The one exception is substitution at N^6, which can enhance affinity at A_1-receptors greatly [3]. Consequently, the search for new adenosine derivatives as ligands for the A_1-receptor has been focussed mainly on the N^6 region. A general model for this region was introduced by Kusachi et al. [4].

The N^6 region was explored extensively and systematically with a number of N^6-substituted adenosine derivatives in three studies [5–7]. In these studies chirality at C_2 of the N^6-substituent, a prominent feature of the A_1-receptor, is heavily emphasized. Less is known about points of interac-

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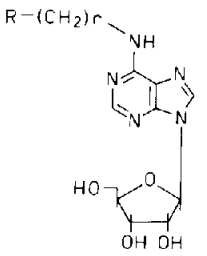

Fig.1. Synthesized compounds. I, R = H; II, R = NH_2; III, R = adenosin-N^6-yl. n = 2–12.

tion more remote from the N^6 region, with the exception of the studies of Jacobson et al. [8,9], using the functionalized congener approach.

In order to extend the knowledge of possible binding sites further removed from the N^6 region, we synthesized three series of N^6-substituted adenosine derivatives, having in common an unbranched alkyl chain with lengths varying from 2 to 12 methylene units, but differing in their ω-alkyl substituents (fig.1). R = H yields N^6-n-alkyladenosines (I) and R = NH_2 yields N^6-ω-aminoalkyladenosines (II). The compounds of the third series, α,ω-di(adenosin-N^6-yl) alkanes (III), have a second adenosine molecule and thus combine two functional groups in one molecule.

Portoghese and co-workers [10] have introduced the term bivalent ligands for this type of compounds. It describes molecules that contain two pharmacophores, joined by a spacer arm. Such molecules might bind with both pharmacophores to two different recognition sites simultaneously [10]. In opioid receptor research, this approach has led to the development of bimorphinans, potent and selective κ-receptor antagonists [11].

2. MATERIALS AND METHODS

Compounds were synthesized according to the well established method of Fleysher et al. [12]. Briefly stated, 6-chloropurineriboside (6-CPR) was refluxed for 2–6 h in ethanol with the appropriate alkylamine to give N^6-substituted adenosines. Triethylamine or excess of the alkylamine were used as proton scavengers. In the synthesis of II and III, α,ω-diamino alkanes were used. With a 5–10-fold molar excess of the diamine, almost exclusively II was formed. With a slight excess of 6-CPR, predominantly III was formed. Compounds were purified by extraction and/or recrystallization. Purity was checked by TLC. Structures were confirmed by 300 MHz NMR spectroscopy.

N^6-(2-Aminoethyl)adenosine, N^6-(4-aminobutyl)adenosine, 1,2-di(adenosin-N^6-yl)ethane and 1,4-di(adenosin-N^6-yl)butane were gifts of Dr J. Zemlicka, Michigan Cancer Foundation, Detroit. Adenosine deaminase was from Boehringer, Mannheim, FRG. 8-Phenyltheophylline was from Janssen Chimica, Beerse, Belgium. [^{3}H]PIA (40 Ci/mmol) was from Amersham, Buckinghamshire, England.

2.1. *Preparation of membranes*

Calf brain (approx. 300 g), obtained from a local slaughterhouse, was placed in 5 mM Hepes-Tris (pH 7.4) (buffer A) within 30 min of slaughter. Cerebral cortex was dissected, disrupted, diluted to a total volume of 2500 ml with buffer A and centrifuged at 10000 × g for 10 min. The supernatant was centrifuged at 30000 × g for 45 min. The P_2 fraction was washed in 600 ml of buffer A and recentrifuged at 30000 × g for 45 min. The pellet was resuspended in 55 ml of buffer A, incubated with 200 IU of adenosine deaminase for 30 min at 37°C and stored in liquid nitrogen. Protein was assayed according to the method of Lowry et al. [13].

2.2. *Binding assay*

[^{3}H]PIA binding assays were performed in 50 mM Tris-HCl (pH 7.4) (buffer B) in a final volume of 400 μl. The standard assay contained ~40 μg membrane protein and 0.3 nM [^{3}H]PIA. Incubations were carried out in duplicate for 120 min at 25°C and were terminated by diluting the samples with 1 ml ice-cold buffer B, followed by rapid filtration through Whatman GF/C glass fiber filters. Subsequently, tubes were washed with 1 ml buffer and filters were washed twice with 3 ml buffer B. Filters were dried for 45 min at 60°C, 3.5 ml of scintillation fluid (LKB OptiPhase MP) was added and radioactivity was determined in an LKB 1214 Rackbeta liquid scintillation counter. Solutions of N^6-substituted adenosines were made in DMSO and were diluted with buffer to a final DMSO concentration of 1%. Non-specific binding was determined in the presence of 5×10^{-6} M

8-phenyltheophylline. K_i values were calculated with a computer program based on the law of mass-action [14], using a K_d for [^{3}H]PIA of 0.16 nM (unpublished).

3. RESULTS AND DISCUSSION

A_1-receptor affinity [15] was determined in a binding assay using calf cerebral cortical membranes and [^{3}H]PIA as the radioligand.

In table 1, K_i values for the various compounds tested are listed. In fig.2, pK_i values are represented as a function of spacer length.

Within the N^6-*n*-alkyladenosine series (I), compounds with relatively short N^6-substituents are the most potent inhibitors of [^{3}H]PIA binding. This is in agreement with the assumption of Daly that hydrophobic interactions play an important role in binding to the N^6 region [5].

In contrast with our results however, Daly (measuring [^{3}H]CHA displacement in rat brain membranes) observed optimum activity with N^6-*n*-propyl and N^6-*n*-butyladenosine. With N^6-*n*-pentyl and N^6-*n*-hexyladenosine, a sharp decline in activity was seen. In our assay, potency in the *n*-alkyladenosine series remains high with chain lengths up to 8 methylene units, and optimum activity is found with N^6-*n*-pentyladenosine (K_i = 0.50 nM). This may be attributed to species differences between A_1-receptors in rat brain and in calf brain. Such differences are especially clear in the case of xanthine adenosine antagonists, which are much more potent in bovine brain than in rat brain [16].

Table 1

K_i values of N^6-substituted adenosine derivatives (nM, ± SE) for displacement of [^{3}H]PIA in calf brain

n	I. N^6-alkyl-adenosines	II. N^6-(ω-aminoalkyl)-adenosines	III. α,ω-di-(adenosinyl)-alkanes
2	3.9 ± 0.3	38 ± 3	76 ± 13
3	1.1 ± 0.1	9.1 ± 1.2	18 ± 1
4	1.5 ± 0.1	8.9 ± 0.7	23 ± 2
5	0.50 ± 0.04	3.7 ± 0.4	4.0 ± 0.9
6	1.8 ± 0.2	0.97 ± 0.10	2.0 ± 0.2
8	1.9 ± 0.2	0.50 ± 0.05	1.3 ± 0.1
9	8.1 ± 0.7	0.32 ± 0.03	0.70 ± 0.05
10	21 ± 2	0.51 ± 0.04	2.0 ± 0.1
11	71 ± 5	–	–
12	147 ± 34	1.7 ± 0.2	5.6 ± 0.4

Values are means of 3 experiments. *n* = number of methylene units

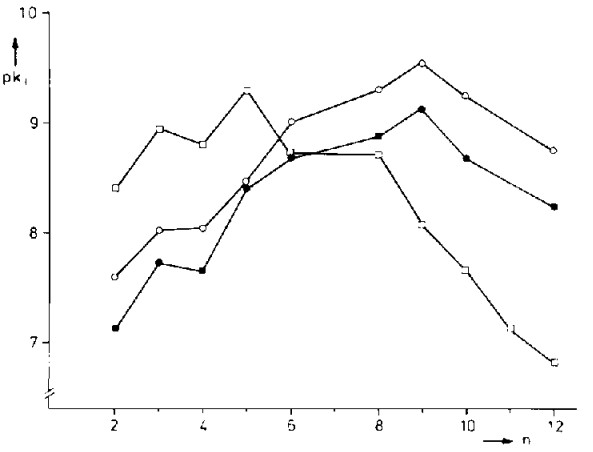

Fig.2. Plot of pK_i values of N^6-substituted adenosine derivatives ([^{3}H]PIA binding to calf brain membranes) versus chain length. (□) N^6-*n*-alkyladenosines, (○) N^6-ω-amino-alkyladenosines, (●) α,ω-di-(adenosin-N^6-yl) alkanes; *n* = number of methylene units.

Our results show that also for agonist ligands, there are significant differences in binding to A_1-receptors of rat brain and calf brain, respectively.

With the compounds of series II and III with n = 2–4, a 6–20-fold decrease in affinity is seen, as compared to the affinities of the corresponding compounds of series I. Again, this may be compared to the findings of Daly. When various polar terminal groups were attached to N^6-ethyladenosine, the resulting compounds all had less affinity for the A_1-receptor than N^6-ethyladenosine itself. N^6-2-Aminoethyladenosine was the least potent, with a 17-fold decrease in affinity [5]. Apparently, hydrophilicity is not tolerated very well in the area close to N^6.

Differences in K_i between series II, with a small and positively charged terminal amino group, and series III, with a bulky adenosine substituent, are quite small, 2–4-fold at the most, which seems to indicate that steric hindrance does not play an important role here.

With increasing chain length, the order of poten-

cy is reversed. The N^6-*n*-alkyladenosines with N^6-substituents larger than 8 carbon atoms steadily lose potency, while the compounds in series II and III show a rapid increase in affinity, with an optimum of nine methylene units in both series. Thus, N^6-9-amino-nonyladenosine is very potent (K_i = 0.32 nM) and has 25-fold higher affinity than N^6-*n*-nonyladenosine. N^6-12-Aminododecyladenosine has even 86-fold higher affinity than N^6-*n*-dodecyladenosine. This reversal of potency order with increasing chain length is best explained if it is assumed that the area further removed from N^6 is essentially hydrophilic in nature. This would be unfavourable for the adenosine analogs with a long and lipophilic alkyl chain. An accessory site for a relatively polar terminal group like an amine or adenosine would overcome this unfavourable effect, yielding the more potent compounds of series II and III with chain lengths from 8 to 12 methylene units. Jacobson observed similar increases in potency with a terminal amine attached to functionalized congeners of N^6-phenyladenosine [8] and xanthine adenosine antagonists [17]. Whether, as was suggested, this can be ascribed to an ionic interaction of the positively charged amine with a negatively charged part of the receptor seems doubtful, since the affinities of the non-ionic compounds in series III constantly parallel the affinities of the charged amines of series II.

These close similarities between series II and III also show that it is unlikely that the α,ω-di(adenosin-N^6-yl) alkanes actually bridge two receptor sites. In that case, a different profile would be expected for the interaction of an adenosine moiety to a second receptor site and the binding of an amino group to a less specific accessory site. Moreover, a bivalent ligand would be expected to bind more strongly, which is evidently not the case for these compounds.

Since lipophilic substituents play an important role in the binding of N^6-substituted adenosine analogs, our choice for a distinctly lipophilic spacer arm seemed justified. However, it is conceivable that via these strong, hydrophobic interactions in the N^6 region, the spacer arm becomes orientated in such a way that the interaction of the terminal adenosine group with a second receptor site is actually hampered. Therefore, it could be of interest to synthesize bifunctional molecules with a less lipophilic spacer arm, in order to investigate if two adenosine receptors can actually be bridged by one bivalent ligand. A prerequisite, however, is that binding sites are organized in a regular recognition pattern, situated on oligomeric subunits. It is likely that this is the case for the earlier mentioned κ-opioid receptors [11], but whether this is also the case for adenosine receptors remains to be shown.

In conclusion, the picture that arises from this work is a hydrophobic region near N^6, developing into a hydrophilic area further removed from N^6, with ample space to accommodate bulky groups like adenosine. Speculatively, these qualifications would be compatible with a situation in which N^6 is pointed away from the receptor, directed into the extracellular space, which of course meets both the requirements concerning hydrophilicity and space.

Interestingly, something similar is seen with several enzymes that have binding sites for adenosine-containing coenzymes. The structures of some of these enzyme-coenzyme complexes, e.g. parahydroxybenzoate-hydroxylase with FAD and dihydrofolatereductase with NADP are known [18,19]. When these structures are examined with the aid of molecular graphics, it is evident that the domain where the N^6 in the adenosine moiety of the coenzyme is binding is invariably on the surface of the protein, with N^6 pointing outwards (R. Wieringa and H. van Vlijmen, personal communication).

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REFERENCES

[1] Daly, J.W. (1982) J. Med. Chem. 25, 197–207.
[2] Stone, T.W. (1984) in: Purines: Pharmacology and Physiological Roles (Stone, T.W. ed.) pp.1–4, Macmillan, Basingstoke.
[3] Daly, J.W. (1985) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 19, 29–46.
[4] Kusachi, S., Thompson, R.D., Bugni, W.J., Yamada, N. and Olsson, R.A. (1985) J. Med. Chem. 28, 1636–1643.

[5] Daly, J.W., Padgett, W., Thompson, R.D., Kusachi, S., Bugni, W.J. and Olsson, R.A. (1986) Biochem. Pharmacol. 35, 2467–2481.
[6] Paton, D.M., Olsson, R.A. and Thompson, R.D. (1986) Naunyn-Schmiedeberg's Arch. Pharmacol. 333, 313–322.
[7] Dunwiddie, T.V., Watt, T.S. and Olsson, R.A. (1986) Naunyn-Schmiedeberg's Arch. Pharmacol. 334, 77–85.
[8] Jacobson, K.A., Kirk, K.L., Padgett, W.L. and Daly, J.W. (1985) J. Med. Chem. 28, 1341–1346.
[9] Jacobson, K.A., Yamada, N., Kirk, K.L., Daly, J.W. and Olsson, R.A. (1986) Biochem. Biophys. Res. Commun. 136, 1097.
[10] Erez, M., Takemori, A.E. and Portoghese, P.S. (1985) J. Med. Chem. 25, 847–849.
[11] Portoghese, P.S., Lipkowski, A.W. and Takemori, A.E. (1987) J. Med. Chem. 30, 238–239.
[12] Fleysher, M.H., Hakala, M.T., Bloch, A. and Hall, R.H. (1968) J. Med. Chem. 11, 717–720.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] IJzerman, A.P., Bultsma, T., Timmerman, H. and Zaagsma, J. (1984) Naunyn-Schmiedeberg's Arch. Pharmacol. 327, 293–298.
[15] Lohse, M.J., Lenschow, V. and Schwabe, U. (1984) Mol. Pharmacol. 26, 1–9.
[16] Ferkany, J.W., Valentine, H.L., Stone, G.A. and Williams, H. (1986) Drug Dev. Res. 9, 85–93.
[17] Jacobson, K.A., Kirk, K.L., Padgett, W.L. and Daly, J.W. (1985) J. Med. Chem. 28, 1334–1340.
[18] Van der Laan, J.M. (1986) in: On the Enzymatic Cycle of *p*-Hydroxybenzoate Hydroxylase, p.12, Thesis, Groningen, The Netherlands.
[19] Filman, D.J., Bolin, J.T., Matthews, D.A. and Krant, J. (1982) J. Biol. Chem. 257, 13663–13672.